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What is the Source of Free Radicals Causing Hemolysis in Stored Blood?

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Summary

The quality of stored blood can be deteriorated by hemolysis caused by free radicals. The purpose of this study was to elucidate whether neutrophile leukocytes are the source of free radicals in stored blood as in hemodialyzed patients. Resuspensions with low (LL) or high (HL) leukocyte concentrations were prepared from samples of twenty healthy volunteers. The samples were incubated for 10 days at 4 °C and then for one day at 37 °C. Markers of hemolysis and free radical metabolism were examined before and after incubation in LL and HL samples. In spite of the difference of leukocytes counts in LL and HL resuspensions ($p<0.0001$), the pre-incubation values of all laboratory parameters were practically identical. In post-storage samples, superoxide dismutase and glutathione peroxidase activities did not differ in either group. Reduced glutathione in erythrocytes and extracellular antioxidant capacity were insignificantly lower in HL resuspensions, but the increase of malondialdehyde was much more pronounced in the HL samples ($p<0.0001$). The degree of hemolysis, expressed as the extracellular increase of potassium ($p<0.001$), hemoglobin ($p<0.05$) and lactate dehydrogenase ($p<0.05$), was higher in the HL samples. Our results support the hypothesis that leukocytes participate in free radical production in stored blood.

Key words
Free radicals • Hemolysis • Leukocytes

Introduction

Hemolysis which occurs in stored blood can lead to deterioration of its quality due to hemoglobin and potassium leakage from erythrocytes. Free radicals (FR) may injure erythrocyte membranes (Niki *et al.* 1988). However, this process can be diminished by supplementation of blood donors with antioxidants, as was shown in our previous study (Racek *et al.* 1997). Similarly, free radicals damage to the erythrocyte membrane followed by hemolysis and anemia can be observed in hemodialyzed patients (Ong-Ajyooth *et al.*1991, Durak *et al.* 1994). This process can also be partially controlled by antioxidants administered to these patients (Kuang *et al.* 1994, Zachée *et al.* 1995).

As the main source of free radicals in patients under regular hemodialysis treatment are leukocytes activated during their contact with the dialysis membrane (Zima *et al.* 1993), we supposed that this process could

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also be responsible for the hemolysis of stored blood. The aim of this study was to confirm this hypothesis.

Material and Methods

Experimental design

Blood samples of 20 donors (450 ml each) collected into quadruple blood bags (Grupo Grofols, Spain) with a CAPD-1 anticoagulation solution (63 ml) were centrifuged at 4 000 x *g* for 10 min and the plasma was removed. The column of blood cells was then divided into two equal parts – the upper one with the buffy coat containing most leukocytes and the lower part with a minimum of white blood cells. Two resuspensions in the SAGM solution (final hematocrit value approximately 0.50) were prepared from each sample; they were marked as low-leukocyte (LL) and high-leukocyte (HL) resuspensions. All these operations were performed within 30 min under sterile conditions. The composition of SAGM solution was as follows (in mmol/l): sodium chloride 150, adenine 1.25, glucose 50, mannitol 29.

Blood resuspensions were stored in "component aliquot bags" from plastics (Medsep Corporation, Ottawa, Canada) at 1-6 °C for 10 days and then at 37 °C for one more day as in our previous study (Racek *et al.* 1997). This scheme was chosen to simulate blood storage before transfusion followed by its warming to body temperature. Laboratory investigations were performed in all samples before and after the incubation period.

Analytical methods

Markers of hemolysis. Supernatant concentrations of hemoglobin were assessed by the cyanide method, Na^+ and K^+ on a Corning 480 flame photometer, lactate dehydrogenase activity by the UV test from Roche Diagnostics (Mannheim, Germany).

Markers of FR metabolism. Malondialdehyde (MDA) in the supernatant was estimated by spectrophotometric determination with thiobarbituric acid as thiobarbituric acid-reactive substances (TBARS). Superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) were determined in erythrocytes, the supernatant antioxidant capacity (AOC) with kits from Randox (Crumlin, Northern Ireland, UK) and reduced glutathione (GSH) in erythrocytes with the kit from Oxis International (Bonnenie, Marue, France). All methods were adapted for a Hitachi 717 analyzer (Roche Analytical Systems, Mannheim, Germany).

Blood cell counts. All hematological parameters, including the differential white blood count, were measured on a Sysmex 9000R analyzer (Toa Medical Electronics, Kobe, Japan).

Statistics

Pre- and post-incubation results were compared between the LL and HL groups and the differences between the changes during the incubation in pairs of LL and HL samples were also evaluated. In all cases, the paired Wilcoxon test was used.

Table 1. Hematocrit and leukocyte count in HL and LL resuspensions.

Resuspension type	Hematocrit	Total leukocytes (x 10^9/l)	Neutrophile granulocytes (x 10^9/l)	Lymphocytes (x 10^9/l)
LL	0.505 ± 0.025	6.36 ± 2.43	4.20 ± 1.85	1.65 ± 0.55
HL	0.522 ± 0.031	25.20 ± 6.00	13.61 ± 2.25	8.06 ± 2.11
Statistical significance	NS	P < 0.0001	P < 0.0001	P < 0.00001

LL – low leukocyte resuspension, HL – high leukocyte resuspension. Data are means ± S.D., n = 20.
NS – not significant.

Results

The average number of leukocytes in LL and HL resuspensions was significantly different (p<0.0001), while the hematocrit values were practically identical. As free radicals are produced mainly in activated neutrophile granulocytes, the differential count of leukocytes was measured in all samples and the number of neutrophile

granulocytes was calculated. It was more than three times higher in HL resuspensions in comparison with LL resuspensions ($p<0.0001$) (Table 1).

Tables 2 and 3 summarize all the results, namely mean pre- and post-storage values of markers of hemolysis and free radical metabolism in LL and HL resuspensions. Before incubation, none of the estimated parameters showed significant difference between the LL and HL groups. On the other hand, there were many significant differences in post-storage values or changes during storage between LL and HL resuspensions (Figs 1 and 2). The degree of hemolysis was significantly more pronounced in HL resuspensions, while LL resuspensions exhibited a lower degree of oxidative stress, expressed as higher antioxidant capacity and lower malondialdehyde increase during the incubation period.

Table 2. Changes of laboratory parameters in LL resuspensions during incubation and their statistical significance.

Parameter	Pre-incubation values	Post-incubation values	Statistical significance
SOD (U/g Hb)	995 ± 103	1001 ± 145	NS
GSHPx (U/g Hb)	50.6 ± 11.4	49.1 ± 12.5	NS
GSH (mmol/l)	2.13 ± 0.31	2.11 ± 0.63	NS
AOC (mmol/l)	0.87 ± 0.20	1.92 ± 0.78	P < 0.0001
MDA (μmol/l)	2.69 ± 0.62	3.30 ± 0.65	P < 0.001
Na^+ (mmol/l)	160.8 ± 5.5	156.7 ± 8.7	P < 0.01
K^+ (mmol/l)	2.93 ± 0.62	26.10 ± 10.35	P < 0.0001
Hb (g/l)	0.68 ± 0.64	1.59 ± 0.86	P < 0.01
LD (μkat/l)	9.85 ± 6.01	67.80 ± 27.36	P < 0.0001

Data are means ± S.D., n = 20. NS – not significant

Table 3. Changes of laboratory parameters in HL resuspensions during incubation and their statistical significance.

Parameter	Pre-incubation values	Post-incubation values	Statistical significance
SOD (U/g Hb)	998 ± 121	973 ± 105	NS
GSHPx (U/g Hb)	50.3 ± 0.8	47.7 ± 9.9	NS
GSH (mmol/l)	2.15 ± 0.42	1.95 ± 0.27	NS
AOC (mmol/l)	0.91 ± 0.31	1.55 ± 0.43	P < 0.0001
MDA (μmol/l)	2.71 ± 0.55	5.09 ± 0.73	P < 0.0001
Na^+ (mmol/l)	161.2 ± 6.1	151.2 ± 5.1	P < 0.001
K^+ (mmol/l)	2.88 ± 0.55	33.40 ± 7.90	P < 0.0001
Hb (g/l)	0.61 ± 0.59	1.91 ± 0.67	P < 0.001
LD (μkat/l)	8.92 ± 5.91	82.2 ± 24.18	P < 0.0001

Data are means ± S.D., n = 20. NS – not significant

Discussion

Formation of free radicals in stored blood is of a complex origin. The high glucose content, high concentration of polyunsaturated fatty acids, hemoglobin-mediated formation of the hydroxyl radical from hydrogen peroxide and other changes should be mentioned (Jain 1988, Chiu *et al.* 1989, Knight *et al.* 1991, 1992).

Leukocytes activated during their contact with the dialyzing membrane seem to be the main site of FR production in hemodialyzed patients (Ong-Ajyooth *et al.*

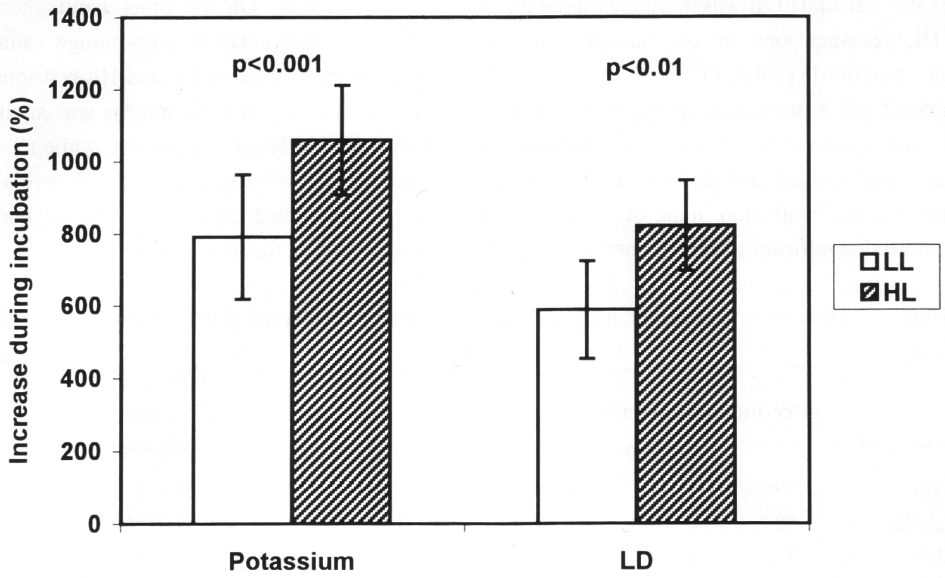

Fig. 1. S*ignificant differences in changes of extracellular potassium concentration and lactate dehydrogenase (LD) activity during the incubation period between LL and HL resuspensions (means ± S.D., n=20), expressed as percentage of the pre-incubation value.*

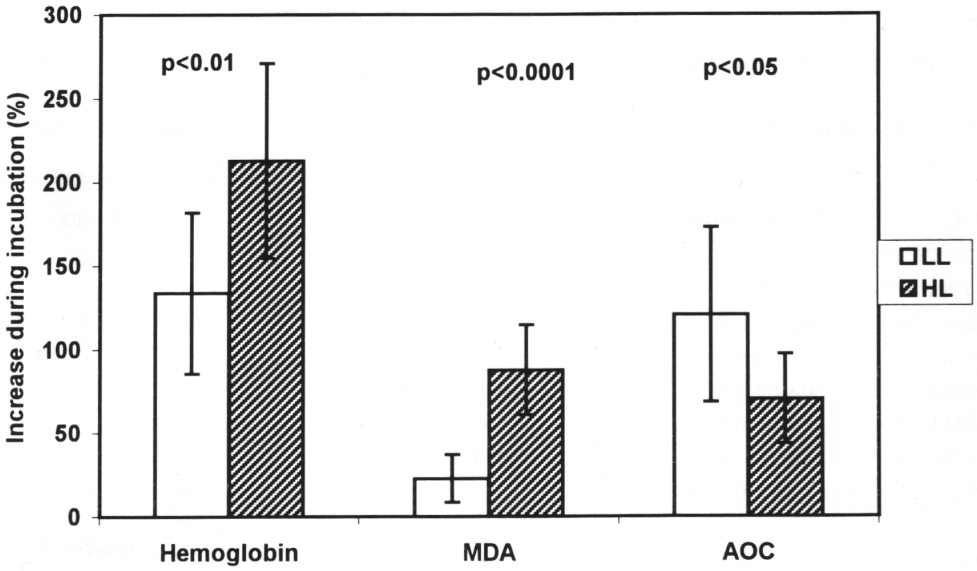

Fig. 2. *Significant differences in changes of extracellular hemoglobin (Hb), malondialdehyde (MDA) and antioxidant capacity (AOC) during the incubation period between LL and HL resuspensions (means ± S.D., n=20), expressed as percentage of the pre-incubation value.*

1991, Zima *et al.* 1993, Durak *et al.* 1994). According to some authors, this source of free radicals may also be important in stored blood, although it is not the only cause of their formation. This opinion is supported by a significantly higher increase of MDA in HL resuspensions; this parameter is an indirect marker of lipid peroxidation. MDA can modify proteins and, together with the changes of membrane lipids during lipid peroxidation, may be the main cause of damage to erythrocyte membranes and the subsequent hemolysis

(Jain 1988, Chiu *et al.* 1989, Knight *et al.* 1992). A moderate, but significant increase of MDA concentration was also observed in LL resuspensions. We could not decide whether free radicals in LL resuspensions originate from residual leukocytes or from some other sources. As expected, the degree of hemolysis, manifested in the form of the leakage of intracellular components from erythrocytes, was much higher in the HL resuspensions. In HL resuspensions, all the monitored parameters increased significantly more than in LL blood samples.

The changes in total extracellular antioxidant capacity are difficult to evaluate. This parameter was significantly higher in both resuspension types, although it was more pronounced in LL blood. In agreement with this finding, the malondialdehyde increase was much higher in HL resuspensions. This shows a higher degree of lipid peroxidation and thus of membrane damage in resuspensions with a higher content of leukocytes. The increase of AOC can preserve erythrocytes from attacks of free radicals, although we have repeatedly shown that only intracellular antioxidants are of basic importance in cell defense against free radicals and the elimination of free radicals is usually accompanied by a decrease of antioxidant capacity in the plasma (Racek *et al.* 1995, 1996). The cause of AOC increase during the incubation and the nature of antioxidants are not clear. As it has not been observed in a similar experiment with whole blood, the resuspension solution may thus contribute to the formation of antioxidants in the samples. Mannitol is considered as a FR scavenger but antioxidant capacity of the SAGM solution itself is present in minute quantities (0.13 mmol/l).

We can conclude that leukocytes may act as one source of FR production in stored blood, thus worsening its quality by hemolysis. It is therefore necessary to stress the importance of using erythrocyte resuspensions with reduced concentrations of leukocytes for blood transfusions. These resuspensions not only represent a weaker antigenic stimulus for the acceptors, but have additionally one more advantage: erythrocytes are more stable and the degree of hemolysis is much lower than if whole blood is used. This seems to be a result of the elimination of leukocytes which are an important source of free radicals. Another possibility of improving the quality of stored blood is to supplement blood donors with antioxidants at least one week before blood collection (Racek *et al.* 1997).

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