STRUCTURAL AND PHYSIOLOGICAL MODIFICATIONS OF HUMAN ERYTHROCYTE INDUCED BY COBALT 60 GAMMA RADIATION

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ABSTRACT

Irradiation of stored blood or its products could be an attractive strategy for prevention both infections or grafts-versus-host disease after transfusion. Sterilization doses are higher than those used for elimination of graft vs. host disease and a increased erythrocytes radioresistance was reported, suggesting those higher doses could be used to improve sterility. We studied structural and physiological alterations of human erythrocytes ^{60}Co irradiated until 1600 Gy, by cytometric techniques, looking for total cell volume, ionic and hemoglobin alterations, and erythrocyte membrane proteins. Human A+ erythrocytes were washed in PBS and submitted to uniform ^{60}Co radiation, with a dose ratio of 5 to 1600 Gy in log intervals. By SDS-PAGE and Western-blot of isolated membranes after radiation, we found significant alterations on proteins, specially spectrin, without isosmotic morphological alteration, but with lower osmotic resistance to higher doses. By cytometry, cell volume was increased, with extracellular K+ increase, suggesting some radiation induced physiological alterations. By ektacytometry, the irradiated samples presented higher low shear resistance as compared to non irradiated cells, despite similar values at higher shear stress. The optimization of this process, possibly with use of specific free radicals scavengers, would result in higher sterility with almost preserved erythrocytes, useful in certain clinical relevant applications.

Key words: erythrocytes, gamma radiation, proteins, cytometry, shear stress

INTRODUCTION

Human blood and its components have many uses in medical therapy, but with receptor risk both of infections or donor cell transplant with graft vs. host diseases (1). The source, treatment and storage of these products are restricted by determinations of each country councils, as FDA in USA or US in Brazil (2). The supply of blood or its components, adhering to those restrict quality control, implies in high costs, besides a delay in its delivery for human use (3). The routine sterilization of dried materials with γ rays is usually performed at 20kGy(4). Blood or components are usually submitted to 50 Gy, despite some reports of higher radioresistance of erythrocyte (5). The main goal is the elimination of donor nucleated cells, for bone marrow recipients transfusions (6,7) to avoid graft vs. host adverse reactions. Blood sterilization or decontamination was not adequately studied, with some reports on platelet function (8) or lymphocyte elimination (9). Reports of erythrocyte irradiation describe stability without major alterations at 35 Gy (10) or increase in osmotic fragility at higher doses on pig erythrocyte (11). Most reports concentrate on alterations induced on erythrocyte membrane proteins or lipids (12, 13), but erythrocyte irradiation was not adequately studied at doses higher than 50 Gy. At 200 Gy, most blood contaminating nucleated parasites are inactivated (14, 15, 16), and, if used, this irradiation could reduced the cost of transfusions. New approaches on erythrocyte physiology, as ektacytometry, allow the study of physiology of erythrocyte at several stress levels, similar to those found in human circulation(17). We study structural and physiological modifications of human erythrocytes induced by 20 – 1600 Gy ^{60}Cobalt irradiation, using new ekta- and cytometric analysis.

METHODS

Human blood, A+, with negative serology for usual pathogens, was collected from volunteers or as stored blood from the Hemocentro of São Paulo, stored in CPD-1 solution and conserved at 4^{O} C. Before use, erythrocyte fraction were washed in phosphate buffered saline pH 7.2(PBS) by centrifugation. Others

reagents were purchased from suppliers, with pro-analysis quality, and solutions prepared with MilliQ ultra pure water. Samples were divided, transported in ice to a panoramic ^{60}Co source (Yoshizawa Kiko Co Ltd) and located exactly 25 cm from the source in a rotary shaker for uniform irradiation, without barriers. This device provide a 0,273 kGy/h dose, allowing irradiation of 5, 10, 25, 50, 100, 200, 400, 800 and 1600 Gy triplicate samples.

Cytometric studies were performed on irradiated erythrocytes washed and suspended on PBS in a automatic cell counter, Technicon H3 RTX system, with analysis of at least 10000 cells/sample, with determination of single cell volume and hemoglobin, percentage of hypochromic cells. Free Na+ and K+ was determined in external medium by a flame photometer.

Osmotic fragility was determined by an in-house microplate adapted assay, with serial dilutions of NaCl (0 to 1x 0,15M) in sodium phosphate pH 7,2 0,02M, applied to a 96 multiwell plate. Erythrocytes at 10% submitted or not to progressive amounts of radiation were added to the wells and incubated with shaking by 30 min at 37^{o}C. The plate was centrifuged at 4000 g and the supernatant carefully transferred to another plate with clear optical flat bottom. Free hemoglobin, as 540 nm O.D. was determined in a microplate reader. The osmotic fragility (50% lysis) was calculated using lower NaCl concentration as total lysis parameters, with sigmoidal dose response analysis performed by Graph Pad Prism software.

Deformability studies were performed on controls and irradiated erythrocytes suspended in PBS, and its deformability induced by shear stress determined in an IZ/OW ektacytometer (Laser assisted optical rotational cell analyzer) with constant temperature (37^{o} C) and medium viscosity [31mPa*s], with 0.3-30 Pa shear stress. The deformability profile was determined in at least 100 cells. The elongation index at each shear stress was carefully determined and analyzed with a LORCA 1.00 software, with comparative deformability at lower shear stress (0,3 Pa) and higher shear stress (10 Pa) for each radiation doses.

Ghosts(membrane preparation) were obtained from erythrocytes by hypotonic lysis and centrifugation, in the presence of protease inhibitors (PMSF 0,1mM, Benzamidine 1 mM) and submitted to ^{60}Co irradiation. Protein from ghosts were submitted to SDS-PAGE using a urea containing sample buffer and 7,5% acrylamide running gel, using a Bio-Rad Mini-Protean II Electrophoresis Cell system. Gels were stained both by Coomassie Brilliant Blue or silver, with scanning of the dried slabs in a Hewlett Packard Scan IV scanner.

RESULTS

We performed the irradiation of fresh or stored blood as described in Methods, without any significant hemolysis at normal osmotic pressure, using cells samples with less than two weeks at refrigerator storage. The quantitative data of cytometric analysis of stored blood are presented as a function of doses, as could be seen in Fig.1.

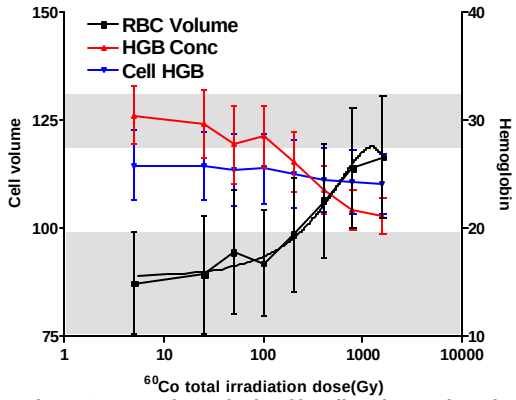

Fig.1 - Cytometric analysis of irradiated stored erythrocytes Dashed area represents normal values

The radiation induced a progressive increase of cell volume, with consequent lowering of cell hemoglobin concentration, clearly significant at 400 Gy or higher doses, but without lysis of the cell. When fresh blood purified erythrocytes were immediately irradiated, the cytometric analysis presented similar effects but with a lower magnitude(Fig.2).

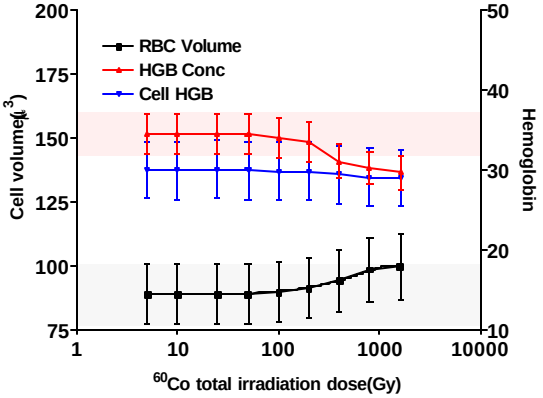

Fig.2 - Cytometric values of irradiated fresh erythrocytes. Dashed area represent normal range.

The concentration of extracellular K+ increased similarly to the cell volume (data not shown). The percentage of hypochromic cells were determined in both preparations, with an increasing proportion of hypochromic cells in stored blood (Fig.3).

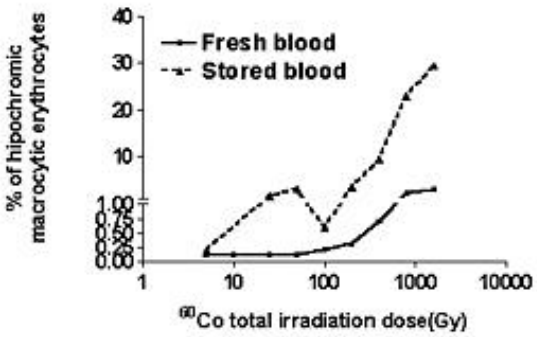

Fig.3 - Percentage of hypochromic cells in irradiated fresh or stored erythrocytes.

The osmotic fragility of stored erythrocytes submitted to irradiation also increases proportionally to the applied doses, with a significant increment in osmotic fragility after 400 Gy, not observed at lower doses (Fig.4).

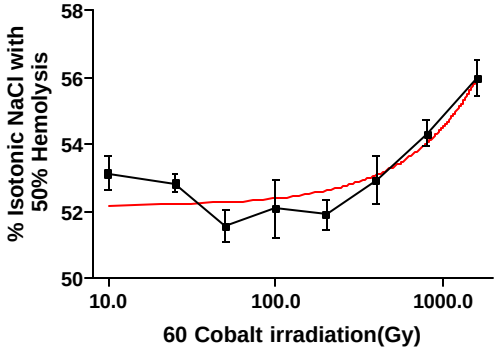

Fig.4 - Osmotic fragility of erythrocytes irradiated at several doses of ^{60}Co irradiation. Exponential fitting is showed by the red line.

The analysis of deformability of fresh blood erythrocytes, by ektacytometry, showed that irradiation induces alterations in elongation index only at low shear stress [0.3 Pa] in doses higher than 400Gy, without similar effect at high shear stress [3 Pa], when the elongation index was not affected by irradiation (Fig.5).

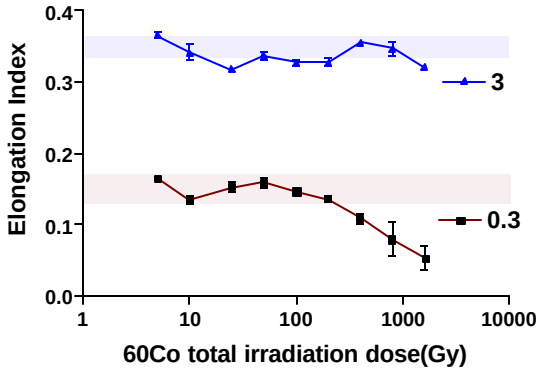

Fig 5. Elongation index of irradiated erythrocytes submitted to 60Co irradiation, at two shear stress.

The SDS-PAGE analysis of erythrocytes ghosts of irradiated erythrocytes (Fig6) show a clear aggregation of spectrin at higher doses, with some quantitative difference in low molecular weight proteins, as band 3.

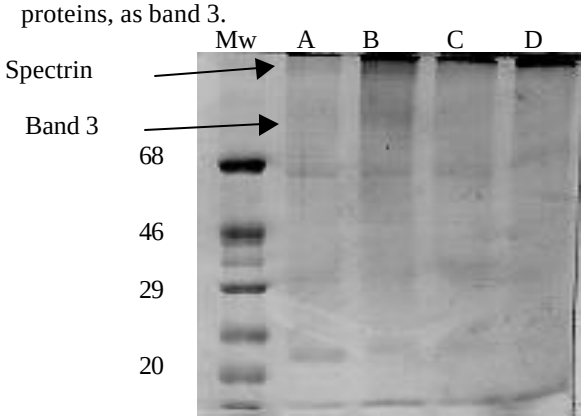

Fig.6- SDS PAGE of irradiated erythrocytes ghosts proteins. Mw Markers. A- Non irradiated. B 400 Gy, C 800 Gy, D 1600 Gy

DISCUSSION

Our data clearly show that erythrocytes could be irradiated at 200Gy, without significant effect in its fragility, cell volume, hemoglobin concentration or cell deformability both at low or high shear stress. In higher doses, the effect of irradiation was significant, but also lower than expected, without significant isosmotic cell destruction. The effect of prolonged storage was also significant, with an increase in radio-sensibility of the erythrocytes. The blood irradiation is mainly used for prevention of transfusion- associated graft-versus- host disease in immunosuppressed bone marrow transplant patients, at 20-30 Gy, enough to destroy any lymphocytes (18), usually by inducing apoptosis (19) but lower parasites or other blood contaminants requires much higher doses for elimination (14). Complete inactivation of T cells was achieved with 25 Gy (7), without effect on red cells, including its life span in the host (6). Our data also confirm the combined effect of storage and irradiation, which affects the cell volume, and probably its survival in the host, at higher doses of 400 Gy (18). The deformability of the cells was curiously affected, showing a lower deformability at low shear stress in our data. This fact could be explained by our findings of aggregation of spectrin, the main cytoskeleton component in the erythrocyte (22), resulting in higher resistance to deformability at low forces, but ineffective in high stresses. This could imply that erythrocytes irradiated with doses higher than 400 Gy could be less efficient in capillary flow, when small forces induce deformation of the red cell, for adequate flow and oxygen exchange (20), which could be also hampered by the volume increase observed in higher doses. Another problem, already described, was the efflux of K+ ions in the extracellular medium, which could be relieved by final

washing before use (1). Despite those limitations, the use of higher irradiation doses for blood could provide a alternative elimination of nucleated infectious agents, like *Plasmodium*, *Toxoplasma* or *Trypanosoma cruzi*, that are transmitted by blood transfusions (21). The control of possible presence of these pathogens in donors blood, specially in tropical countries, results both in high cost and high discard rate of collected blood, by limited serological analysis. The irradiation of blood, at safe doses or with the use of specific scavengers of free radicals, could result in a cheaper and safer blood or components.

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REFERENCES

[1]Cable, Alan W. and col. .Blood Irradiation: A User's Guide American Association of Blood Banks Bethesda, Maryland 1966

[2]Sazama K Current good manufacturing practices for transfusion medicine Transfus Med Rev 10(4):286-295 1996

[3]Schmunis GA, Zicker F, Pinheiro F, Brandling-Bennett D - Risk for transfusion-transmitted infectious diseases in Central and South America. Emerg Infect Dis 4(1):5-11, 1998

[4]Grecz N, Brannon RB, Killgore G. Radiation sterilization of surgical instruments with a consideration of metal shielding on sterilization efficiency. Am J Infect Control Jun;15(3):101-106 1987

[5] Leitman SF, Holland PV Irradiation of blood products. Indications and guidelines. Transfusion 25(4):293-303 1985

[6] Button LN, DeWolf WC, Newburger PE, Jacobson MS, Kevy SV The effects of irradiation on blood components.Transfusion 1981 Jul;21(4):419-426

[7] Fagiolo E, Pozzetto A, Cellini N Valentini V Determination of the radiation dose on blood components to prevent post-transfusional graft-vs-host reaction.Haematologica 68(5):573-580 1983

[8] Sweeney JD, Holme S, Moroff G Storage of apheresis platelets after gamma radiation. Transfusion 34(9):779-783 1994

[9] Pelszynski MM, Moroff G, Luban NL, Taylor BJ, Quinones RR Effect of gamma irradiation of red blood cell units on T-cell inactivation as assessed by limiting dilution analysis: implications for preventing transfusion-associated graft-versus-host disease. Blood 83(6):1683-1689 1994

[10] Hillyer CD, Tiegerman KO, Berkman EM Evaluation of the red cell storage lesion after irradiation in filtered packed red cell units. Transfusion 31(6):497-499 1991

[11] Helszer Z, Jozwiak Z, Leyko W Osmotic fragility and lipid peroxidation of irradiated erythrocytes in the presence of radioprotectors. Experientia 36(5):521-524 1980

[12] Cantafora A, Ceccarini M, Guidoni L, Ianzini F, Minetti M, Viti V Effects of gamma-irradiation on the erythrocyte membrane: ESR, NMR and biochemical studies. Int J Radiat Biol Relat Stud Phys Chem Med 51(1):59-69 1987

[13] Soszynski M, Schuessler H Effect of X-irradiation on erythrocyte membrane proteins. Primary radicals.Int J Radiat Biol 60(6):859-875 1991

[14] Hiramoto, R. (1998) Efeitos da radiação ionizante sobre a estrutura, metabolismo e infectividade de um protozoário patogênico, \. Dissertação de Mestrado – Instituto de Pesquisas Energéticas e Nucleares – São Paulo – 1998.

[15} Dubey JP, Jenkins MC, Thayer DW, Kwok OC, Shen SK Killing of Toxoplasma gondii oocysts by irradiation and protective immunity induced by vaccination with irradiated oocysts.J Parasitol 82(5):724-727 1996

[16] Waki S, Yonome I, Suzuki M Plasmodium falciparum: attenuation by irradiation. Exp Parasitol 56(3):339-345 1983

[17] Mohandas N, Chasis JA Red blood cell deformability, membrane material properties and shape: Regulation by transmembrane, skeletal and cytosolic proteins and lipids. Sem. Hematol. 30:171-192, 1993.

[18] Davey RJ, McCoy NC, Yu M, Sullivan JA, Spiegel DM & Leitman SF The effect of prestorage irradiation on posttrasnfussion red cell survival. Transfusion 32:525-528, 1992

[19] Lemes M. O uso da apoptose de linfócitos periféricos humanos como método alternativo de dosimetria biológica dos efeitos da radiação do cobalto-60. Dissertação de Mestrado – Instituto de Pesquisas Energéticas e Nucleares – São Paulo - 1997

[20] Stuart J & Nash GB – Technological advances in blood rheology. Crit.Rev.Clin.Lab.Sci. 28:61-93, 1990

[21] Chamberland M, Khabbaz RF Emerging issues in blood safety Infect.Dis.Clin. North Am. 12(1)217-229(1998)

[22] Nash GB, Gratzer WB Structural determinants of the rigidity of the red cell membrane.Biorheology 30:397-407, 1993