No Early Effect of Storage Time of Transfused Red Blood Cells on Fatigue and Plasma Cytokines in Patients with Anaemia from Non-Acute Gastrointestinal Bleeding

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Key Words

Anaemia · IL-1 · Immunomodulation · Storage, TNF- α · Transfusion · Fatigue

Summary

Background: Fatigue in anaemia is empirically reduced by blood transfusion. Long storage time of red cells may be associated with immunomodulatory effects, and blood stored for a long time may cause tissue hypoxia upon transfusion. **Patients and Methods:** 22 patients admitted with haemoglobin < 6.0 mmol/l, complaints of fatigue and no active bleeding were included. Eleven patients received two red cells units (SAGM) stored less than 1 week (short storage time, S), and 11 patients received two units stored more than 3 weeks (long storage time, L). Fatigue was self-estimated on a visual analogue scale. Clinical observations and blood samples were obtained before transfusion was started, and were repeated 2–8 h after transfusion of the 2nd unit. Measured plasma parameters included IL-1 β , IL-6, IL-8, IL-10, IL-12 and TNF- α . **Results:** There were no significant differences between group S and L (nsSL) in demographic data, observational data and blood plasma values. Haemoglobin increased from mean ($\pm$ SD) 5.2 ± 0.6 to 6.4 ± 0.7 mmol/l after transfusion (nsSL). Fatigue score significantly decreased from a pre-transfusion median 6.6 (range 0.1–9.9) to post-transfusion 4.7 (0.6–10.0) ($p = 0.02$) for all patients (nsSL). Beside increase in haemoglobin the only significant change in blood parameters after transfusion was a decrease in thrombocyte count (nsSL). No significant differences were seen in concentrations of cytokines before and after transfusion. **Conclusion:** Transfusion of two units of red cells relieved self-estimated fatigue, independent of blood storage time. Thrombocyte count decreased after transfusion, probably due to dilution by transfused blood. Aged red cells may not, or only sparsely, directly trigger the interleukin cascade.

Schlüsselwörter

Anämie · IL-1 · Immunomodulation · Lagerung · TNF- α · Transfusion · Fatigue

Zusammenfassung

Hintergrund: Fatigue bei Anämie kann, wie empirisch belegt, durch eine Bluttransfusion reduziert werden. Eine lange Lagerungszeit der Erythrozyten ist möglicherweise mit immunmodulatorischen Effekten verbunden ebenso wie lang gelagertes Blut möglicherweise eine Hypoxie bei Transfusion verursacht. **Patienten und Methoden:** 22 Patienten, die in unsere Klinik mit einem Hämoglobinwert < 6,0, Fatigue-Beschwerden, aber ohne aktive Blutung eingewiesen wurden, wurden in die Studie eingeschlossen. Elf Patienten erhielten je zwei Erythrozytenkonzentrate (SAGM), die weniger als 1 Woche (kurze Lagerungszeit; S) gelagert worden waren, die anderen 11 Patienten je zwei Erythrozytenkonzentrate, die mehr als 3 Wochen (lange Lagerungszeit; L) gelagert worden waren. Der Grad der Fatigue wurde mittels Selbsteinschätzung auf einer visuellen Analogskala bestimmt. Klinische Untersuchungen und Blutprobenentnahme wurden vor Beginn der Transfusion und 2–8 h nach der Transfusion des zweiten Erythrozytenkonzentrats durchgeführt. Folgende Plasmaparameter wurden bestimmt: IL-1 β , IL-6, IL-8, IL-10, IL-12 und TNF- α . **Ergebnisse:** In Bezug auf die demographischen Daten, die Untersuchungsergebnisse und die Blutwerte gab es keine signifikanten Unterschiede zwischen Gruppe S und L (nsSL). Der Hämoglobinwert stieg von $5,2 \pm 0,6$ (Mittelwert $\pm$ SD) vor auf $6,4 \pm 0,7$ mmol/l nach der Transfusion (nsSL). Der Fatigue-Score reduzierte sich signifikant von einem prätransfusionellen Median von 6,6 (Bereich 0,1–9,9) auf 4,7 (0,6–10,0) nach der Transfusion (nsSL). Der einzige Veränderung der Blutwerte neben dem Hämoglobinanstieg war die Abnahme der Thrombozytenzahl (nsSL). Die Konzentrationen der Zytokine waren durch die Transfusion nicht signifikant verändert. **Schlussfolgerung:** Die Transfusion von zwei Erythrozytenkonzentraten verbessert den durch Patienten selbst eingeschätzten Fatigue-Wert unabhängig von Lagerungsdauer des Bluts. Die Thrombozytenzahl nimmt nach der Transfusion ab – möglicherweise aufgrund der Verdünnung durch das transfundierte Blut. Ältere Erythrozyten scheinen die Interleukinkaskade gar nicht, oder nur sehr gering, zu triggern.

Introduction

One of the indications for red cell transfusion in non-acute anaemia is to relieve symptoms such as fatigue, headache, dyspnoea and palpitations. It is known that increasing the haemoglobin concentration by erythropoietin in cancer patients decreases the level of fatigue [1]. In the clinical everyday practice, blood transfusion may relieve the symptoms, but not always so. Red blood cell transfusions may improve subjective well-being in only 50% of terminally ill cancer patients, and this is not clearly associated with relieve in fatigue [2].

Long storage of red cells may change the cells and hence generate immunomodulation in the recipient [3]. The studies of immune parameters after blood transfusion are however primarily based on in vitro and animal studies. Previous human in vivo studies have often been done in patients undergoing surgery. The patients studied have not been stratified to post-operative infectious complications and varying storage time of transfused blood [4, 5]. The potential immunomodulatory effect of blood transfusion is probably only detectable after stimulation of the immune system by danger signals [3], i.e. surgery, infection, or trauma. Red cells units may, however, under certain other circumstances trigger the immune system as leucocytosis within a few hours after transfusion is more frequent in non-septic than in septic intensive care patients [6].

Immunological markers previously shown to be affected by blood transfusion are TNF- α , IL-2 and IL-10 [3]. Several cytokines are indicated to be involved in fatigue associated with cancer [7]. We, therefore, performed this study of the impact of storage time of transfused blood on anaemia-associated fatigue and on immunological markers

Patients and Methods

The Scientific Ethical Committee of Copenhagen and Frederiksberg approved the study (KF 01–212/03), and patients were included on the basis of informed written consent.

26 patients admitted to our surgical gastroenterology department with haemoglobin < 6.0 mmol/l, complaints of fatigue and no active bleeding (<300 ml/day) or signs of blood pressure instability were consecutively included from April 2004 to February 2005. The following categories of patients were not included: patients with known immunological disorders or infections, patients not expected to understand the visual analogue scale (VAS), and patients who had undergone surgery or received blood transfusion within 2 months. Randomisation was done immediately after admission and inclusion. Each patient received two units of red cell suspension; 13 patients received two units stored less than 1 week (group S) and 13 patients received two units stored more than 3 weeks (group L).

A unit of blood is produced by drawing 450 ml of whole donor blood into a blood bag with 63 ml CPD solution (Baxter A/S, Allerød, Denmark). The bag is subsequently subjected to centrifugation, plasma and buffy coat are removed, and red cells are transferred to a separate blood bag containing 100 ml saline, adenine, glucose and mannitol (SAGM) solution. This results in a red cell suspension of approximately 275 ml with a 65–75% leucocyte reduction and a haematocrit of 60%.

Table 1. Demographic data and disease characteristics of the 22 patients receiving transfusion of blood of short or long storage time

	Group S	Group L
Age, median (range), years	73 (31–89)	68 (54–88)
Female/male	6/5	6/5
ASA group		
I	2	1
II	6	7
III	3	3
Bleeding source		
Peptic	2	6
Colon/rectum	6	4
Unknown	3	1
Diagnoses (yes/no)		
Cancer	5/6	3/8
CVD	8/3	8/3
CLD	0/11	3/8

ASA = American Society of Anaesthesiologists; CVD = cardiovascular disease; CLD = chronic lung disease.

Fatigue was self-estimated on a VAS (no fatigue = 0, maximal fatigue = 10) before and 2–8 h after transfusion. VAS score of fatigue (lack of energy) is one of 10 items in the Edmonton Symptom Assessment Scale validated for graphic display of symptom intensity in patients e.g. cancer patients [8]. The patient and the interviewer were blinded of the unit storage time. Clinical observations and blood samples were obtained immediately before transfusion and 2–8 h after transfusion of the 2nd unit, which was started within 3 h after the start of first transfusion.

Measured blood and plasma parameters included total leucocyte count and differential count, thrombocyte count, international normalized ratio (INR), activated partial thromboplastin time (APTT), antithrombin-3, D-dimer, fibrinogen and C-reactive protein measured using the Roche Tina-quant CRP Immunoturbidimetric assay (Roche Diagnostics GmbH, Mannheim, Germany) on a Hitachi 917.

Collection and Storage of Blood Samples

Blood samples were collected by venipuncture into K2 EDTA-containing Vacutainer Tubes (BD Bioscience, San Jose, CA, USA). Blood was centrifuged at $3,000 \times g$ for 10 min at 4 °C. Plasma was then separated and subsequently stored at –80 °C in aliquots.

Cytokine Assay

Plasma concentrations of IL-1 β , IL-6, IL-8, IL-10, IL-12 (p70), and TNF- α were measured using the Beadlyte™ human multicytokine detection system 2 from Upstate Cell Signalling Solutions (Lake Placid, NY, USA). The assay was performed according to the manufacturer's instructions. Briefly, the lyophilized multicytokine standard was re-suspended in human Beadlyte serum standard diluents and then 1:3 serially diluted from 5,000 to 6.9 pg/ml. To each designated pre-wetted well of a 96-well filter plate 50 μ l standard or 1:1 diluted sample (diluted in Beadlyte human serum diluents) and 25 μ l of the bead solution were added. After 2 h incubation at room temperature the reporter solution was added. The streptavidin-phycoerythrin solution diluted 1:25 in assay buffer was added after an additional incubation of 1.5 h at room temperature. After a final incubation at room temperature for 30 min and the addition of the stop solution, the plate was placed on the XY platform of the Luminex100 (Luminex Corp., Austin, TX, USA) and analysed using StarStation v.2 software from Applied Cytometry Systems (Sheffield, UK). From each well, a minimum of 100 analyte-specific beads was analysed for both bead designation and RPE fluorescence.

Table 2. Physical, biochemical and haematological pre- and post-transfusion data from the 22 patients pre- and post-transfusion of blood of short or long storage time

	Group S		Group L	
	pre-transfusion	post-transfusion	pre-transfusion	post-transfusion
Systolic pressure, mm Hg	130 ± 31	148 ± 23	125 ± 26	131 ± 26
Diastolic pressure, mm Hg	64 ± 11	75 ± 8*	66 ± 12	70 ± 9*
Heart rate	74 ± 15	72 ± 11	85 ± 17	87 ± 15
Temperature, °C	36.7 ± 0.5	37.1 ± 0.5	37.1 ± 0.9	37.1 ± 0.5
Haemoglobin mmol/l	5.1 ± 0.6	6.3 ± 0.7*	5.2 ± 0.6	6.6 ± 0.7*
Leucocytes × 10 ⁹ /l	8.3 ± 2.2	8.3 ± 2.7	9.1 ± 3.2	8.9 ± 3.4
Neutrophils	5.9 ± 2.0	5.7 ± 2.2	6.7 ± 2.8	6.5 ± 3.4
Eosinophils	0.2 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.3
Basophils	0.04 ± 0.04	0.05 ± 0.05	0.05 ± 0.03	0.03 ± 0.03
Lymphocytes	1.6 ± 0.5	1.7 ± 0.7	1.7 ± 0.9	1.5 ± 0.9
Monocytes	0.6 ± 0.3	0.7 ± 0.2	0.7 ± 0.4	0.8 ± 0.4
Thrombocytes × 10 ⁹ /l	312 ± 132	284 ± 116*	345 ± 126	308 ± 115*
C-reactive protein, mg/l	38 ± 52	38 ± 52	49 ± 55	56 ± 68
TNF-α, pg/ml	10 ± 9	9 ± 10	12 ± 8	11 ± 6
IL-1β, pg/ml	15 ± 11	13 ± 11	17 ± 10	17 ± 10
IL-6, pg/ml	14 ± 13	16 ± 16	31 ± 26	28 ± 15
IL-8, pg/ml	28 ± 38	27 ± 43	18 ± 26	20 ± 28
IL-10, pg/ml	5 ± 4	5 ± 4	7 ± 3	7 ± 6
IL-12, pg/ml	32 ± 23	30 ± 25	36 ± 27	33 ± 23

* = Significant difference ($p < 0.05$) between pre- and post-transfusion within group S or group L.

Statistical Analysis

With an expected standard deviation of 20% and risk of type 2 error of 10%, it was calculated that to detect a difference in VAS score of 30%; 20 patients (2×10) had to be included. Differences between cytokine concentrations before and after transfusion were tested using paired samples t-tests. Independent samples t-tests were used to compare differences in cytokine concentrations in group S vs. group L. Normal distribution of plasma cytokine values was tested with one-sample Kolmogorov-Smirnov's test. Differences in VAS scores were analysed with non-parametric tests. The results are expressed as mean ± SD when not otherwise indicated, and a p value less than 0.05 was considered significant. All statistical analyses were performed with the SPSS 13.0 software program.

Results

Initially 26 patients were included. Four patients were subsequently excluded, two because of transfusion with blood of incorrect storage time, one had sudden active bleeding during transfusion, and one had X-ray-verified pneumonia. Excluded patients were equally belonging to group S and group L, resulting in a total of 22 patients with 11 in each group. In all, 8 patients suffered from cancer, 1 with breast cancer and 7 with colon cancer. There were no significant differences between groups S and L (nsSL) with regard to demographic data (table 1). Median storage time of blood transfused in group S was 4.5 days (range 1–7 days), and in group L median storage time of blood was 27 days (range 23–34 days). Each patient received two units of blood with less than 2 days difference in storage time.

Haemoglobin increased in all patients from mean 5.2 ± 0.6 to 6.4 ± 0.7 mmol/l after transfusion (nsSL). The only other significant change in blood parameters after transfusion was a decrease ($p < 0.01$) in thrombocyte count in all patients from 330 ± 127 to $296 \pm 113 \times 10^9/l$ (nsSL). The only significant change in clinical observational data after blood transfusion was an increase ($p < 0.01$) in diastolic pressure in all patients from 65 ± 11 to 73 ± 9 mm Hg (nsSL) (table 2).

Self-estimated fatigue score significantly decreased from median 6.6 (range 0.1 – 9.9) to 4.7 (range 0.6 – 10.0) ($p = 0.02$) for all patients. The fatigue score decreased in group S from 6.2 to 3.4 and in group L from 6.8 to 6.4 (nsSL). Although this difference may seem significant, it was not; due to variability and equally distribution of individuals reporting *increase* in fatigue (fig. 1).

Plasma cytokine levels showed some variation, but pre-transfusion levels were not significantly different from post-transfusion levels. Likewise, there were no significant differences within group S and group L (table 2). As an example of the variation in cytokine concentration the values of IL-1β are shown in figure 2.

There was a significantly higher plasma level of C-reactive protein in patients with cancer (table 3). Higher levels of IL-1β, TNF-α, IL-6, IL-8 and IL-12 also reflected inflammation, but significant differences were only seen with IL-1β and IL-6. There was no significant change in any inflammatory marker after transfusion, even after stratification for diagnosis (table 3).

Table 3. Physical, biochemical and haematological pre- and post transfusion data on the 22 patients; 14 without cancer diagnosis and 8 with cancer diagnosis

	Non-cancer patients		Cancer patients	
	pre-transfusion	post-transfusion	pre-transfusion	post-transfusion
Systolic pressure, mm Hg	123 ± 29	134 ± 29	136 ± 25	148 ± 15
Diastolic pressure, mm Hg	63 ± 10	69 ± 8	70 ± 13	79 ± 5 [#]
Heart rate	75 ± 17	78 ± 16	86 ± 16	83 ± 15
Temperature, °C	36.7 ± 0.7	37.0 ± 0.6	37.2 ± 0.7	37.4 ± 0.3
Haemoglobin, mmol/l	4.9 ± 0.6	6.2 ± 0.6	5.6 ± 0.4 [#]	6.9 ± 0.4 [#]
Leucocytes × 10 ⁹ /l	7.6 ± 1.9	8.3 ± 2.7	10.6 ± 3.0 [#]	10.1 ± 3.3
Neutrophils	5.4 ± 2.0	5.3 ± 2.8	7.7 ± 2.3 [#]	7.3 ± 2.6
Eosinophils	0.2 ± 0.2	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1
Basophils	0.05 ± 0.04	0.05 ± 0.05	0.04 ± 0.02	0.03 ± 0.01
Lymphocytes	1.5 ± 0.7	1.5 ± 0.9	1.9 ± 0.7	1.8 ± 0.8
Monocytes	0.5 ± 0.2	0.6 ± 0.2	0.8 ± 0.4	0.8 ± 0.4
Thrombocytes × 10 ⁹ /l	263 ± 87	234 ± 75	439 ± 106 [#]	398 ± 90 [#]
C-reactive protein, mg/l	19 ± 39	24 ± 56	80 ± 50 [#]	81 ± 49 [#]
TNF-α, pg/ml	9 ± 9	7 ± 8	14 ± 8	14 ± 7
IL-1β, pg/ml	13 ± 10	11 ± 8	21 ± 9	22 ± 11 [#]
IL-6, pg/ml	19 ± 25	14 ± 12	30 ± 16	35 ± 14 [#]
IL-8, pg/ml	13 ± 21	12 ± 23	40 ± 42	43 ± 46
IL-10, pg/ml	5 ± 4	5 ± 5	8 ± 5	8 ± 5
IL-12, pg/ml	28 ± 21	24 ± 20	44 ± 28	35 ± 26

[#]Significant difference ($p < 0.05$) between cancer and non-cancer patients within 'pre-transfusion' and 'post-transfusion'.

Discussion

Blood units may accumulate bioactive substances during storage which, in theory, could modulate the recipient's immune system after transfusion [3]. Increasing concentrations of TNF-α, IL-1β, IL-6 and IL-10 in SAGM units after long storage have previously been reported, however, not exceeding in vivo plasma levels [3]. In contrast, IL-8 is reported to attain concentrations of 300 pg/ml [9], which is 5–10 times higher than normal plasma levels.

In the present study no significant changes in immunological markers were observed after infusion of two units of erythrocyte suspension. This supports previous results indicating that SAGM blood during cold storage do not accumulate cytokines in amounts sufficient to increase post-transfusion plasma levels [3]. Obviously, additive amounts with transfusion of cytokines like IL-8 may have been diluted and metabolised within a few hours. The only observed direct effect of blood transfusion was an increase in haemoglobin and a decrease in thrombocyte count. A decrease in thrombocyte count had to be expected when using buffy coat-depleted SAGM erythrocyte suspension, which practically is devoid of thrombocytes.

With infusion of antigens to humans, response time to peak levels of cytokines like TNF-α, IL-6 and IL-8 are within a few hours [10–12]. Therefore, if the blood transfusions had exerted a direct antigenic effect we would have expected a significant cytokine response within the time frame of the study. Previous studies have shown that substances released from

disintegrating granulocytes during storage of blood, e.g. myeloperoxidase from neutrophils and eosinophilic cationic protein from eosinophils, also have a potential of modulating cytokine profiles [3]. Similarly, although in vitro, it has been shown that stored red cells may cause immunomodulation and IL-8 release. This has been suggested to be due to stimulation by red cell membrane-derived lipids which are accumulated in the supernatant of blood units during storage [13]. These effects could not be demonstrated in our in vivo study by transfusion of two units of blood of approximately 550 ml red cell suspension. It is possible that transfusion of a higher number of units could have induced a detectable secretion of cytokines. However, it has previously been shown in in vitro studies that only one erythrocyte unit could cause a reduction in stimulated TNF-α release with a concomitantly increased IL-10 secretion from whole blood-derived recipient cells [14]. The reason for not finding this effect in the present study may have been an insufficient stimulation of immune cells. There was no additional antigen stimulation simultaneously with the in vivo blood transfusion in the present study, but the transfused donor blood definitely served as an alloantigenic stimulus. There was an increased C-reactive protein level (normal upper limit < 10 mg/ml), probably due to disease and anaemia. It was possible to detect differences in inflammatory status between cancer and non-cancer patients. This indicates that it would have been possible to detect expectable effects on immune parameters within the study size, but it also indicates that in vitro study results may not be directly transferable to in vivo conditions.

Fig. 1. VAS scores of self-estimated fatigue before and after transfusion in 11 patients receiving >21 days old red cells and in 11 patients receiving red cells stored <7 days.

Fig. 2. Plasma IL-1 β (pg/ml) before and after transfusion in 11 patients receiving >21 days old red cells and in 11 patients receiving red cells stored <7 days.

Despite of the quality of the present study, several limitations deserve consideration. First of all, the study population may be too small and too heterogeneous to find a low impact of red cell storage time on post-transfusion cytokine concentrations and fatigue. Secondly the study time frame may be too short and thus not detect long-term effects of transfusion. It should therefore be stressed that the present study set-up does not exclude that cytokines and other inflammatory markers may be elevated when measured in a larger group and especially after longer post-transfusion observation periods. Other cytokines such as vascular endothelial growth factor [15, 16] might be sensitive to transfusion with red cells of different

storage time. Thirdly fatigue was self-estimated, and objective measures of e.g. walking distance would have added further strength to our study results.

Haemoglobin concentration between 4.5 and 6.0 mmol/l is only a relative indication for transfusion in normovolaemic patients with no cardiovascular disease in our institution. Self-estimated fatigue was relieved by transfusion of two units of red cells in these mildly anaemic patients, and this was independent of blood storage time. The result should, however, be interpreted in the light of no blinding of the patient receiving red cells. It has previously been shown that blood transfusion may improve well-being and weakness in cancer patients [2,

17], but, with no blinding, it may only be a psychological benefit [18]. Still, it is plausible that the volume effect from blood transfusion may decrease the cardiac and physiologic workload and thereby reduce fatigue. This was supported by our finding that transfusion resulted in improvement of the haemodynamic status, indicated by an increased diastolic pressure. It is reasonable to assume that this 'volume effect' effect may have been identical in the groups receiving blood of short or long storage time, and thereby it could have interfered with a possible difference in fatigue between them. It was interesting to see that not all patients in both groups reported a decrease in fatigue after transfusion, some patients even reported an increase. It should be investigated how the immediate volume-expanding effect of red cell suspension affects fatigue by repeated assessments in the days after transfusion.

In conclusion, this study showed that transfusion of two units of red cells to patients with mild anaemia relieved self-estimated fatigue, but most importantly we found no difference

between transfusion of short- and long-stored red cells. Within 2–8 h after transfusion there was no detectable change in plasma concentrations of the cytokines IL-1 β , TNF- α , IL-6, IL-8, IL-10 and IL-12, indicating no immediate antigenic stimulatory effect from stored transfused red cells. Caution in conclusions should be drawn from the restricted patient number, but the results are useful in the planning of further studies. An indirect effect from stored red cells on cytokine secretion caused by tissue hypoxia or a late stimulation has to be examined by measurements on samples collected more than 8 h post transfusion. The results are readily applicable in the clinical setting in searching for diagnoses when presented to blood sample abnormalities in a patient right after transfusion.

Acknowledgment

Bloddonorernes Forskningsfond is thanked for financial support.

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