Influence of Pre-Storage Irradiation on the Oxidative Stress Markers, Membrane Integrity, Size and Shape of the Cold Stored Red Blood Cells

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Keywords

Red blood cell · Gamma irradiation · Storage · Oxygen-free radical · Flow cytometry

Summary

Background: To investigate the extent of oxidative damage and changes in morphology of manually isolated red blood cells (RBCs) from whole blood, cold stored (up to 20 days) in polystyrene tubes and subjected to pre-storage irradiation (50 Gy) and to compare the properties of SAGM-preserved RBCs stored under experimental conditions (polystyrene tubes) with RBCs from standard blood bag storage. **Methods:** The percentage of hemolysis as well as the extracellular activity of LDH, thiobarbituric acid-reactive substances, reduced glutathione (GSH), and total antioxidant capacity (TAC) were measured. Changes in the topology of RBC membrane, shape, and size were evaluated by flow cytometry and judged against microscopy images. **Results:** Irradiation caused significant LDH release as well as increased hemolysis and lipid peroxidation, GSH depletion, and reduction of TAC. Prolonged storage of irradiated RBCs resulted in phosphatidylserine exposure on the cell surface. By day 20, approximately 60% of RBCs displayed non-discoid shape. We did not notice significant differences in percentage of altered cells and cell volume between RBCs exposed to irradiation and those not exposed. **Conclusion:** Irradiation of RBC transfusion units with a dose of 50 Gy should be avoided. For research purposes such as studying the role of antioxidants, storage of small volumes of RBCs derived from the same donor would be more useful, cheaper, and blood-saving.

Introduction

Aging of red blood cells (RBCs) is characterized by accumulation of structural, metabolic, and functional modifications. RBC shrinkage, membrane remodeling, microvesiculation, and exposure of surface removal markers that triggers erythrophagocytosis are some of the typical changes occurring in senescent cells. Powerful removal signals include externalization of phosphatidylserine (PS) and the binding of autologous immunoglobulin G (IgG) to senescence-specific neoantigens that originate from structural changes in band 3 protein. The RBC aging is associated with an apoptosis-like programmed cell death that could be induced by Ca^{2+} influx and prevented by calpain and caspase inhibitors [1]. The suicidal death may be elicited by several cell stressors, including osmotic shock, oxidative stress, and energy depletion [2].

It has been generally accepted that under blood bank conditions RBCs undergo major biochemical and mechanical changes referred to as 'RBC storage lesion' which could affect their after-transfusion performance. During storage the RBC membrane undergoes various modifications such as lipid peroxidation, PS externalization, decline of critical antigenic markers, protein aggregation, membrane-hemoglobin (Hb) association and oxidation, which are accompanied by an increase of intracellular calcium and metabolic depletion [1, 3]. Several of these factors are potent regulators of membrane skeletal organization [4]. Therefore, events that progressively occur in stored RBCs include defective deformability, loss of the surface area, spherocytosis transformation, and microvesiculation leading to hemolysis of a subpopulation of the oldest/damaged RBCs. It has been suggested that erythrocyte physiological aging may be accelerated by storage conditions [5]. Among several hypotheses, the oxidative stress/free radical theory offers the best mechanistic elucidation of in vitro aging of RBCs [6].

Gamma irradiation of the RBC transfusion units (25–50 Gy) is the procedure of choice to prevent transfusion-associated graft-versus-host disease (TA-GvHD) [7, 8]. However, when RBCs are irradiated, a notable alteration of their properties has been noted accounting for a shortened RBC transfusion unit shelf life [9–12]. Studies have demonstrated that irradiation of RBCs enhanced the degree of lipid peroxidation and oxidative protein damage in the membranes of stored RBCs [13–16]. Consequently, irradiation can affect membrane integrity, accelerate leakage of K⁺ ions, LDH and Hb, and reduce ATP and other purine nucleotides, resulting in decreased recovery of transfused RBCs [11, 17–19]. These changes suggest that some portions of additional RBC damage may occur or become evident after extended storage following irradiation [20]. Mechanisms responsible for these lesions have not been fully understood. On the other hand, studies conducted by Cicha et al. [21] showed that lipid peroxidation was not significantly increased during the storage of RBCs and by irradiation (35–50 Gy). Generally, numerous studies have focused on the lesions in RBC transfusion units accumulating over increasing storage time but relatively less attention has been devoted to the RBC storage lesions caused by pre-storage irradiation.

The aim of the present study was to investigate whether gamma irradiation at a dose of 50 Gy, and if so to which extent, could induce oxidative damage, membrane alterations, and morphological changes in RBCs, manually isolated from citrate-phosphate-dextrose(CPD)-preserved whole blood and stored for up to 20 days at 4 ± 2 °C. The RBCs were irradiated at the first day after collection (pre-storage irradiation) and stored in polystyrene tubes. The percentage of hemolysis and extracellular LDH activity served as determinants of the membrane damage. The amount of the thiobarbituric acid-reactive substances (TBARS) and total antioxidant capacity (TAC) were measured to assess the extent of membrane lipid peroxidation and oxidative stress/antioxidant balance in the RBC medium, respectively. The concentration of non-protein thiols (glutathione; GSH) was estimated as a marker of the endogenous antioxidant defense. Changes of the asymmetry of the RBC membrane phospholipid bilayer were evaluated by measurement the amount of PS exposure on the outer leaflet. Additionally, morphology-related forward scatter (FSC) and side scatter (SSC) parameters measured, and phase contrast microscopic observations were conducted. These methods have largely been used by others to show the progression of cell injury in irradiated RBCs. Nevertheless, the published findings are fragmentary; the extent of radiation-induced changes significantly differs between the studies due to the variety of experimental conditions. Different irradiation doses and variability of preserve/additive solutions make the data difficult to compare.

The novelty of the present study is that we used a broad panel of in vitro assays in the same study to assess the impact of a high radiation dose (50 Gy). We focused on the selection of some reliable indices which could be used as biomarkers of the in vitro aging of RBCs. Examining irradiation and storage injury in this model system would be helpful for understanding the mechanisms of the in vitro senescence of RBCs. This is of high importance for the devel-

opment of storage conditions in blood banks by increasing the time of viability of stored RBCs [22]. One approach to improve the RBC quality during storage is to reduce oxidative stress by addition of antioxidants. Using this model system, we have recently shown a protective effect of trolox (manuscript in press) and to less extent quercetin [23] against oxidative damage of RBCs cold stored for up to 20 days. Although storage of RBCs, manually isolated from CPD-preserved whole blood, in plastic tubes does not represent a prevalent method utilized in blood banks, the usage of RBCs derived from one donor for screening different antioxidants in broad concentration ranges seems to be advantageous.

The model system produces worse conditions than the standard blood bags. The additional aim of the present study was to examine the impact that containers had on the in vitro quality of the blood bank-manufactured saline-adenine-glucose-mannitol(SAGM)-preserved RBCs. The features (hemolysis rate, LDH activity, lipid peroxidation level, and TAC) of the RBC aliquot taken from the transfusion unit and stored in a polystyrene tube were compared with conventional blood bag storage of the unit.

Material and Methods

Material

Human blood from 18 single donors and SAGM-preserved RBC transfusion units derived from 3 donors were obtained from the Regional Center for Transfusion Medicine in Lodz (Poland). Blood was collected in CPD anticoagulant preservative solution. This study was approved by the local Ethics Committee (no KBBN-UL/I/4/2011).

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), hydrogen peroxide, nicotinamide adenine dinucleotide (NADH), perchloric acid (PCA), potassium persulfate, sodium pyruvate, and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Chemical Co. (Warsaw, Poland). Annexin V-FITC Apoptosis Kit was obtained from BioVision, Inc. (Milpitas, CA, USA). Drabkin's reagent was purchased from Aqua-Med (Lodz, Poland). Other chemicals, all of the analytical grade, were obtained from POCh (Gliwice, Poland).

Preparation of RBC Suspensions and Storage Conditions

RBCs were separated from plasma and leukocytes by centrifugation (2,800 × g for 10 min), then the RBC concentrate of 70% hematocrit (Ht) was prepared by addition of the autologous plasma to the packed cells. From the RBC suspensions (Ht = 70%), two equal aliquots (volume 6 ml) were taken and placed into polystyrene tubes; one was non-irradiated, the other one was used to test the effects of the 50 Gy irradiation dose. The irradiated and non-irradiated RBC concentrates were stored at 4–6 °C, and RBC samples were analyzed after 1-, 10- and 20-day storage. Before the assays, the taken aliquots of RBCs were washed three times with phosphate-buffered saline (PBS) (140 mmol/l NaCl in 10 mmol/l sodium phosphate, pH 7.4).

The blood bank-manufactured, SAGM-preserved RBC transfusion units (leukocytes < 10⁶/unit) received from 3 donors (n = 3) were used to examine the impact of containers on the in vitro quality of the stored RBCs. Samples (6 ml) were removed aseptically from each RBC unit and placed in polystyrene tubes. RBCs in blood bags and in tubes were stored under the same conditions (4 ± 2 °C). The RBC samples were analyzed after 1-, 10-, 20- and 30-day storage.

Gamma Irradiation

The RBC concentrates (Ht = 70%) were irradiated with a dose of 50 Gy at the Institute of Applied Radiation Chemistry (Technical University, Lodz, Po-

land) using a ^{60}Co source (dose rate 0.392 ± 0.027 Gy/min; first category Irradiator BK-10000 ZZUJ Polon, Poznan, Poland). The dosimetry was performed by using alanine dosimeters ES200–2106 (Bruker, Poznan, Poland) and radi-ochromic film dosimeters B3000 (Gex Corporation, Centennial, CO, USA).

Hemolysis Measurement

The rate of hemolysis was calculated based on the measurement of Hb released from the cells, relatively to the total amount of Hb in the RBC suspension. Free Hb concentration was determined by the cyano hemoglobin method using Drabkin's reagent. The percentage of hemolysis was calculated as described previously [24] using the following formula: % hemolysis = (the measured free Hb concentration (g/dl) $\times$ supernatant volume (dl) / total Hb (g)) $\times$ 100.

LDH Activity Measurement

Extracellular LDH activity was measured by following a decrease in absorbance at a wavelength of $\lambda = 340$ nm (Spectrophotometer Ultraviolet/Visible (UV/Vis) Helios alpha Unicam, Cambridge, UK) resulting from the NADH oxidation [25]. To 0.5 ml of the RBC suspension an equal volume of PBS was added, and the suspension was centrifuged at $1,800 \times g$ for 5 min. The supernatant was collected for further study. LDH activity was calculated in international units per liter (IU/l).

Lipid Peroxidation Measurement

Lipid peroxidation was quantified by measuring the concentration of the TBARS [26]. Briefly, equal volumes of the RBC suspension (Ht = 4%), 15% (m/v) trichloroacetic acid (TCA) containing 0.25 mol/l HCl, and 0.375% (m/v) thiobarbituric acid (TBA) containing 0.25 mol/l HCl were mixed, incubated at 95°C for 10 min, and cooled. The sample was centrifuged at $6,000 \times g$ for 20 min, and absorbance was measured at 535 nm (Spectrophotometer UV/Vis Helios alpha). The TBARS concentration was calculated using the molar extinction coefficient ($\epsilon = 156,000 \text{ M}^{-1} \text{ cm}^{-1}$).

Total Antioxidant Capacity Measurement

TAC was estimated by the method of Erel [27]. ABTS $^{+}$ stock solution (10 mmol/l) was prepared in 30 mmol/l acetate buffer, pH 3.6, containing 2 mmol/l H_2O_2 . The TAC assay was performed in a 96-well plate. Briefly, 200 μl of 0.4 mol/l acetate buffer, pH 5.8, was mixed with 5 μl of the supernatant (RBC medium) or trolox standard (final concentration of 0.063–0.5 mmol/l), and the first absorbance of the assay was taken at 414 nm (as sample blank, A_0) (SPECTROstarNano, BMG LABTECH, Ortenberg, Germany). Alternatively, 205 μl of the acetate buffer was taken as a blank (to evaluate a spontaneous ABTS $^{+}$ discoloration rate). Then, 20 μl of the 10 mmol/l ABTS $^{+}$ stock solution was added to each well and the last absorbance at 414 nm (A) was taken at the end of the incubation period (5 min after the mixing). $A - A_0$ was calculated for samples and trolox standards; TAC expressed in mmol trolox equivalent/l, was calculated based on the standard curve ($A - A_0$ as a function of concentration of standard trolox solution).

Glutathione and Total Non-Protein Thiol Measurement

Concentrations of total non-protein thiols (SH groups) in RBCs and plasma (RBC medium) were measured spectrophotometrically (Spectrophotometer UV/Vis Helios alpha) with Ellman's reagent [28]. Briefly, an aliquot of 100 μl of 25% TCA was added to 900 μl of RBC suspension (Ht = 2%) or plasma and centrifuged (5 min, $10,000 \times g$). Then, 250 μl of the supernatant was mixed with 750 μl of 0.5 mol/l sodium phosphate buffer, pH 8.0 and 50 μl of the Ellman's reagent (10 mmol/l DTNB in 0.5 mol/l sodium phosphate buffer, pH 8.0). After incubation (20 min in the dark at 37°C) the absorbances were recorded at 412 nm. The thiol concentration was calculated using the molar extinction coefficient ($\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$).

Reduced GSH concentration was determined according to the modified published high performance liquid chromatography (HPLC) procedure [29]. Briefly, 150 μl of RBC suspension was diluted with 150 μl of 0.2 mol/l phosphate buffer, pH 7.5 and derivatized with 10 μl of 0.1 mol/l 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) at room temperature. After 3 min reaction

mixture was acidified with 150 μl of 3 mol/l perchloric acid (PCA) followed by centrifugation ($12,000 \times g$, 10 min). A 10 μl of supernatant was transferred into the HPLC system (Hewlett-Packard 1100 Series system, Waldbronn, Germany).

Flow Cytometry

The RBC concentrates were analyzed by flow cytometry (LSR II; Becton Dickinson, San Jose, CA, USA). The flow cytometry gate on the RBCs has been established for data acquisition, and the cell size and shape were determined with simultaneous separate detection of low angle (FSC-A) and right angle (SSC-A) light scattering. The light scattered near the forward direction (low angle) is expected to be proportional to the size (volume) of the particle (cell), whereas scattering at the right angle depends on the cell shape (internal properties of the scattered particles). The data were recorded for a total of 30,000 events per sample.

Measurement of Phosphatidylserine Externalization

The level of PS exposure on the outside of RBCs was measured using an annexin V-FITC assay, according to the procedure given by the manufacturer (Annexin V-FITC Apoptosis Kit, Biovision, Inc). Briefly, the RBCs were resuspended in annexin binding buffer (Ht = 0.05%) and then stained with annexin V-FITC from human placenta at a dilution of 1:250. After 15 min, the intensity of fluorescence was measured in 30,000 cells using flow cytometer (LSR II), with excitation of 488 nm and a 530 nm emission filter. Data was analyzed using Flowing Software ver. 2.5.0 (Perttu Terho, Turku, Finland).

Phase Contrast Microscopy and Cell Counting

The RBC concentrates were diluted to a Ht of 2% with 0.1% glutaraldehyde and placed in Petri dishes. Then, the samples were observed at the magnification of 600 using phase contrast microscope (Olympus IX 70, Tokyo, Japan). In addition, the percentages of shape-changed cells were evaluated by counting approximately 350 RBCs in randomly chosen fields.

Statistical Analysis

The data points in figures are the means of 3–18 independent experiments, each performed in triplicate. The data were analyzed using statistical software 'Statistica' ver.10 (StatSoft Inc., Tulsa, OK, USA). The results were tested for normal distribution by means of the Kolmogorov-Smirnov test. The differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or by the non-parametric Kruskal-Wallis test. The significance of differences between the mean values of irradiated versus appropriate non-irradiated samples was analyzed by paired Student's t-test or by the non-parametric Wilcoxon test. A level $p < 0.05$ was accepted as statistically significant.

Results

Radiation-Induced Changes in RBC Membrane Integrity and Oxidative Stress Markers

At day 1 of storage, no significant differences in the degree of hemolysis and lipid peroxidation levels between RBCs exposed to gamma irradiation versus those not exposed could be observed; on the other hand, irradiation resulted in elevation of the LDH activity and TAC depletion by approximately 20 and 25% ($p < 0.05$), respectively; (fig. 1). Importantly, the effect of pre-storage irradiation was more prominent after longer storage. Both at 10 and 20 post-irradiation days the percentage of hemolysis, LDH release, and TBARS levels were significantly higher in RBC exposed to gamma irradiation (by almost 50, 30 and 25–30%, respectively, at day 10 ($p < 0.05$, $p < 0.01$, $p < 0.05$) and day 20 post irradiation ($p < 0.001$,

Fig. 1. Changes in the percentage of hemolysis (**A**), extracellular LDH activity (**B**), TBARS concentration (**C**), and TAC (**D**) of control and gamma-irradiated (50 Gy) RBCs during cold long-term storage. Values are given as mean $\pm$ SD of 18 independent experiments in each group (n = 18); (square bracket) non-irradiated versus irradiated at the appropriate day of storage; **p < 0.01, ***p < 0.001 compared to non-irradiated at 1 day; ##p < 0.001 compared to irradiated at 1 day, n = a number of donors.

p < 0.001 and p < 0.01)), when compared to those not irradiated. At the beginning and at the end of storage, TAC in the supernatant was constantly lower by approximately 25% in irradiated than in non-irradiated RBCs (day 1 post irradiation p < 0.05; (at day 10 and 20 post irradiation p < 0.01).

Our results showed a significant storage time-dependent increase of hemolysis rate, extracellular LDH activity, and TBARS concentration as well as a decrease of TAC in the supernatant. At day 20 of storage the percentage of hemolysis, LDH activity and TBARS levels raised 4- (p < 0.001), 5- (p < 0.001) and more than 3-fold (p < 0.01) in non-irradiated RBCs, respectively, when compared to appropriate controls (day 1). At the same storage period approximately 2.5-fold (p < 0.01) lower TAC levels were noted in the supernatant of non-irradiated RBCs.

In the RBCs GSH represents more than 98% of the all low-molecular-weight thiols, and therefore determination of their concentration with Ellman's reagent, which reacts with all non-protein cellular thiols, can be approximately related to the concentration of GSH. Our results showed a small but significant decrease of GSH concentration in RBCs subjected to 50 Gy irradiation when compared to non-irradiated cells (by approximately 5 and 10% at day 1 and 10, respectively (p < 0.05)) (fig. 2A). Moreover, a decline of the GSH levels over storage time in both non-irradiated and irradiated RBCs was noted (by approximately 30% at day 20 compared to appropriate controls at the first storage day (p < 0.001)). Due to its small size, GSH can leak from the cell interior to the external environment. To check if this is the case, the concentration of GSH was also measured in the supernatant of the stored RBCs (total plasma non-protein thiols) using Ellman's reagent (fig. 2B). At the beginning of the storage, the concentration of GSH in the RBC medium was low, but quadrupled (p < 0.05) until the end of storage (20 days). In irradiated RBCs, the GSH levels tended to be slightly higher compared to those not exposed to irradiation, but there were no significant differences between groups.

The spectrophotometric measurement of GSH can be affected by errors caused by the non-specificity of the reaction. Therefore, GSH measurement was performed using HPLC technique. The data calculated as percent of control, where the amount of GSH in non-irradiated RBCs at day 1 of storage was taken as 100%, are presented in figure 2C. Before irradiation, the intracellular GSH level in control RBCs (at day 1) was 24.1 ± 6.2 nmol/ml. A dramatic decrease in GSH levels over storage time was noted (by almost 95 and 97% at day 10 and 20, respectively; p < 0.001).

Effects of Pre-Storage Irradiation on Phosphatidylserine Externalization, RBC Size and Shape

Changes in the position of PS (measured by the binding of FITC-labeled annexin V) in the RBC membrane is shown in figure 3A. The percentage of PS-exposing RBCs increased with storage time. Only at day 20 there were significant differences in PS exposure between gamma-irradiated versus non-irradiated RBCs; an irradiation-induced PS exposure by 10% (p < 0.05) was seen.

A flow cytometry method was used to analyze size and shape of the RBCs. Quantitative alterations in SSC-A and FSC-A parameters are shown in figure 3B and C. Control samples (fresh RBCs) were not older than 6 h and not treated with low temperature. The analysis of the FSC-A parameter (proportional to the volume of the particles) revealed alterations in the shape of RBCs after longer storage (day 10 and 20). We observed an increase of the FSC parameter compared to fresh RBCs by 6 and 12% at day 10 and 20, respectively (p < 0.05). The SSC parameter (which is dependent on cell shape and internal properties) was changed over storage time (by 10, 18 and 22% compared to fresh RBCs at day 1 (p < 0.01), 10 (p < 0.001) and 20 (p < 0.001), respectively). We did not observe any significant effects of irradiation on both parameters.

To confirm the results obtained by flow cytometry, changes of the RBC morphology were also analyzed using contrast phase microscopy. The collected images of the non-irradiated and irradiated

Fig. 2. Changes in total RBC non-protein thiols (**A**), total plasma non-protein thiols (**B**) and GSH concentration (**C**) of control and gamma-irradiated (50 Gy) RBCs during cold long-term storage. Values are given as mean $\pm$ SD of 18, 3 and 4 independent experiments in each group ($n = 18$, $n = 3$ and $n = 4$), respectively; (square bracket) non-irradiated versus irradiated at the appropriate day of storage; * $p < 0.05$, *** $p < 0.001$ compared to non-irradiated at 1 day; * $p < 0.05$, *** $p < 0.001$ compared to irradiated at 1 day, $n =$ a number of donors.

RBCs at the same day of storage were very similar. A typical image is presented in figure 4. Additionally, the percentage of non-beconcave disc-shaped (shape-changed) RBCs was calculated. Results are reported as mean $\pm$ SD of non-discoid cells given as percentages of the overall number of cells taken into account in randomly chosen fields (fig. 3D). The number of altered, non-discoid cells significantly increased over storage time. At the end of storage (day 20) a twofold higher amount of changed RBCs was measured when compared to day 1 ($p < 0.001$). We did not observe any significant differences in the percentage of altered cells between RBCs exposed to irradiation and those not.

Storage of the SAGM-Preserved RBCs – Standard Blood Bags versus Tubes

A comparison of the selected indices of the blood bank-manufactured-SAGM-preserved RBCs during storage in blood bags or polystyrene tubes is shown in figure 5. The mean rate of hemolysis, the LDH release, and the lipid peroxidation were significantly higher in RBCs that had been stored in tubes than in those stored in blood bags; at the end of storage period (day 30), the rate of hemolysis was almost doubled ($p < 0.001$), whereas both LDH activity and TBARS levels were decreased by more than 50% ($p < 0.01$). In case of TAC of the supernatants, no differences could be detected.

Discussion

In the present study, the extent of oxidative damage and changes in morphology of the cold-stored RBCs subjected to pre-storage irradiation with a maximal recommended dose of 50 Gy have been evaluated. RBCs that had been manually isolated from whole blood and stored in polystyrene tubes were used. In our study the most evident early irradiation-induced changes noted at first day of storage include LDH release from the RBCs and reduction of TAC in the supernatant. Other modifications caused by irradiation such as increased hemolysis and lipid peroxidation appeared after longer storage (fig. 1). Hemolysis is a very important parameter for assessing the quality of stored RBCs [3]. In RBC transfusion units hemolysis is manifested by the presence of free Hb in RBC suspending media. The hemolysis rate increases with time of storage. The United States Food and Drug Administration (FDA) has approved and licensed additive solutions for long-term storage of RBC units, with less than 1% hemolysis at the end of the storage period. In contrast to the FDA, the official guideline in Europe for hemolysis in RBC units is 0.8% [24, 30]. Abnormal hemolysis in an individual RBC unit can be caused by different factors (inappropriate handling during processing or storage conditions, bacterial contamination) [24]. Our results show that gamma irradiation further enhanced Hb leakage to the medium. These data are consistent with other findings that irradiation (with a dose lower than in our study; 25 Gy), filtration, and combined irradiation and filtration before storage can cause significant damage in RBCs and intensify the RBC storage lesions such as increased free Hb and K^+ [31]. Similar to our results, the increase in free Hb plasma levels in the irradiated RBC units (30 Gy) was found less evident during the first days of storage [7].

The present study shows that, concomitant with an increased rate of hemolysis, pre-storage irradiation resulted in a significant LDH release and lipid peroxidation. These events were already seen and widely discussed in our earlier studies [14, 23]. During storage, oxidative damage to lipids and proteins contributes to RBC injury. An irradiation-induced increase in hemolysis, LDH release, and lipid peroxidation may be indirectly associated with reactive oxygen species (ROS) which have an impact on the RBC membrane. Production of these harmful, highly reactive molecules

Fig. 3. Changes in exposition of PS (**A**), parameter SSC (**B**), parameter FSC (**C**) and shape change (**D**) of control and gamma-irradiated (50 Gy) RBCs during cold long-term storage. Values are given as mean $\pm$ SD of $n = 6$ independent experiments in each group ($n = 6$); (square bracket) non-irradiated versus irradiated at the appropriate day of storage; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to non-irradiated at 1 day; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to irradiated at 1 day, $n =$ a number of donors.

can lead to peroxidation of membrane lipids, oxidation of membrane-bound proteins, and consequently to increased membrane permeability and/or disruption. Irradiation was shown to reduce the RBC deformability and rendered them more susceptible to metal-catalyzed oxidative stress [32]. The ROS are formed in irradiated blood products, but their exact source is not known. Gamma irradiation in aqueous media results in the generation of ROS. Moreover, packed RBC units contain some white blood cells that might contribute to ROS formation. The release of leukocyte-associated enzymes and cytokines has been associated with the RBC storage lesions, and macrophages are capable of generating ROS once activated [1].

Direct correlations of the lipid peroxidation marker malondialdehyde (MDA) and Hb oxidation with membrane damage, as reflected by increasing plasma Hb concentrations in RBC units over the storage period of 28 days were noted [33]. The increase in Hb oxidation during storage of RBCs could be due to a decrease in their antioxidant capacity, resulting in oxidation and deterioration of membrane lipids and proteins which can ultimately lead to irreversible damage to the membrane. In our study a storage-dependent decrease of the TAC, most likely due to the reduction/loss of the endogenous RBC antioxidant defense, was noticed (fig. 1D). Irradiated RBCs are exposed to an additional oxidative stress – directly from the ROS produced by irradiation and indirectly from the iron in the heme (released from a free Hb) due to the Fenton reaction. As a result, we observed a drop of TAC just after irradiation. Interestingly, the reduced TAC in the irradiated versus non-irradiated RBCs was kept until the end of the storage. Since the RBCs are constantly exposed to ROS produced by Hb, the oxygen carrier protein that undergoes auto-oxidation to produce the superoxide anion, the antioxidant system have to eliminate such oxidants. Therefore, a level of oxidative stress in stored blood depends

on increased production of ROS and secondary radicals derived from their reaction with vital cellular molecules (lipids, proteins) as well as a decreased antioxidant defense system [34]. Studies by Ogunro et al. [35] on the effects of RBC storage duration on the RBC antioxidant defense system have shown a decrease in the plasma total antioxidant status (by 27% at day 20 compared to day 1). In addition, GSH peroxidase, superoxide dismutase, and catalase activities decreased at day 15–20 (by above 17%, 17% and 12%, respectively).

Reduced GSH is generally considered to be the most robust antioxidant in RBCs. GSH protects membrane proteins and preserves their stability. Decreased levels of GSH could result in oxidation of membrane SH groups and loss of membrane stability. Since the main source of ROS in RBCs is Hb, if erythrocytes are depleted of GSH, the release of iron is accompanied by lipid peroxidation and hemolysis. Maintaining cellular GSH in banked RBCs protected membrane proteins from oxidative damage [36]. Our data show irradiation- and storage-induced GSH depletion (fig. 2A). Moreover, the HPLC analysis revealed almost total loss of GSH (by 95%) already after 10 days of storage (fig. 2C). These differences could arise from the specificity of the methods. A high background in a colorimetric method can result from the presence of other low-molecular thiols, i.e. cysteine and homocysteine that react with DTNB (approximately 2% of total) and from the nonspecific reaction products. The reliability of thiol determination with Ellman's method can be influenced by some factors. It has been shown that GSH reductase affects the background signal by the slow reduction of DTNB [37]. Reducing agents, such as ascorbic acid, mercaptoethanol, dithiothreitol and cysteine, or thiol reactive compounds could interfere with the DTNB assay as well [38]. Decreased GSH levels could be due to the leakage and/or oxidation to GSSG (oxidized GSH). Since we observed some amounts of GSH in the RBC

Fig. 4. A micrograph of the fresh (**A**) and long-term cold stored RBCs at 1 day (**B**), 10 day (**C**), and 20 day (**D**) of storage. Microscope magnification $\times 600$.

medium (plasma) that increased over storage time (fig. 2B), it can be suggested that to some extent GSH release is involved in the depletion process. GSH oxidation could be a result of a direct oxidative degradation and/or due to repair processes requiring GSH, such as the reduction of oxidized membrane protein thiol groups [13].

During storage morphological changes occur in RBCs that results from remarkable RBC membrane remodeling and vesicular formation. As in other cells, phospholipids are asymmetrically distributed across the RBC membrane. PS is mainly localized in the cytoplasmic membrane leaflet, but during RBC ageing it flip-flops to the external leaflet – a process that is increased in certain pathological conditions (β -thalassemia). Mature, enucleated RBCs do not undergo classical apoptosis, they experience PS externalization which is thought to be a major signal for phagocytosis by macrophages and removal from the circulation [39]. Our studies indicate a progressive time-dependent externalization of PS in stored RBCs which can reduce their post-transfusion survival. The effect of pre-storage irradiation was not noticeable up to day 20 of storage (fig. 3A). Studies conducted by Dinkla et al. [40] on the donor-dependent variations of the PS exposure on stored RBCs showed an increased PS exposure that was found to be associated with hemolysis and vesicle concentration. The percentage of PS-exposing RBCs was positively correlated with the free Hb plasma level of the donor. In consistence with our results, it was demonstrated that prolonged storage (up to 45 days) of the RBCs irradiated with a

cobalt teletherapy unit (30 and 40 Gy) resulted in loss of membrane phospholipid asymmetry, exposing PS on the cells' surface in a time- and dose-dependent manner. The magnitude of this effect did not seem to be clinically relevant [7].

Morphological changes were evaluated using flow cytometry and contrast phase microscopy. We could notice that by day 20 approximately 60% of RBCs displayed non-discoid shape, and 1 day storage at low temperature resulted in twice as much shape-changed cells when compared to fresh RBCs (fig. 3D). Part of these changes most likely would be reversible after warming. Changes of the SSC parameter were in parallel to the results obtained from cell counting (fig. 3A). An increase in RBC volume was found to be the most obvious at day 20 of storage (fig. 3C). However, no significant differences were found in morphology parameters between RBCs subjected to pre-storage irradiation and those not. Progressive, storage-induced morphological changes were also reported by Blasi et al. [41]. Ran et al. [31] using confocal laser scanning microscopy reported disappearance of the normal structure of RBCs with increasing storage time and appearance of cells with thin sheet morphology and shrinkage, with a large number of pseudopodia, debris, and impurities. This effect was enhanced in the groups treated with irradiation.

Usage of the polyvinyl chloride blood bags plasticized with di(2-ethylhexyl)phthalate guarantee reduction of the rate of hemolysis during RBC storage [3]. As expected, the percentage of hemolysis and LDH activity in the supernatant of the SAGM-pre-

Fig. 5. Comparison of the percentage of hemolysis (**A**), extracellular LDH activity (**B**), TBARS concentration (**C**), and TAC (**D**) between SAGM-preserved RBC transfusion units cold stored in blood bags and samples (6 ml) of the same RBCs stored in polystyrene tubes. Values are given as mean $\pm$ SD of 3 independent experiments in each group ($n = 3$); (square bracket) non-irradiated versus irradiated at the appropriate day of storage; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to non-irradiated at 1 day; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to irradiated at 1 day, $n =$ a number of RBC transfusion units.

served RBCs stored in plastic tubes were significantly higher compared to that of stored in blood bags (fig. 5A, B). Interestingly, lipid peroxidation was also elevated in our experimental conditions (tubes). Considering that the TAC level was similar in both RBCs, this could be explained by better oxygen availability in the small volume (fig. 5C, D).

In conclusion, our results suggest that irradiation of RBC transfusion units with a dose of 50 Gy should be avoided. Although, storage of RBCs manually isolated from CPD-preserved whole blood in plastic tubes does not represent a prevalent method utilized in blood banks and used in transfusion medicine, our model system allowed to assess the net effect of an irradiation dose; many indices of in vitro quality of the 50 Gy irradiated RBCs were compared with those of non-irradiated RBCs stored the same way. The current time limit for banked RBC transfusion units (42 days for non-irradiated and 28 days for irradiated units) is determined mainly by the life span of RBCs in the circulation and by alterations in several biochemical parameters (pH, free Hb, ATP and 2,3-diphosphoglycerate levels, hemolysis rate). Our results imply that parallel measurement of additional indices such as LDH, lipid peroxidation, and other oxidative stress markers would provide

better control of the quality of stored RBC units, especially of those exposed to irradiation. In addition, better RBC storage would require oxidative stress reduction. Therefore, the role of antioxidants in the prevention of the deleterious effects of storage deserves investigation. Although quantitative changes between RBCs stored under our experimental storage conditions and conventionally stored RBC units are noticeable, the qualitative alterations are largely the same. Thus, our storage conditions provides a useful, cheaper, and blood-saving experimental system for studying the role of antioxidants as components of additive solutions.

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Disclosure Statement

The authors declare that there is no conflict of interest associated with this manuscript.

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