Deformability and viability of irradiated red cells

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Summary. The irradiation of blood components with X or gamma rays is necessary to prevent the graft-versus-host disease, but it also provokes untoward effects. In particular, red cells are damaged and have a decreased in vivo recovery, an increased in vitro haemolysis, and a leakage of potassium in the supernatant. The results of the clinical studies show that the loss of viability progressively increases with the storage after irradiation. On the other hand, the storage before irradiation is inconsequential. The mechanism through which irradiation causes the loss of viability is unknown, but a critical examination of the literature and our results indicate that the erythrocyte deformability is the only parameter related to viability to show sufficiently precocious and important changes. We also tried to identify the mechanism by which irradiation influences deformability and examined, in particular, the changes in the mean cell volume (MCV) and vesiculation. However, the temporal behaviour of both suggests no causal relationship.

Key words: irradiation, erythrocyte viability, erythrocyte deformability, erythrocyte filterability.

INTRODUCTION

In 1989, 10.1% of all blood components transfused in the USA were irradiated to prevent the graft-versus-host disease, but it also provokes untoward effects. In particular, red cells are damaged and have a decreased in vivo recovery, an increased in vitro haemolysis, and a leakage of potassium in the supernatant. The results of the clinical studies show that the loss of viability progressively increases with the storage after irradiation. On the other hand, the storage before irradiation is inconsequential. The mechanism through which irradiation causes the loss of viability is unknown, but a critical examination of the literature and our results indicate that the erythrocyte deformability is the only parameter related to viability to show sufficiently precocious and important changes. We also tried to identify the mechanism by which irradiation influences deformability and examined, in particular, the changes in the mean cell volume (MCV) and vesiculation. However, the temporal behaviour of both suggests no causal relationship.

IRRADIATION AND RED BLOOD CELL VIABILITY

The kinetics of in vivo survival of red cells, transfused after a long storage in vitro, is usually composed of a steep initial decrease, followed by a slower linear decline. It is a classic two-component curve, with the first component due to the rapid removal of non-vital red cells from circulation, whilst the second is caused by progressive aging. The two...
components are influenced by different factors \cite{4} and therefore they are (partially) independent. The initial component is estimated 24 h after transfusion (24-h recovery). The second requires measurements more prolonged in time (long-term survival).

The first studies on the effects of irradiation on red cell viability were performed during experimental research on extracorporeal irradiation of blood as a therapy for leukaemia \cite{5}. The dose levels varied from 350 to 2000 Gy, one or two orders of magnitude greater than those used today. Autologous blood was irradiated in the extracorporeal circulation, labelled with $^{51}$Cr and immediately reinfused. Schiffer et al. \cite{5} noticed the two-component curve. The 24-h recovery correlated linearly with the dose. On the contrary, the relationship with long-term survival was exponential, with a modest variation up to 500 Gy and marked effects at higher doses.

The first study on stored blood was conducted by Button et al. \cite{6} (Table 1). The viability of packed red cells or whole blood, stored 21 days in citrate-phosphate-dextrose (CPD) and irradiated before transfusion, was measured in 17 and 16 patients, respectively, and compared with non-irradiated controls. The dose ranged from 50 to 200 Gy. The authors observed no significant difference in the 24-h recovery, but long-term survival was impaired at all dose levels, although the difference was statistically significant with 200 Gy only ($T_{50}$: 23.2 days, vs 28.7 in the controls).

Considering the first results, it is surprising that long-term survival was reported in two further studies only \cite{7, 8}. In both cases, the dose was 25 Gy and survival was slightly reduced \cite{7} or not varied \cite{8}.

Studies reporting the 24-h recovery were more numerous (Table 1). In most cases, authors utilized volunteers who were transfused, at an appropriate interval of time, with autologous irradiated or non-irradiated red cells. This kind of experimental design permits to lessen the influence of the variability between subjects and to obtain significant results studying just a few cases. Friedman et al. \cite{9} used red cells stored in Nutricel (AS3), irradiated (20 Gy) on day 1 and transfused after 21 or 28 days of storage (6 volunteers). They observed that the 24-h recovery was significantly decreased by irradia-

### Table 1 | Summary of available data on 24-h recovery of irradiated red blood cells

<table>
<thead>
<tr>
<th>Author</th>
<th>Storage solution</th>
<th>Dose (Gy)</th>
<th>Day of Irradiation</th>
<th>Day of Transfusion</th>
<th>24-h Recovery (%)</th>
<th>P</th>
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<td>84.3</td>
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<td>86.0</td>
<td>83.2</td>
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</tr>
</tbody>
</table>

NS = Not significant.
NR = Not reported.
tion (21 day storage: 82.7 vs 90.4%; 28 day storage: 80.7 vs 85.0%), although it remained higher than 75%. A similar study, with red cells stored in Adsol (AS1), irradiated on day 0 (30 Gy) and transfused on day 42, confirmed the decreased recovery (68.5 vs 78.4%) [10]. In 6 out of 8 irradiated blood units, recovery was lower than 75%, whilst that only happened in 1 case out of 8, with control red cells. These results were substantially confirmed by Moroff et al. [7], again with red cells in AS1 and a dose of 25 Gy (Table 1). On the contrary, Mintz and Anderson [11], in similar conditions (red cells in AS1, 30 Gy) found no significant difference after 35 days of storage from irradiation.

A more complex experimental design was used by Moroff et al. [8], in order to verify the influence of storage before and after irradiation. Results showed convincingly that storage before irradiation entailed no difference in viability with the controls. On the contrary, the more the storage after irradiation was prolonged, the more viability was decreased (Table 1). In three studies, red cells were irradiated before [12, 13] or during [14] the frozen storage. In those experiments, the dose levels ranged from 15 to 35 Gy and the storage after irradiation was at most 6 days. No difference in recovery was observed. Finally, only one study evaluated the effect of leucoreduction [15]. Red cells from 7 volunteers were collected in AS3, irradiated on day 0 with 25 Gy and transfused on day 42. Half of the blood units had been leucoreduced by filtration before irradiation. They showed a slightly but significantly better recovery (78.0 vs 72.3%).

Altogether, it may be concluded that 24-h recovery is impaired by irradiation and the difference between irradiated and control red cells increases as storage after irradiation is prolonged (about 2% per week [16]). On the other hand, storage before irradiation seems to be inconsequential. In the light of these facts, the recommendation by Food and Drug Administration (FDA) that red cells should not be stored more than 28 days after irradiation seems appropriate [17]. On the contrary, the European guidelines [18] (and the Italian law [19]) appear unjustified in forbidding irradiation after the 14th day of storage: data clearly show that it is better to irradiate the blood units after 28 days of storage, rather than storing up to day 28 red cells irradiated on day 14.

The few data on survival suggest that at the current dose levels the effect of irradiation is minimal or absent. Finally, it may be noticed that all studies cited in Table 1 come from North America: this explains why red cells stored in SAGM, the solution used in Europe, were not studied.

**FACTOR(S) RESPONSIBLE FOR THE DECREASED VIABILITY**

From the above-mentioned considerations, some characteristics emerge, which must be possessed by the factor(s) responsible for the decreased viability. In principle, the following characteristics could be used to identify the factor(s): it must differ between irradiated and control red cells; the difference must increase with the dose and the length of storage after irradiation; however, it must not vary immediately after irradiation; moreover, it should presumably be a factor also influencing viability prescinding from irradiation.

**Role of adenosine-triphosphate (ATP)**

Bearing in mind the data accumulated on the relationship between ATP and erythrocyte viability [20], ATP concentration or total adenine nucleotide concentration are the most plausible candidates. However, many studies found no significant difference between irradiated and control red cells [9, 15, 21-23]. Moroff et al. [8] reported that ATP was decreased, in comparison to the controls, in red cells irradiated on day 14 and measured on day 24. However, in the same study, the difference was not significant in blood units irradiated on day 1 and measured on day 28. In both cases, in vivo recovery was significantly different. Vice versa, ATP was significantly decreased in blood units irradiated on day 14 and measured on day 28, but in vivo recovery was not. A discrepancy between in vivo recovery and ATP concentration, with the first one significantly decreased and the second not, was also found by Friedman et al. [9] and Davey et al. [15]. A decreased ATP concentration, in comparison to controls, was described by Moore and Ledford [24] (40 Gy) and Leitner et al. [25] (30 Gy), after 21 days of storage post-irradiation; by Hillyer et al. [26] (35 Gy) and Davey et al. [10] (30 Gy), after 42 days. Notwithstanding the statistical significance, Moore and Ledford [24] commented that the difference was small, similar to that encountered between different data sets collected in the same conditions. They supposed, therefore, that it would not have been biologically significant. Finally, in the three studies in which ATP concentration was measured systematically every 7-14 days [24-26], the difference between irradiated and control red cells did not increase progressively in time. This last fact, and the modest variation in comparison to the controls, indicates that ATP is not the factor responsible for the impaired viability.

**In vitro haemolysis**

Irradiation causes immediate haemolysis of red cells, but only at much higher doses than currently used [27]. In those circumstances, the cell membrane is damaged, ions can freely diffuse across and this causes an osmotic lysis. At usual doses, haemolysis does not appear immediately after irradiation [24, 25, 28]. During the storage post-irradiation, haemolysis increases more markedly in the irradiated red cells than in the controls, but the difference is only significant after 4 [8], 5 [24, 25] or 6 weeks [10, 26]. In fact, we found that the irradiated samples showed
a greater haemolysis since the first week of storage, in comparison to the controls (Figure 1). However, haemolysis was very low in the first weeks of storage and, in any case, values remained lower than 1% even after 5 weeks. Therefore, haemolysis represents no more than a small percentage of the red cells that do not survive after 24 hours.

**Osmotic resistance**

In a classic study on red cell storage, osmotic resistance (more exactly, haemolysis in 0.6% NaCl) was the parameter best correlated with the in vivo recovery, excepting ATP concentration [29]. Osmotic resistance actually measures the surface/volume ratio, which decreases during storage because of the loss of part of the cell membrane [30]. One of the first studies on irradiation of red cells reported surprising results about the osmotic resistance: immediately after irradiation there was no difference with the controls, but after an incubation at 37 °C for 24 hours, osmotic resistance increased with doses of 500-1000 Gy and markedly decreased with 2000 Gy [5]. The authors offered no explanation for this phenomenon but, 40 years later, we are able to formulate a plausible conjecture: the decreased resistance at the higher dose probably reflects the severe membrane damage also responsible for immediate haemolysis (see above). The increased resistance at lower doses is probably provoked by a less severe damage but sufficient to activate, during the 37 °C incubation, the K-Cl cotransport [31]. This, on its part, causes dehydration and an increase in the surface/volume ratio. The only other study [32] that reported data on osmotic resistance, utilized a dose of radiation much nearer (50 Gy) to the recommended one. Before measurement, red cells were incubated at 37 °C for an hour. Contrarily to the study cited above [5], irradiation caused a modest but significant increase of haemolysis in 0.65% NaCl. The difference with the controls was already evident after a week of storage but did not increase afterwards. In the presence of a powerful antioxidant (tirilazad mesylate), haemolysis in 0.65% NaCl halved in both the irradiated sample and the control. Autologous plasma (final haematocrit 42-47%) was even more effective (six fold reduction). Authors attributed the erythrocyte damage by irradiation and storage to oxidative processes, and the protective effects of plasma to antioxidant properties. In conclusion, osmotic resistance is influenced by factors not related to erythrocyte viability [4] and, therefore, it cannot be directly responsible for the loss of viability after irradiation.

**Deformability and other rheologic parameters**

A close relationship between erythrocyte deformability and viability was described four decades ago by Haradin et al. [33]. They used a rudimentary filterability technique and the measurement of viscosity. However, examining their published graphs, the inferred relationship could be explained by the fact that both viability and the rheologic modifications are time-dependent. A more convincing demonstration was offered by Card et al. [34], who studied a subject whose red cells lose viability at an accelerated pace during storage. All haematological and biochemical parameters (including ATP) were normal, except deformability (measured with the ektacytometer), which worsened much faster than in the controls. There are just a few studies on the rheology of irradiated red cells. A Japanese group evaluated the elongation of erythrocytes under various shear stresses (deformation index) [21, 35, 36]. Red cells in mannitol, adenine, phosphate (MAP), irradiated with 15 or 35 Gy of X rays, showed a lower deformation index, in comparison to controls, starting from

![Free haemoglobin (haemolysis), expressed as a percentage of the total haemoglobin content, after irradiation with 25 Gy of gamma rays. Irradiation was performed on day 1. Data are medians of 6-12 samples. Error bars connect the first and the third quartile. (Closed circles: irradiated; open squares: non irradiated)](image)
2-3 weeks of storage [35]. The difference in terms of percentage of erythrocytes not deformable at low shear stress (33 dyn/cm²) was even more evident. Similar results were obtained with gamma rays (35 Gy) [36]. Surprisingly, with the same experimental apparatus, red cells irradiated with 50 Gy (X rays) and stored up to 4 weeks in CPD did not show any difference with the controls [21]. In this last study, the authors did not find variations of mean cell volume (MCV) during storage. On the contrary, in the studies with MAP, MCV decreased progressively during storage. Presumably, this happened partly because of the hypertonicity of MAP, and partly because of the potassium leakage (see below). We consider it probable that this kind of deformability measurement be mainly a function of MCV. An alternative explanation for the difference between the results with CPD and with MAP is the presence of plasma in the first. A study already quoted above showed that plasma protects the red cells against the oxidative damage [32]. In any case, considered the results with MAP, which certainly preserves the erythrocytes better than CPD, we may conclude that this deformability test is not correlated with viability. Another kind of experimental apparatus, according to Barjas-Castro et al. [37], measures the elasticity of single erythrocytes. In fact, however, it is based on the deformation of cells dragged along the direction of flow. Up to 14 days of storage in citrate-phosphate-dextrose-adenine-1 (CPDA-1), there was no difference between red cells irradiated with 25 Gy and controls (not even in comparison to day 0). The differences appeared after 21 days and were very marked after 28 days. Despite the opinion of the authors, the results leave many doubts about the sensitivity of this test.

We studied the deformability of leucoreduced red cells, irradiated with 25 Gy and stored in saline-glucose-mannitol (SAGM). Our technique measures the filterability through 3 µm pores and was designed to eliminate the influence of known disturbing factors: the red cell suspension is free of leucocytes, platelets and plasma; the test is performed at 37 °C in a buffer at pH 7.4 and normal osmolarity [38]. With our technique, we evidenced differences of deformability already after a week of storage in several anticoagulant and nutrient solutions [39]. Figure 2 shows the results obtained with irradiated red cells and controls (the same blood units were divided in two aliquots). On the day after irradiation the differences are not evident, but they appear already after a week and increase progressively in time (note that the data reported in Figure 2 are logarithmically transformed). Judging from the graph (Figure 2), irradiated red cells present deformability values, after a week of storage, similar to non-irradiated red cells after 3 weeks. Altogether, erythrocyte deformability, as measured by our filterability technique, has a temporal behaviour corresponding to what is known about viability.

**IN WHAT WAY IRRADIATION INFLUENCES DEFORMABILITY?**

If deformability is really the factor responsible for the loss of red cell viability after irradiation, it is appropriate to look for the mechanism that connects the two. Obviously, none of the factors discussed above can be the missing link. In this part, we will examine the relationship between irradiation and some characteristics that may influence deformability, like MCV and vesciculation. Moreover, we will consider the leakage of potassium, which has a temporal behaviour much like deformability. First of all, however, we will discuss the primary effects of irradiation.

![Fig. 2](image-url) | Red cell filterability (deformability) after irradiation with 25 Gy of gamma rays. Filterability is the time necessary for a 10ml volume of a diluted red cell suspension to pass through a 3um pore filter. A higher value means a lower deformability. Raw data were logarithmically transformed and expressed as percentages with reference to the initial sample (day 0). Irradiation was performed on day 1. Data are means of 6-12 samples. Error bars represent 1 SD. (Closed circles: irradiated; open squares: non irradiated)
Primary effects of radiation on red cells

Mature erythrocytes are relatively radioresistant in comparison to other blood cells [27] and the type of damage is different: in nucleated cells, the main target of radiation is DNA. Therefore, leucocytes are exquisitely sensitive to alpha particles. Instead, red cells prevalently suffer an indirect damage, caused by water radiolysis that generates reactive oxygen species (ROS). For this reason, X or gamma rays are more effective [40]. Further indications that the damage is indirect are the inverse dose-rate effect [41], the protection provided by freezing or dehydration [42] and, vice versa, the enhancement due to the presence of oxygen [43]. An inverse dose-rate effect means that it is more damaging to administer the total dose in a longer time, rather than in a shorter. The explanation for this paradoxical phenomenon just lies in the generation of ROS: if ROS are produced in a low concentration, i.e. when the dose rate is low, they prevalently react with the surrounding protein or lipid molecules. In this way, they produce the radical chain reaction that leads to protein oxidation and lipid peroxidation [42].

\[ \text{e.g.}, \text{ up to 20 different amino acids can be damaged by a single ROS [44].} \]

When the dose rate is higher, the concentration of ROS is greater and they tend to react with each other. In this way, the propagation of the radical chain reaction is limited and termination reactions are prevailing [41]. However, the relationship between total dose, dose-rate, time and biological effects is more complex: at the same total dose and dose rate, the administration in two parts, divided by 3.5 hours of interval, conferred a certain protection: 2.4 times less haemolysis, less metHb, less damage to membrane lipids, but the damage to proteins was not changed [45]. Freezing or dehydration very effectively protect biological molecules from the effects of irradiation so that, to obtain the same injury, doses up to 100 times higher are needed [42]. In these conditions, ROS cannot freely diffuse and the radical chain reaction is blocked. Therefore, direct effects of radiation prevail [42]. On the contrary, the presence of oxygen enhances the effect of ROS 16 times in comparison to anaerobic conditions [46]. The radiolysis of water, i.e. the dissociation of its molecules, provokes the formation of several ROS: hydroxyl radicals (OH), hydrated electrons and hydrogen atoms [43]. In the presence of air, hydrated electrons and hydrogen atoms rapidly react with oxygen forming peroxide radicals. These last ones react more slowly and are easily inactivated in intact cells because of the presence of endogenous catalase [40]. Therefore, hydroxyl radicals are the main responsible for the indirect effects of irradiation. In anaerobic conditions, the dominating process induced on red cells by radiation is the aggregation of membrane proteins, caused by the formation of S-S bridges. In aerobic conditions, oxygen suppresses the cross-linking and catalyses an intense protein oxidation and lipid peroxidation [46]. In the presence of antioxidants, red cells are partially protected against lipid peroxidation and the decrease in the osmotic resistance [32]. However, the effects on potassium leakage are modest (-10% to -20%) [47] and data on red cell viability are lacking. Altogether, the primary effects of radiation do not offer an immediate explanation of the effects on deformability.

**Red cell volume (MCV)**

In the paragraph on rheologic parameters, we commented that some deformability tests are probably influenced by MCV. Presumably, our filterability test also is influenced by MCV or, at least, this is a plausible explanation for the sensitivity to pH (at acidic pH, red cells swell) and osmolarity changes.

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**Fig. 3** | Mean cell volume (MCV) of red cells after irradiation with 25 Gy of gamma rays. Irradiation was performed on day 1. Data are means of 6-12 samples. Error bars represent 1 SD. (Closed circles: irradiated; open squares: non irradiated)
(increasing the osmolarity of the medium, the erythrocytes decrease their volume) [38]. Data on MCV changes after irradiation are scanty and contradictory. Brugnara and Churchill [48] found that MCV increased and mean corpuscular haemoglobin concentration (MCHC) decreased during storage, as expected [20], but there were no differences between irradiated and control red cells. Suzuki et al. used red cells in CPD, as in the experiment cited above, but they did not observe any change, neither caused by storage, nor by irradiation [21]. The same group, using red cells in MAP, found significant differences between irradiated and control red cells since the first week of storage, but in these experiments MCV decreased and MCHC increased with time [35, 36]. Finally, we and others [23] observed significant differences between irradiated and control red cells, but in our case MCV increased during storage (Figure 3). Actually, the increase in the MCV during storage is a matter of fact [4], at least with the common storage solutions. However, according to some studies [49], the swelling is reversible and it masks a real decrease in the cell volume that appears when the erythrocytes are incubated at 37 °C for 24 hours in autologous plasma. These volume changes are blocked inhibiting the K-Cl cotransport, but not inhibiting the Na-K pump, nor the Na-K-Cl cotransport, nor the Ca-activated K channel [31]. These opposite changes in the cell volume according to the manipulation of the erythrocytes could explain the different results reported above. However, in the quoted articles [35, 36] there is no mention of any incubation at 37°C before the measurement of the red cell indices. In any case, in our experiments the differences between irradiated and control red cells appeared no sooner than after two weeks (Figure 3); therefore they were not so early as to explain the loss of deformability.

**Vesiculation**

During storage, erythrocytes lose their normal discocytic form and become echinocytes. These morphological changes are only partially reversible, because microvesicles are released from the extremity of the spicules [20]. These vesicles are rich of membrane but contain very little haemoglobin [50]. Since in this way the surface/volume ratio decreases, vesiculation could represent a plausible mechanism for the loss of deformability after irradiation. To our knowledge, there are no further data on this argument, apart from those collected by us (Figure 4). We measured the true acetylcholinesterase activity in plasma, which is exclusively associated with cell membranes and therefore is an excellent marker of vesiculation. The release of microvesicles is more pronounced in the irradiated samples, but the difference is small in comparison with the variability among the different subjects. Moreover, it appears too late (not less than 21 days) to be responsible for the loss of deformability.

**Potassium leakage**

The release of potassium into the supernatant is the only parameter we have studied to show differences between irradiated and control red cells as precocious as deformability (Figure 5). This phenomenon was described in the first studies on irradiation [6] and subsequently confirmed by all others. However, the leakage of potassium is not characteristic of irradiation, because it also appears during the storage of non-irradiated red cells. Brugnara and Churchill demonstrated that the Na-K pump is not damaged but only inactive, owing to the low temperature of storage [48]. Once incubated at 37 °C, the red cells recover intracellular potassium and at the same time the MCHC. The difference between irradiated and control red cells is already evident after a week and is probably due to an increase of the passive permeability of the
Deformability of Irradiated Red Cells

It is interesting to note from Figure 5 that the difference between irradiated and control cells reaches a maximum early (about 7 days) and, afterwards, it tends slowly to decrease. This behaviour was reported by others, too [36, 48], and clearly shows that potassium is not released because of haemolysis only (compare Figure 1 and Figure 5). Despite some similarity, the curves of extracellular potassium (Figure 5) and deformability (Figure 2) are sufficiently different to suggest that there is no cause-effect relationship between the two. Moreover, there is no prima facie mechanism to link these two parameters.

CONCLUSIONS

The storage of red cells after irradiation decreases viability in a linear way, approximately 2% per week [16]. The mechanism by which irradiation influences viability is not clear, but deformability is the only known parameter related to viability that possesses all the characteristics of the culprit. Deformability is clearly impaired by irradiation but, again, we ignore by what mechanism. However, what we know about the effects of irradiation on viability suggests a number of practical measures:

- viability is not impaired immediately after irradiation, therefore the best practice is to irradiate just before transfusion;
- all known experimental data suggest that the effects of irradiation are not influenced by the prior storage of red cells. Accordingly, there is no point in limiting the permissible storage age before irradiation;
- red cells show the inverse dose-rate effect. Therefore, the total dose should be reached as fast as possible to limit the damage to red cells. In general, more attention should be paid to the intensity (the duration) of the irradiation, besides the total dose: e.g., the total dose is usually delivered in a few minutes using a $^{137}$Ce source, but may require more than 60 minutes with a teletherapy unit. However, to increase the dose-rate further, it would be necessary to use a high-energy source or to replace it earlier than customary;
- at the same dose, alpha particles should be more damaging to white cells and less to red cells, than gamma or X rays. Unfortunately, alpha particles have such a low penetration that their use in our context is not practical.

In any case, we need a much greater understanding of the mechanisms activated by the primary effects of radiation on red cells. It is not improbable that those mechanisms are the same involved in the loss of viability after storage. If this is the case, irradiation could be viewed as inducing a sort of accelerated aging.

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