Vijith Vijayan, Robert Greite, Sebastian Schott, Julian Doricic, Kukuh Madyaningrana, Pooja Pradhan, Jörg Martens, Rainer Blasczyk, Sabina Janciauskiene and Stephan Immenschuh*

Determination of free heme in stored red blood cells with an apo-horseradish peroxidase-based assay

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Abstract: Transfusion effectiveness of red blood cells (RBCs) has been associated with duration of the storage period. Storage-dependent RBC alterations lead to hemolysis and release of toxic free heme, but the increase of free heme levels over time is largely unknown. In the current study, an apo-horseradish peroxidase (apoHRP)-based assay was applied to measure levels of free heme at regular intervals or periodically in supernatants of RBCs until a maximum storage period of 42 days. Free heme levels increased with linear time-dependent kinetics up to day 21 and accelerated disproportionally after day 28 until day 42, as determined with the apoHRP assay. Individual time courses of free heme in different RBC units exhibited high variability. Notably, levels of free hemoglobin, an established indicator of RBC damage, and those of total heme increased with continuous time-dependent linear kinetics over the entire 42 day storage period, respectively. Supernatants from RBC units with high levels of free heme led to inflammatory activation of human neutrophils. In conclusion, determining free heme in stored RBCs with the applied apoHRP assay may become feasible for testing of RBC storage quality in clinical transfusion medicine.

***Corresponding author: Stephan Immenschuh**, Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany, E-mail: immenschuh.stephan@mh-hannover.de
Vijith Vijayan, Pooja Pradhan, Jörg Martens and Rainer Blasczyk, Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany
Robert Greite, Sebastian Schott and Julian Doricic, Department of Nephrology, Hannover Medical School, D-30625 Hannover, Germany
Kukuh Madyaningrana, Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany; and Faculty of Biotechnology, Universitas Kristen Duta Wacana, 55224 Yogyakarta, Indonesia
Sabina Janciauskiene, Department of Pulmonology, Hannover Medical School, D-30625 Hannover, Germany

Keywords: apoHRP assay; free heme; red blood cells; storage lesion; transfusion.

Introduction

Transfusion of RBCs is a common life-saving procedure in clinical medicine. RBCs can be stored for extended periods of time under defined conditions that is, in general, a maximum of 42 days in most blood banks. The maximum duration of the RBC storing period has been scrutinized in recent years (Belpulsi et al. 2017; Flegel et al. 2014; Lee and Kim-Shapiro 2017) because transfusion of older compared to younger RBCs has been associated with decreased blood quality for transfusion and adverse clinical outcomes in surgical and critical ill patients (Chasse et al. 2016; Francis et al. 2020; Gauvin et al. 2010; Goel et al. 2016; Kanias et al. 2017; Roubinian et al. 2022; Tinmouth et al. 2006; Wagener et al. 2018). Notably, the levels of harmful alterations in RBCs towards the end of the 42 day storage period appear to vary in blood donors and are dependent on genetic and non-genetic factors that may compromise transfusion effectiveness (Chasse et al. 2016; Donadee et al. 2011; Francis et al. 2020; Kanias et al. 2017; Rapido et al. 2017; Roubinian et al. 2022; Wagener et al. 2018). The potential toxicity of long-term stored RBCs has been associated with the so-called "storage lesion" that causes biochemical and functional alterations with subsequent RBC damage ("in-bag" hemolysis) (Bennett-Guerrero et al. 2007; Kim-Shapiro et al. 2011) and release of cell-free hemoglobin (fHb) and free heme (Immenschuh et al. 2017; Schaer et al. 2012). Free heme can be toxic via its prooxidant and pro-inflammatory effects and has been proposed to be a damage-associated molecular pattern (DAMP)(Immenschuh et al. 2017; Kumar and Bandyopadhyay 2005; Soares and Bozza 2016). Moreover, excess free heme may aggravate RBC-dependent mortality in experimental animal disease models (Baek et al. 2012; Graw et al. 2016; Stapley et al. 2015) and in patients with severe sepsis (Adamzik et al. 2012; Larsen et al. 2010). Increase of fHb and free heme that indicates hemolysis in long-

term stored RBCs has previously been measured with a complex absorbance spectrum deconvolution method (Oh et al. 2016) that does not seem to be feasible for applications in clinical transfusion medicine. Thus, it was the goal of the current study to evaluate the diagnostic potential of a previously established apo-horseradish peroxidase (apoHRP)-based assay for measuring free heme levels in supernatants of long-term stored RBCs as an indicator of the storage lesion.

Results

Kinetics of free heme level increase in long-term stored RBCs as determined with the apoHRP assay

A recently developed apoHRP-based heme assay has been adapted for determining levels of free heme in supernatants of stored RBCs (Atamna et al. 2015). In parallel to optical signs of hemolysis the levels of free heme increased time-dependently during the 42 day storage period in two representative RBC units, as measured with the apoHRP assay (Figure 1A). In the following, free heme concentrations were determined in supernatants of 14 individual RBC units over the maximum storage period of 42 days. With the exception of one donor the levels of free heme in fresh RBC units (<7 days) (0–200 nM) of 13 RBC units increased only marginally until day 21 of storage (22–245 nM) (Figure 1B). After day 28 of storage free heme levels in the RBS units began to rise (165–1665 nM) and were markedly higher after 35 (350–4640 nM) and 42 (664–8245 nM) days of storage, respectively (Figure 1B and C). The individual time courses of increased free heme levels from weekly collected supernatants of various RBC units exhibited high variability over the entire 42 day storage period (Figure 1B). Comparison of mean levels of free heme in weekly collected supernatants of the 14 RBC units revealed a statistically significant increase at days 35 and 42 in comparison to fresh RBCs (<7 days) (Figure 1C).

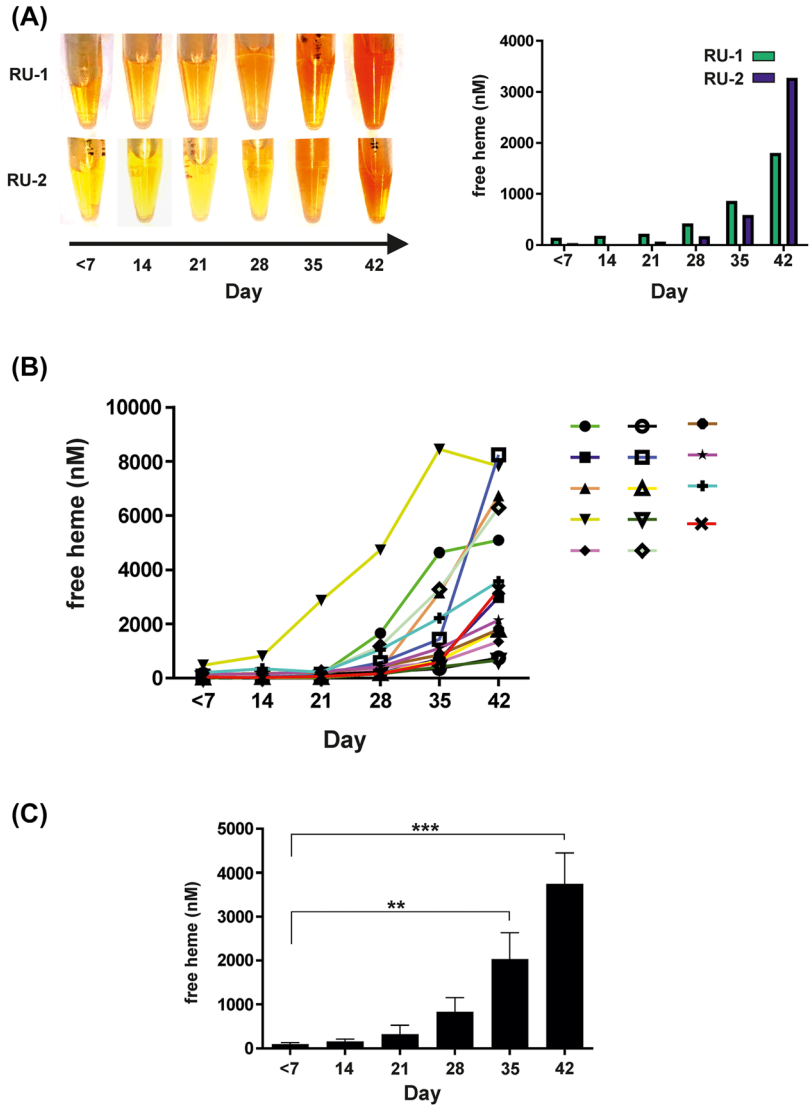

Figure 1: Free heme concentrations increase upon storage of RBC units.
20 mL of blood from two independent RBC units (RU-1, RU-2) at the indicated days was centrifuged at 200 x g and the supernatant was collected in Eppendorf tubes. (A) Visual comparison of supernatants from RU-1 and RU-2 (left panel). Levels of free heme were determined in supernatants from RU-1 and RU-2 at the indicated times with the apoHRP heme assay, as detailed in MATERIALS AND METHODS (right panel). (B) Supernatants collected from 14 independent RBC units at the indicated time points were subjected to the apoHRP heme assay. Levels of free heme in individual fresh (<7 day) versus stored RBC units are given over time (days 14, 21, 28, 35 and 42, respectively). Each color and symbol represents one RBC unit. (C) Means of free heme levels at the indicated times are presented as bar graphs. One way ANOVA was performed for statistical analyses, $^{**}p < 0.01$, $^{***}p < 0.001$. RU, RBC unit.

In summary, prolonged duration of RBC storage leads to a time-dependent increase of free heme levels with accelerated kinetics after day 28 exhibiting major variations between individual RBC units.

Biological activity of free heme in supernatants from stored RBCs

Functional tests were used to evaluate the biological activity of free heme in supernatants from RBCs after 42 days of storage. To this end, RBC supernatants were incubated with hemopexin, a serum scavenger protein known to irreversibly bind and neutralize the toxicity of heme (Immenschuh et al. 2017; Muller Eberhard 1970), before determining heme with the apoHRP assay. Free heme concentrations in supernatants pre-incubated with hemopexin were significantly lower relative to untreated supernatants indicating that the apoHRP assay detected free, but not protein-bound heme (Supplementary Figure 1). Next, a neutrophil activation assay was applied to assess putative pro-inflammatory effects of supernatants from fresh (<day 7 of storage; low concentration of free heme) and long-term stored RBCs (day 42 of storage; high concentration of free heme). Indeed, supernatants from long-term stored, but not from fresh RBC units, caused activation of neutrophils, as indicated by an increase of the surface activation marker CD11b (Figure 2A) and secretion of the pro-inflammatory cytokine interleukin (IL)-6 in the presence of the inflammatory stimulus lipopolysaccharide (Figure 2B). Collectively, the data indicate that free heme levels in supernatants of long-term stored RBCs exert pro-inflammatory effects in primary human neutrophils.

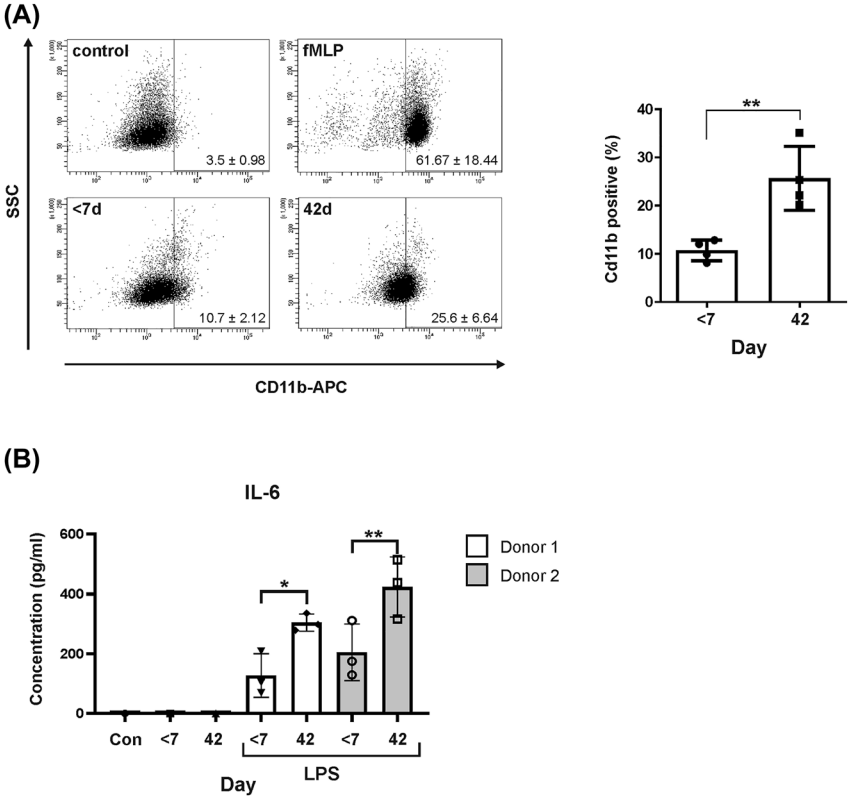

Figure 2: Biological activity of supernatants collected from stored RBCs.
Freshly isolated neutrophils were incubated with 15% supernatants from four different RBC units collected at the indicated times or with freshly prepared hemin (3 µM) for 1 h or with the positive control fMLP (2 µM) for 30 min. Neutrophils were stained with CD11b-APC antibody followed by flow cytometry. (A) The representative dot blots and the % of cells positive for CD11b in relation to the untreated control +/− SD from three independent experiments is shown (left). Statistical significance was assessed for the ability of fresh versus long-term stored supernatnats to up-regulate CD11b from 3 independent experiments using Student's *t*-test, $^{**}p < 0.01$ (right). (B) Freshly isolated neutrophils from two independent healthy donors (donor 1 and 2) were left unstimulated (Con) or incubated with 15% supernatants from three different RBC units collected at the indicated times in the presence or absence of LPS (1 µg/mL) for 24 h as indicated. Supernatants from cell cultures were collected and the concentration of IL-6 was determined by ELISA.

Time courses of fHb and total heme levels in long-term-stored RBCs

Levels of free heme in supernatants of long-term stored RBCs were also directly compared next to each other with concentrations of fHb and total heme. Mean concentrations of fHb increased in four different RBC units with a linear time-dependent kinetics (1.2–1.5 fold weekly) that was statistically significant after 28 days of storage (Figure 3; left panel). For a comparison, the time-dependent increase of free heme in stored RBCs was more variable between individual RBC units (ranging from 1.5- to 4-fold between day 28–42; Figure 3; right panel). While fHb levels after day 28 were within a narrow range of 73–90 mg/dL, free heme concentrations exhibited markedly higher variations from 660 to 2150 nM (Figure 3). Finally, total heme (free heme plus protein-bound heme) was determined in supernatants of four RBC units. Similar to fHb, levels of total heme exhibited a linear increase in weekly intervals ranging from a relative fold up-regulation between 1.2 and 1.5 (Table 1). Moreover, in contrast to free heme, total heme concentrations of stored RBC units were in a similar range after 42 days of storage (21–26 μM) (Table 1). In summary, the time-dependent increased levels of fHb and total heme in stored RBCs are less variable in comparison to free heme at the end of the 42 day storage period.

Discussion

In the current study an apoHRP assay was adapted for measuring free heme in supernatants from stored RBCs. It is demonstrated that levels of free heme increased with an accelerated time-dependent kinetics in long-term stored RBCs towards the end of a 42 day storage period exhibiting major variations in individual RBC units.

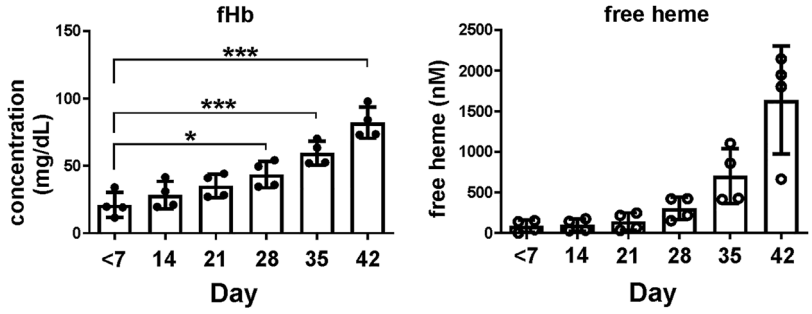

Figure 3: Comparison of free hemoglobin (fHb) and free heme in stored RBCs.
Supernatants from four independent RBC units at the indicated times were analyzed for fHb and free heme, respectively. Levels of fHb were determined by a spectrometric method with a Shimadzu spectral photometer and free heme with the apoHRP heme assay, as described in Materials and Methods. (A) fHb and (B) free heme levels are presented as depicted bar graphs as mean ± SEM. Statistical analysis was performed with one way ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 1: Total heme versus free heme in stored RBC units.

Sample	<Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
			A) Total heme (μM)			
RU1	4.09	5.53	8.59	8.44	15.48	21.53
RU2	5.48	7.15	8.7	14.29	18.21	25.62
RU3	11.61	12.8	14.78	17.9	18.96	24.52
RU4	9.37	11.64	11.61	14.35	17.44	26.05
			B) Free heme (nM)			
RU1	6.04	16.83	22.68	160.4	650.54	1772
RU2	108.36	123.82	144.12	1665.45	4640	5086
RU3	15.03	115.03	120.79	312.58	3155	6730
RU4	237.82	348	294.88	1055.03	2221	4359

Supernatants were collected from four independent RBC units at the indicated times and total heme (A) and free heme (B) at the different time points was measured.

Determination of free heme levels in long-term stored RBCs with an apoHRP assay

Transfusion of RBCs after prolonged storage duration have been associated with the incidence of adverse effects in high-risk patients and decreased transfusion effectiveness (Chasse et al. 2016; Francis et al. 2020; Gauvin et al. 2010; Goel et al. 2016; Graw et al. 2016; Kanias et al. 2017; Roubinian et al. 2022; Stapley et al. 2015; Tinmouth et al. 2006; Wagener et al. 2018). In clinical practice, no indicators are currently available to assess the extent of the storage lesion in individual units of stored RBCs. Because senescence of RBCs leads to in-bag hemolysis, we hypothesized that free heme levels may correlate with the extent of cellular damage in RBCs over a time period of 42 days. An apoHRP assay that has previously been used for determining free heme in cell cultures (Atamna et al. 2015; Yuan et al. 2016) and in tissues (Wang et al. 2019) has been adapted for measuring free heme levels in supernatants of stored RBCs. Notably, the biophysical nature of free heme that is the metabolically active fraction of heme is not well understood (Chiabrando et al. 2014; Schaer et al. 2012). However, an excess of free heme causes prooxidant and pro-inflammatory effects (Dutra and Bozza 2014; Kumar and Bandyopadhyay 2005; Soares and Bozza 2016). Our current findings indicate a pronounced acceleration of the time-dependent increase of free heme levels after day 28 of the 42 day storage period (Figure 1). Our data correspond with a previous report, in which fHb and free heme have been determined with an absorbance spectrum deconvolution approach (Oh et al. 2016; Wagener et al. 2018), a sophisticated method for measuring free heme (for a review see (Hopp et al. 2020)). The presence of biologically active heme in RBC supernatants was confirmed by limiting the availability of free heme for apoHRP to form holoHRP using the heme-binding protein hemopexin (Supplementary Figure 1) and in a functional assay determining activation of primary human neutrophils (Figure 2)(Janciauskiene et al. 2017). However, it needs to be pointed out that the absolute concentrations of free heme that are calculated from the apoHRP assay were not compared to another method of free heme determination in this study. Thus, differences in absolute free heme concentrations, if determined with alternative methods, cannot be ruled out. Importantly, time-courses of free heme levels in supernatants of RBCs over the 42 day storage period were different to those observed for fHb and total heme, respectively (Figure 3 and Table 1). The reason for the different time kinetics are currently not clear.

Free heme as an indicator of RBC storage quality in clinical transfusion medicine

The maximum RBC storage duration that is usually 42 days in most blood banks is controversially discussed (Belpulsi et al. 2017; Flegel et al. 2014; Lee and Kim-Shapiro 2017), because the extent of RBC damage appears to be disproportionally higher towards the end of the storage period (Donadee et al. 2011; Rapido et al. 2017; Wagener et al. 2018). This assumption is supported by the current findings showing an accelerated increase of the time-dependent kinetics of free heme in supernatants from stored RBCs after 28 days of RBC storage (Figure 1). Moreover, our data revealed marked variations in individual RBC units (Figure 1) indicating that storability of RBCs may be affected by a number of blood donor-specific factors (Chasse et al. 2016; Francis et al. 2020; Roubinian et al. 2022). Notably, ethnicity, sex, age and hereditable variants such as glucose-6-phosphate dehydrogenase deficiency or spherocytosis can be associated with such differences (Francis et al. 2020; Kanias et al. 2017). Currently, no specific markers for transfusion quality have been established for testing individual long-term stored RBC units. Thus, determination of free heme levels in single RBC units directly prior to transfusion of high-risk patients may help to identify potentially hazardous stored RBC units with toxic heme levels. The herein applied apoHRP assay may hold promise as a relatively simple and rapid method for determining free heme in clinical transfusion medicine ("point-of-care testing"). To evaluate the feasibility of this approach further studies with higher numbers of RBC units are required.

In conclusion, free heme levels determined with an apoHRP assay may serve as an indicator for transfusion quality in long-term stored RBCs and further studies on the applicability of this method in clinical transfusion medicine are warranted.

Materials and methods

Ethical approval

The study has been approved by the Institutional Review Board of Hannover Medical School (Ethical Approval: MHH 6895).

Materials

N-Formylmethionine-leucyl-phenylalanine (fMLP) was purchased from Sigma (St. Louis, MO, USA), apoHRP from BBI Solutions (Gwent, UK), lipopolysaccharide from InvivoGen (SA, CA, USA) and

hemin from Frontier Scientific (Logan, UT, USA). Human hemopexin was purchased from Athens Research and Technology (Athens, GA, USA) and prepared as previously described (Taketani et al. 1998). Peroxidase Substrate Kit TMB-ONE was from Kem-En-Tec Diagnostics A/S (Copenhagen, Denmark) and RPMI 1640 medium from Life technologies (Carlsbad, CA, USA).

RBC units

Blood bags manufactured by Haemonetics with leukocyte filters (Product Code WBT 434GCB) were utilized for collection of 450 mL blood in 63 mL citrate phosphate dextrose from 14 different donors in the Institute for Transfusion Medicine and Transplant Engineering at Hannover Medical School with the whole blood filtration method (Semple et al. 2012). Blood collection was performed by certified technicians with standard operating procedures and stored in a monitored refrigerator. RBC aliquots were taken in weekly intervals.

Hemin stock

Hemin stock solution was prepared in dimethyl sulfoxide and the concentration was determined using absorbance at 400 nm in 40% dimethyl sulfoxide and the molar extinction coefficient of 180 using Synergy 2 multi-mode plate reader (BIOTEK).

ApoHRP stock solution

The concentration of apoHRP was determined using a molar extinction coefficient of 20,000 at 280 nm and stored in aliquots at −20 °C until use.

Establishing the standard curve of apoHRP reconstitution with hemin (free heme assay)

The stock solution of hemin was diluted in dimethyl sulfoxide to 25 nM and immediately used for the labile heme assay. Hemin standards were prepared in Hank's balanced salt solution (HBSS) with 0.5% bovine serum albumin (BSA) at the following concentrations 0.25, 0.5, 1, 1.5, 2 and 2.5 nM in a final volume of 100 µL per reaction which contains 0.75 µM of apoHRP and the assay was performed as previously described (Atamna et al. 2015; Sudan et al. 2019). In brief, the reaction mixture was incubated for ten minutes in 4 °C, after which 5 µL out of each reconstitution reaction was added to 96-well plate wells followed by 200 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Holo-HRP activity was measured by absorbance at 652 nm for the enzymatic oxidation of TMB in a kinetic fashion immediately following addition of the substrate until the absorbance value for the highest standard was between 1.2 and 1.4. The absorbance data at 652 nm was plotted against concentration of hemin to establish the standard curve. As shown in Supplementary Figure 2, the assay using this protocol was consistently linear and reproducible. Although the standards worked in HBSS alone the presence of low concentrations of BSA appeared to stabilize the hemin standards.

Sample preparation from RBC units for free heme measurement with the apoHRP assay

20 mL from stored RBC units was collected at regular intervals (the day the RBC units was received followed by day 7, 14, 21, 28, 35 and 42 days from the date of production) and centrifuged at 200 × g for 10 min. The supernatant was transferred to Eppendorf tubes and centrifuged at 200 × g for 10 min to remove residual RBCs. The supernatants obtained were aliquoted and immediately stored at −80 °C until further use. For the apoHRP assay, supernatants were diluted in HBSS in a range between 1:50 to 1:16,000. Dilutions with an absorbance in the range of the hemin standards (0.25–2.5 nM) were used for calculating the final concentration of free heme in the sample.

Determination of fHb

fHb was measured by a spectrophotometric method with a Shimadzu spectral photometer as previously described (Kahn et al. 1981).

Flow cytometry

Primary neutrophils were isolated from fresh blood obtained from healthy donors using polymorphprep (Axis-Shield, Oslo, Norway), as described previously (Janciauskiene et al. 2017). 3×10^5 cells were incubated with 15% supernatant from fresh and long-term stored RBCs or freshly prepared hemin (3 µM) in RPMI medium. After 1 h of incubation in the cell culture incubator (37 °C, 5%CO_2, 100% humidity) cells were washed with PBS and incubated for another 20 min with an antibody against CD11b conjugated with APC (Biolegend) or isotype control, respectively. Cells were analyzed by flow cytometry using BD FACS CANTO II and FACS analysis was performed with BD FACS diva software.

IL-6 ELISA

At the end of the experiment supernatants were collected, centrifuged at 200 × g for 5 min, and transferred to fresh Eppendorff tubes that were stored at −80 °C until use. The IL-6 ELISA (R&D Systems, MN, USA) was performed according to the manufacturer's instructions.

Determination of total heme with the pyridine hemochromagen assay

Pyridine hemo-chromagen assay was performed, as previously described (Barr and Guo 2015). Briefly, the supernatants were mixed with solution I (0.2 M NaOH, 40% (v/v) pyridine, 500 µM potassium ferricyanide) in a 1:1 ratio, followed by the addition of 10 µL of solution II containing 0.5 M sodium dithionite in 0.5 M NaOH. The absorbance was recorded kinetically at 557 nm up to 5 min. The absorbance with the highest peak was taken as the reduced pyridine-heme spectrum and the concentration was calculated using Beer's law, $A = \varepsilon\ c\ l$ (Absorbance = extinction coefficient × concentration × path length) with an extinction coefficient of 34.7 $mM^{-1}cm^{-1}$.

Statistical analysis

Statistical analysis was performed by One way ANOVA or Student's *t*-test using Prism 6.0 software (GraphPad, San Diego, CA, USA). A $p < 0.05$ was considered significant.

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