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ORIGINAL ARTICLE

The effect of gamma radiation on the lipid profile of irradiated red blood cells

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Abstract An investigation into the effects of irradiation and of the storage time on aging and quality are a relevant issue to ensure the safety and the efficiency of irradiation in the prevention of transfusion-associated graft-versus-host disease (TA-GVHD). In this work, the biochemical properties and alterations presented by erythrocyte membranes, up to 28-days post-irradiation, with a dose of 25 Gy, were studied as a function of storage and post-irradiation time. There was a considerable variation in the total of phospholipid content, when comparing the control and irradiated samples, mostly from the third day onwards; and at the same time, the effect occurred as a function on the storage time of blood bags. The levels of total cholesterol decreased 3–9 days after irradiation. TBARS levels were increased after irradiation and 7 days of storage, but no increment of catalase activity was observed after the irradiation. Furthermore, the protein profile was maintained throughout the irradiation and storage time, until the 21st day, with the presence of a protein fragmentation band of around 28 kDa on the 28th day. In conclusion, although gamma irradiation is the main agent for the prevention of TA-GVHD, a better understanding of the physical and biochemical properties of erythrocytes are necessary to better assess their viability, and to be able to issue more secure recommendations on the shelf life of blood bags, and the safe use of the irradiated red cells therein.

Keywords Lipid peroxidation · Gamma radiation · TA-GVHD · Cholesterol · Phospholipids

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Introduction

Transfusion-associated graft-versus-host disease (TA-GVHD) is a pathological condition that occurs when severely immunocompromised or immunodepressed patients receive blood transfusions containing immunologically competent lymphocytes [1–4]. Although TA-GVHD is a rather unusual complication of blood transfusions, it presents a high-mortality risk because its symptoms, such as fever, cutaneous erythema, hepatitis, and diarrhea (among others), are easily confused with those of other diseases [5–7]. Gamma irradiation is routinely used for the prevention of TA-GVHD because irradiation halts the proliferation and inactivates the immune response of donor lymphocytes that are the causal agents of TA-GVHD, thus preventing the onset of the pathology [2, 4, 5].

The difference in susceptibility to gamma irradiation between human red cells and lymphocytes is the key behind the strategy for inactivating T-lymphocytes that are present in red cell concentrates (with $1.0–2.0 \times 10^9$ cells per unit). The recommended shelf life of hemoconcentrates after irradiation is 28 days, and the dose applied should be between 25 and 50 Gy [8]. Recently, it was revealed that the radiation damaged the function of Na, K-ATPase [9] and modified the hemoglobin conformation due to alterations in oxidative stress in the erythrocytes [10].

The ability of biological membranes to control cell interactions and signaling as well as the subsequent metabolic routes depends on their physical and chemical integrity. Therefore, radiation-induced lipid and protein membrane oxidation might be responsible for damaging red cell membranes, leading to an increase in permeability to monovalent and divalent ions [11, 12].

Lipid peroxidation is recognized as one of the main mechanisms of membrane damage, and it is driven by reactive oxygen species (ROS). Some iron complexes are known to induce extensive lipid peroxidation in a process initiated by the classical Fenton reaction [13]. Lipid peroxidation is a cytotoxic event that can be defined as a cascade of biochemical events arising from free-radical attack of unsaturated fatty acids on membrane lipids, which generates several products and initiates a sequence of structural and functional alterations, thus resulting in an imbalance of cell permeability, membrane ion exchange, and homeostasis that might lead to apoptosis [14].

Alterations of erythrocyte membrane ionic permeability and increases in lipid peroxidation or proteolysis are events that can be triggered by an increase in oxidative stress, and these events have been well described. However, few studies have examined modifications in erythrocyte energy metabolism or explored which enzymes are more sensitive to oxidative stress [15].

For the proper balance of ion flux across the erythrocyte membrane, it is mandatory that its organization, composition, fluidity, and asymmetry are preserved to ensure the selectivity of ion channels and pumps on the lipid bilayer [16].

For the adequate arrangement and function of cell membranes, the type, localization, and proportions of lipids found on each leaflet of the membrane must be tightly controlled, and alterations in these characteristics can generate changes in the erythrocytes. Such alterations might occur in several cell events, including physiological or pathological processes. The lipids, aside from being partially responsible for the correct structure of the erythrocyte, can also modulate enzyme activity and participate directly in cell signaling [17]. Therefore, an investigation of the effects of irradiation and storage time on the aging and quality of blood bags, with the aim of preserving the integrity and function of the erythrocytes, is relevant to ensure the safety and efficiency of irradiation for the prevention of TA-GVHD. In this work, the biochemical properties of and alterations in erythrocyte membranes were studied up to 28 days post-irradiation with a dose of 25 Gy as a function of storage and post-irradiation time.

Materials and methods

Preparation of red cell membranes (ghosts)

Calmodulin-depleted, cytoplasm- and hemoglobin-free membrane ghosts were prepared from the stored blood concentrate bags according to Rega et al. [18] with some modifications. At each previously defined day, 20 mL of blood concentrates were spun at 6,500*g* for 10 min at 4 °C, and the precipitates were resuspended to a final volume of 40 mL in a solution of 20 mM Tris–HCl (pH 7.4), 130 mM KCl, and 0.6 mg/mL phenyl methyl sulfonyl fluoride (PMSF). This suspension was centrifuged at 6,500*g* for 10 min at 4 °C. The cell pellets were lysed by freezing in liquid nitrogen and subsequently thawing at room temperature (25 °C). The lysed cells were then resuspended in 40 mL of 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES; pH 7.4), 1 mM ethylenediamine tetra acetic acid (EDTA) and 0.6 mg/mL PMSF and centrifuged at 9,000*g* for 10 min at 4 °C. This washing step was repeated four times, and the resulting pellets were resuspended in 40 mL of 10 mM HEPES (pH 7.4), 130 mM KCl, 0.5 mM $MgCl_2$, and 0.05 mM $CaCl_2$. The solutions were spun again at 9,000*g* for 10 min and resuspended in 5 mL of 10 mM HEPES (pH 7.4), 130 mM KCl, 0.5 mM $MgCl_2$, and 0.05 mM $CaCl_2$. Lastly, the pellets were stored in liquid nitrogen until use. The total protein concentration was determined according to Hartree [19].

Lipid peroxidation

Lipid peroxidation was assayed using samples of hemoglobin-free ghost membranes according to Buege and Aust [20]: 100 μg of protein was incubated for 1 h at 37 °C in a final volume of 100 μL of 10 mM Tris (pH 7.0). After the addition of 200 μL of 1 % TBA (*w*/*v*) and 100 μL of 12 % TCA (*w*/*v*), the samples were incubated at 95 °C for 30 min. The samples were then cooled on ice for 5 min and centrifuged for 5 min at 13,000*g*. The supernatant was removed, and the absorbance was determined at 532 nm using a spectrophotometer (Genesys S10 UV/VIS; Thermo Scientific, Waltham, MA, USA).

Determination of Fe^{3+} in residual plasma samples

Fe^{3+} quantification was performed as described by Adams [21] with slight modifications. In a test tube, 150 μL of plasma from the stored blood bags was mixed with 1.5 mL of 1 M KSCN, and the volume was adjusted to 3.0 mL with 0.9 % NaCl. The samples were then homogenized, and the optical density at 480 nm (OD_{480}) was read using a spectrophotometer. A standard curve was constructed using a 1 M $FeCl_3$ solution as a standard. The OD was stable for 15 min.

Determination of catalase activity

Catalase activity was determined in erythrocyte hemolysates based on the method of Aebi [22] with some modifications. Hemolysate supernatants (20 μL) were added to a cuvette containing 2 mL of 50 mM phosphate buffer (pH 7.0). The reactions were initiated by the addition of 3.4 μL of freshly

prepared 30 % H_2O_2. The decomposition of H_2O_2 was followed spectrophotometrically at 240 nm (Genesys S10 UV/VIS; Thermo Scientific). The activity was estimated from the slope and expressed as micromoles of H_2O_2 decomposed per minute per milligram of protein.

Membrane lipids

Total ghost membrane lipids were extracted with a chloroform/isopropanol (7:11) mixture as described by Rose and Oklander [23] and Vokurková et al. [24]. In a capped glass tube, 7 mL of isopropanol was added to 1 mL of ghost membranes and incubated for 60 min on ice with occasional agitation. In sequence, 11 mL of chloroform was added, and the mixture incubated for another 60 min on ice. After phase separation, the organic phase was dried and suspended in a known volume. Total phospholipids and total cholesterol were quantified as described by Chen et al. [25] and Higgins [26], respectively.

Protein electrophoresis

The protein profile of control and post-irradiated membranes was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [27] using 10 % acrylamide for the running gel. The gels were stained with Coomassie Brilliant Blue G, and the molecular weight markers ranged from 10 to 225 kDa.

Statistical analysis

The results were analyzed using GraphPad Prism 5. For markers with normal distribution, analysis of variance (ANOVA) was used followed by Tukey's multiple comparison test and the two groups were compared by Student's *t* test. The significance was set at $p < 0.05$.

Results

After blood collection and gamma irradiation, we measured the lipid peroxidation of erythrocyte membranes as a result of the production of ROS and free radicals, and we achieved positive results.

The levels of thiobarbituric acid reactive substances (TBARS) were not altered on the third or seventh day post-irradiation. However, on the fifth day post-irradiation and from the ninth day forward, lipid peroxidation with increased TBARS levels was observed in the irradiated samples. The peroxidation was significantly greater in irradiated samples, as shown in Fig. 1.

In the lipid samples extracted from erythrocyte concentrates, there was considerable variation in the total

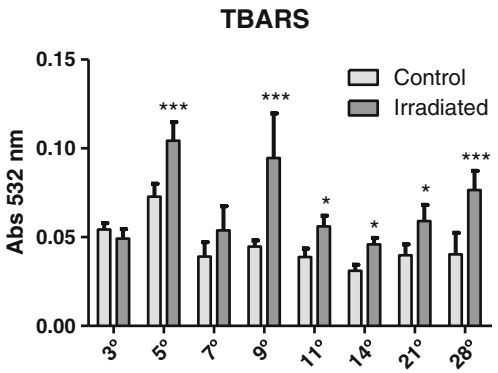

Fig. 1 Lipid peroxidation analysis after storage and irradiation. RBCs were irradiated and stored in clinical condition for 28 days and the TBARS levels were measured. Data are mean ± SE ($n=3$). *$p<0.05$ and ***p 0.001; results are significantly different from controls, as evaluated by ANOVA test

phospholipid content in the initial period after blood collection and irradiation (days 3, 5, and 7) when comparing the control and irradiated samples, mostly from the third day onward (Fig. 2). Figure 2 shows the total phospholipid content of the red blood cells as a function of the storage time of the blood bags. For the non-irradiated samples, an initial decrease in the total content was observed, averaging 40.1 % by the 5th day and 46.5 % by the 9th day and remaining low from then on until the 28th day. A small peak was observed at day 11 when compared with the other days.

In the irradiated samples, the phospholipid content was reduced by approximately 35 % at day 7 of storage and increased 60.9 % on the 11th day. After day 14, the phospholipid content fell and was maintained at the same level until day 28.

As shown in Fig. 3, by the third day post-irradiation, there was a 36.0 % average decrease in the total cholesterol in the irradiated samples compared with the non-irradiated samples. On days 5 and 9, the total cholesterol of the irradiated samples increased by approximately 43.0 and 77.0 %, respectively. No

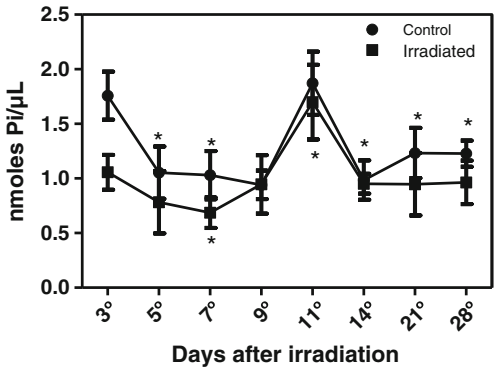

Fig. 2 Phospholipid analysis after storage and irradiation. RBCs were irradiated and stored in clinical condition for 28 days and the total levels of phospholipids were measured. Data are mean ± SE ($n=6$). *$p<0.05$; results are significantly different from controls, as evaluated by ANOVA test

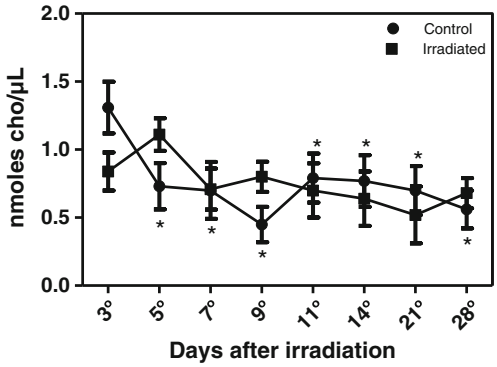

Fig. 3 Cholesterol analysis after storage and irradiation. RBCs were irradiated and stored in clinical condition for 28 days and the total levels of cholesterol were measured. Data are mean ± SE ($n=6$). *$p<0.05$; results are significantly different from controls, as evaluated by ANOVA test

significant changes were observed on the other days of the analysis. A significant decrease in the total cholesterol was observed in the non-irradiated samples, using day 3 as a control.

The Fe^{3+} content in the irradiated red cell concentrates did not show any major differences compared with the amount found in the non-irradiated samples or as a function of storage time. However, variations were observed in the Fe^{3+} content of residual plasma regardless of whether it was irradiated (Fig. 4). The irradiated samples showed markedly reduced Fe^{3+} content on days 5, 7, 9, 21 and 28 after irradiation compared with the controls. The non-irradiated samples presented a significant increase as a function of storage time from the 14th to the 28th day. The irradiated samples showed a decrease during the initial storage period and an increase by the 28th day.

No significant differences were observed in the catalase activity between the control and irradiated samples. However, the catalase levels significantly decreased after the 5th day post-irradiation, with a recovery to normal levels observed on the 11th and 28th day post-irradiation in both conditions (Fig. 5).

The protein profile of irradiated and non-irradiated erythrocyte membranes was very similar until the 21st day. However, on the 28th day, protein fragmentation was observed, and an additional band of approximately 28 kDa was observed in both the irradiated and non-irradiated samples (Fig. 6).

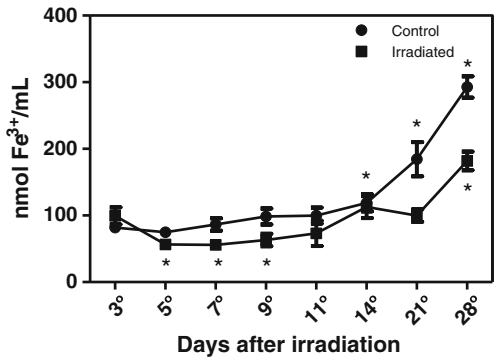

Fig. 4 Oxidized iron analysis after storage and irradiation. RBCs were irradiated and stored in clinical condition for 28 days and the Fe^{+3} levels were measured from residual plasma after centrifugation of red blood cells. Data are mean ± SE ($n=7$). *$p<0.05$; results are significantly different from controls, as evaluated by ANOVA test

Discussion

The rheological properties of erythrocytes are essential for their function as gas transporters, their movement through small capillaries, and many of their main characteristics, such as deformability and viscosity. The presence of ROS might impair these properties because changes in the protein and lipid composition of erythrocyte membranes could alter cell rheology [28, 29].

Variations in cholesterol and/or lipid content might influence ion transport and membrane permeability [30–32]. Alterations in the structure of cell membranes can occur due to ROS-induced lipid peroxidation. During the storage of transfusion blood bags, metabolic deterioration of erythrocytes due to lipid loss alters the protein content and the formation of microvesicles. These changes could affect the rheological

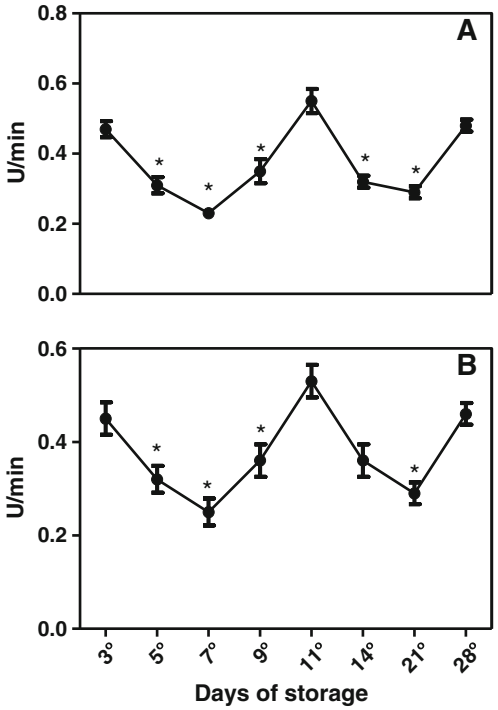

Fig. 5 Evaluation of catalase activity after storage and irradiation. Irradiated (**a**) or control (**b**) red blood cells were stored in clinical condition for 28 days and the catalase activity were measured. Data are mean ± SE ($n=3$). *$p<0.05$; results are significantly different from controls, as evaluated by ANOVA test. Considering U: 1 µmol of H_2O_2 decomposed/min

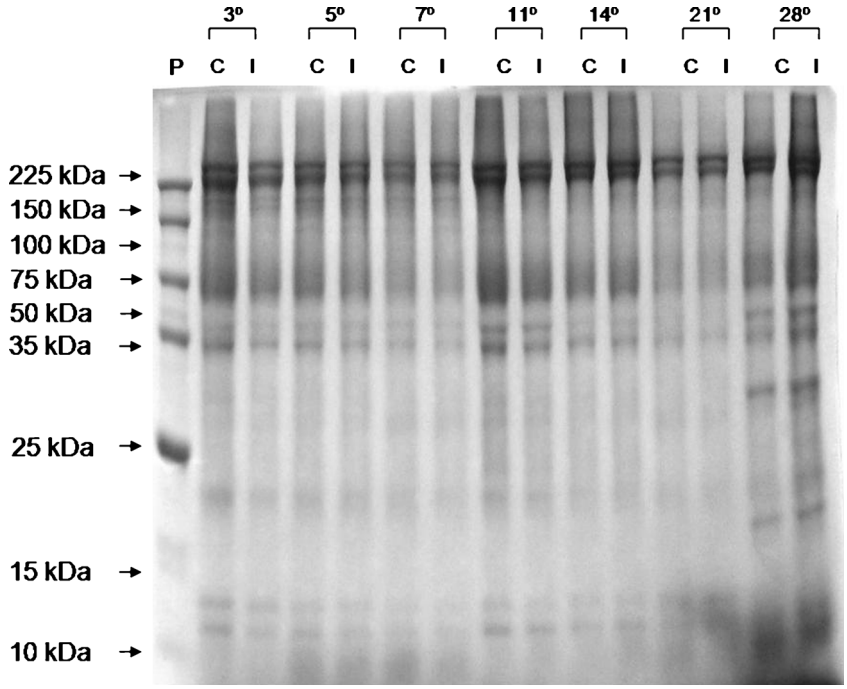

Fig. 6 Protein profile of red blood cells after storage and irradiation. Irradiated (*I*) or control (*C*) red blood cells were stored in clinical condition for 28 days. The proteins of ghost preparation were eletrophoretically separated in a SDS-PAGE gel of 10 %

properties and undermine the functional efficiency of the erythrocytes, as alterations in the elasticity of the membrane could hamper the passage of these cells through narrow capillaries, where the erythrocytes must circulate to perform their tasks [33].

In our assay, we observed several alterations in the membrane lipid content of erythrocytes from irradiated and non-irradiated blood concentrates. These alterations are most likely attempts to maintain the integrity of the membrane as a consequence of damage generated by lipid peroxidation, as suggested by our results in Figs. 1–4.

We found that alterations in red cell membrane lipids were not only due to gamma irradiation but were also a consequence of storage time. This result indicates once again that, at the pace that erythrocytes age, some events, such as the formation of microvesicles or lipid reorganization of the membrane, naturally occur as a cell attempts to maintain its functionality for a longer period. When erythrocytes are senescent or damaged, intracellular Ca^{2+} increases and activates the enzyme scramblase, which swaps phosphatidylserine from the inner to the outer leaflet of the membrane to signal to the macrophages that the cell is no longer viable [34]. Shifts in the cell membrane lipid composition might also alter the activity of the membrane pumps, such as Na^{+} and K^{+}-ATPase [24, 35].

We also observed a peak in the total lipid content on day 11. These changes are certainly an attempt to reorganize the membrane to maintain its integrity as a result of damage. The changes that occur in the lipid bilayer of the erythrocyte are not only due to exposure to radiation but are also a function of storage time, indicating once again that as the erythrocyte ages, some events happen naturally, such as the formation of microvesicles or the reorganization of the lipid membrane in an attempt to preserve function. Microvesicle release depends on microscopic physical properties of the membrane, such as lipid order, fluidity, and composition. Microvesicles are phospholipid-rich vesicles smaller than 0.5 μm that are emitted by red blood cells (RBCs) and many other cell types in vivo. Although microvesicle emission appears to be a controlled process, the functions of these vesicles are still unknown; they might serve as a means for viable cells to get rid of deleterious compounds that would otherwise induce cell lesions or target the cells for degradation. Lion et al. [36] found a significant increase in the formation of plasma membrane projections called microvesicles in stored erythrocytes and determined that this increase begins to accentuate starting at the tenth day of storage.

The red cell lipid bilayer, similar to any other plasma membrane, is enriched in phospholipids, which are prone to oxidation and are preferentially oxidized by the ROS formed by gamma irradiation. Lipid peroxidation of the erythrocyte membrane is characterized by an increase in the production of TBARS and malondialdehyde (MDA) as a consequence of the oxidation of phospholipids in the bilayer by ROS [37].

Benderitter et al. [38] reported that blood samples irradiated with doses of 2, 4, or 8 Gy (which are much lower than those used in clinics to prevent TA-GVHD) presented high levels of MDA after a post-irradiation period of 3 h. This result is indicative of lipid peroxidation, which, in marked contrast, was not observed after 3 days post-irradiation. Conversely, doses of up to 0.4 Gy are considered as conditioning doses because they do not generate products of lipid

peroxidation, and the use of a 2.7 Gy dose generated unwanted side effects on membranes and altered their fluidity.

In our assay, we observed that irradiated samples (25 Gy) presented more severe lipid peroxidation than samples not subjected to irradiation. This phenomenon was already observed on day 3 post-irradiation and was maintained until day 28 post-irradiation, with a significant difference between the irradiated and non-irradiated samples. This result provides evidence that alterations and perturbations in the lipid content are caused by free radicals generated by irradiation (Figs. 2 and 3).

Erythrocytes possess a variety of antioxidant defenses, including catalase and other enzymes that protect against ROS [38, 39]. Benderitter et al. [38] reported a decrease in erythrocyte catalase activity at 3-h post-irradiation with a dose of 8 Gy. As shown in Fig. 5, we also observed a marked decrease in catalase activity as a consequence of storage time after day 5. Nevertheless, we were unable to identify any significant differences attributable to the effects of gamma irradiation. Both the irradiated and non-irradiated samples presented a similar decrease and recovery of activity profiles with similar storage times.

The reduced (ferrous) iron of the heme groups in hemoglobin is another strong contributor to ROS generation because it can trigger the formation of free radicals through the Fenton reaction, giving rise to Fe^{3+}, a strong oxidant [13]. However, an intriguing result was obtained with residual plasma: The free metal concentration profile was similar for both groups (irradiated or not) as the storage time increased (Fig. 4). From day 14 on, an abrupt increase in the Fe^{3+} concentration was noted.

A higher Fe^{3+} content was expected in the irradiated samples, in agreement with the results from the TBARS assay (Fig. 1). The levels of TBARS were unaltered on the third and seventh days post-irradiation but were increased on the fifth day and on day 9 and later. However, we visualized higher values of TBARS on the third and seventh days, compared to the others days. This effect may be due to antioxidant mechanisms that are effective in the beginning of radiation exposure but are later depleted in an environment of sustained, high ROS levels. Glutathione is one of the most important antioxidant molecules in erythrocytes, and adequate levels of nicotinamide adenine dinucleotide phosphate (NADPH) are important for the maintenance of reduced glutathione levels [40]. NADPH is generated by the pentose phosphate pathway metabolism through ATP-dependent processes [41]. It is possible that in the early stages of ROS generation, the levels of glutathione, and NADPH were high enough to prevent lipid peroxidation. The depletion of glutathione and NADPH caused an increase in the TBARS levels on the fifth day post-irradiation; however, sustained ROS generation modulated NADPH synthesis, leading to a decrease in TBARS on the seventh day post-irradiation. However, this modulation cannot be sustained by the erythrocyte, and the depletion of glutathione and NADPH caused a sustained increase in TBARS on the ninth day post-irradiation and later.

Nevertheless, we obtained a divergent result that led us to believe that non-irradiated samples might have greater protection against ROS, especially considering that a higher Fe^{3+} content would make the cells more prone to lipid peroxidation, which was not observed. Apart from catalase and its Fe^{3+}-protective effect, other antioxidant mechanisms present in the red cells might become activated by irradiation.

Lipid peroxidation directly affects membrane fluidity, a paramount feature that maintains conditions that are ideal for erythrocyte function. Membrane fluidity ensures homeostatic control of the flux of O_2, H_2O, and ions, such as Na^+, K^+, and Ca^{2+} (among others), through the membrane, ensuring equilibrium between the intracellular and extracellular milieus.

During the early days of storage, the concentration of extracellular K^+ might increase considerably [12, 35]. This increase might potentially be dangerous to newborns and patients with cardiac complications, increasing the risk of arrhythmia [42].

We also observed alterations in the protein profile of the red cell membranes, although these alterations were apparently more closely related to cell storage time and aging than to irradiation (Fig. 6). This alteration became more apparent on the 28th day, as shown by the appearance of an additional band at ≈28 kDa. This band might represent one of the β subunits of spectrin [43], which is one of the structural proteins that form the junctional complex that contributes to the stabilization of the red cell membrane. Fragmentation of this protein most likely contributes to the alterations in the structure and fluidity of membranes, thereby perturbing the roles of membrane-embedded proteins, such as Na, K-ATPase, which was previously demonstrated to be affected by irradiation [35].

In conclusion, although gamma irradiation is the main agent used for the prevention of TA-GVHD, a better understanding of the eventual collateral effects on red blood cell viability and the physical properties of the cell membrane is required, as gamma irradiation causes a physical modification of red blood cell viscosity and other membrane parameters. The changes in the biochemical properties of erythrocytes must also be characterized to better assess their viability and to issue more secure recommendations regarding the shelf life of blood bags and the safe use of the irradiated red cells therein.

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