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The effect of packed red blood cell storage on arachidonic acid and advanced glycation end-product formation

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Abstract

Introduction: The transfusion of packed red blood cells (PRBCs) is a significant risk to blood recipients. Blood banking procedures permit the storage of PRBCs for up to 42 days. Storage of PRBCs can cause polymorphonuclear granulocytes (PMNs) activation and the development of neutrophil-mediated transfusion-related acute lung injury. The aim of our study was to determine if PRBC storage has an influence on the formation of arachidonic acid (AA) and advanced glycation end products (AGEs).
Materials and Methods: Twenty units of PRBCs were used to measure AA and AGE levels. The samples were taken on the 0th, 14th, 28th, and 42nd days of PRBC storage. The AA level was analyzed by gas-liquid chromatography-mass spectrometry and AGE level by an immunoenzymatic test.
Results: During the first 14 days of PRBC storage, the AA level significantly increased and then slowly decreased. The AGE level increased continuously during the whole time of the study. In a model experiment, the AA glycoxidation product trans-2-nonenal (T2N) formed adducts in reaction with hemoglobin which were detectable with the test for AGE.
Conclusions: It is highly probable that the observed increase in AGE level is related to the decrease in AA in PRBCs, which can be associated with the formation of toxic aldehydes, especially T2N and 4-hydroxynonenal (HNE), from AA. Glucose in the PRBCs (preservative solution) can contribute to AGE formation as well. The formation of AGEs, HNE, and T2N in PRBCs, their influence on PMNs *in vitro*, and confirmation of our assumption need further studies.

Key words: packed red blood cells, polymorphonuclear granulocytes, arachidonic acid, 4-hydroxynonenal, trans-2-nonenal, advanced glycation end products.

Abbreviations: AA – arachidonic acid, ADSOL – adenine, dextrose, sodium chloride, and mannitol, AGE – advanced glycation end product, BSA – bovine serum albumin, CML – N^{ε} (carboxymethyl)lysine, CPD – citrate/phosphate/dextrose, ELISA – enzyme-linked immunosorbent assay, HETES – hydroxyeicosatetraenoic acids, HNE – 4-hydroxynonenal, HRP – horseradish peroxidase, IL-1β – interleukin 1β, IgG – immunoglobulin G, MOF – multiple organ failure, PBS – phosphate-buffered saline, PLA_2 – phospholipase A_2, PMN – polymorphonuclear granulocyte, PRBC – packed red blood cell, PUFA – polyunsaturated fatty acid, SH – sulphydryl, TBS-T – tris-buffered saline-Tween, T2N – trans-2-nonenal, T2N-Hb – T2N-hemoglobin, T2N-Mb – T2N-myoglobin, TNF-α – tumor necrosis factor α, TRALI – transfusion-related acute lung injury, WBC – white blood cell.

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INTRODUCTION

Blood transfusion can be a significant risk to blood recipients. Severe reactions after transfusion, such as acute hemolysis, fever, allergic reactions, or lung edema resulting from non-cardiac causes of transfusion-related acute lung injury (TRALI), are associated with antigen-antibody interaction. This complication of blood transfusion was first reported and defined in the 1970's. It is thought that despite improvements in intensive care and

methods of therapy which allow keeping critically ill patients alive longer, allogenic blood transfusion can lead to the development of multiple organ failure (MOF) [17, 36]. It is known that excessive activation of polymorphonuclear granulocytes (PMNs) plays a central role in the pathogenesis of MOF [3, 21, 23, 24, 37, 38, 39].

TRALI was the post-transfusion complication which focused the attention of investigators on the processes occurring in stored packed red blood cells (PRBCs). Numerous studies showed that the reason of this complication was not only the antigen-antibody reaction, but also the production of different mediators which lead to the priming or activation of PMNs. These mediators are tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, and biologically active lipids, predominantly arachidonic acid (AA) [11, 18, 19, 22, 28, 30].

AA is an essential polyunsaturated fatty acid (PUFA) and a constituent of the phospholipid domain of the cell membrane. AA is released from membranes by phospholipase A_2 (PLA_2), and its subsequent conversion into leukotrienes, prostaglandins, and other eicosanoids is an important catalytic event in the processes leading to inflammation [2]. The AA metabolite tromboxane A2 escalates the adhesion of PMNs to the endothelium [36] and their diapedesis [8]. AA influences many intracellular processes, including the regulation of protein kinase C function, and can play an important role in vessel damage and as a second messenger [13, 16]. AA can be metabolized to eicosanoids via activation of three distinct pathways: a) the cyclooxygenase pathway, resulting in the formation of prostaglandins, prostacyclins, and thromboxanes; b) the lipoxygenase pathway, resulting in the formation of lipoxins and HETES; and c) the cytochrome P-450-dependent epoxygenase pathway, resulting in the formation of epoxy metabolites [13].

In biological systems, the PUFAs of membrane phospholipids are also utilized in non-enzymatic lipid peroxidation initiated by free radicals [26], which in turn can cause non-enzymatic protein modifications [33]. Lipid peroxidation associated with oxidative stress in cells and tissues is implicated in a wide range of pathophysiological disorders by the formation of reactive compounds such as fatty aldehydes [20, 26, 40]. The potential targets of lipid peroxidation products are erythrocytes. The high concentration of PUFAs in membrane phospholipids, which provides a rich substratum for oxidants and transition metals capable of serving as redox agents, hemoglobin, and other heme-containing proteins, can augment lipid peroxidation [40].

Among the reactive end products of the peroxidation of cellular membrane lipids (linoleic and AAs) are trans-2-nonenal (T2N) and 4-hydroxynonenal (HNE) [1, 9, 25]. Siems et al. [27] found that at least some of the damage observed in free-radical pathology is mediated by HNE and other aldehydes, which may act as "second toxic messengers" of the primary free-radical event. HNE exhibits a wide range of biological activities, including inhibition of protein and DNA synthesis, inactivation of enzymes, stimulation of phospholipase C, reduction of gap junction communication, and stimulation of neutrophil migration. Many of these *in vitro* effects, which are observed at low micromolar or even submicromolar HNE concentrations, have been attributed to the modification of cellular proteins by HNE [20]. It was shown that HNE accumulates in erythrocytes [29]. Selley et al. [26] demonstrated that HNE can cause changes in the structural arrangement of phospholipidic membranes, thereby promoting activation of PLA_2. Subsequent inactivation of the enzyme can occur as a result of the accumulation of HNE and its reaction with membrane sulphydryl (SH) groups facing the interior of the platelet [26]. Upon reaction with proteins, HNE reacts with the SH group of cysteine, the imidazole nitrogen(s) of histidine, and the ε-amino group of lysine, resulting in enzyme inactivation [15]. With cysteine and histidine residues, HNE forms Michael addition-type products having a hemiacetal structure, whereas with lysine residues, HNE forms pyrroles and, to a lesser extent, fluorescent cross-linked products [20].

The other compound structurally related to HNE is the less extensively studied T2N [6]. It is mainly formed from oxidative degradation of palmitoleic acid [10]. This aldehyde possesses a reactive 2-alkenal functional group which specifically uncouples mitochondria as HNE does. Echtay et al. [6] believe that this reactive alkenal group modifies specific residues in the uncoupling proteins and adenine nucleotide translocase, leading to increased proton transport activity. Sakuma et al. [25] suggest that T2N can be a modulator of platelet AA metabolism by affecting the activity of cyclooxygenase and 12-lipoxygenase.

The ability of HNE to bind with proteins can probably lead to the formation of advanced glycation end products (AGEs). Most of these "glycoxidation" reactions usually require the presence of oxygen and trace redox metal ions, and the products are collectively referred to AGEs [4]. AGEs are formed in physiological systems by the reaction of highly reactive α-oxoaldehydes with proteins, basic phospholipids, and nucleotides and by the degradation of fructosamines. α-Oxoaldehydes are formed by the degradation of glucose, Schiff's base adducts, and fructosamines, by glycolytic intermediates and lipid peroxidation [33, 34]. Khalifah [12] identified *in vitro* the pathways of AGE formation involving lipid oxidation. The most abundant AGE is probably N^{ε}(carboxymethyl)lysine (CML) [4], a product of both lipid peroxidation and glycoxidation reactions. Fu et al. [7] consider lipoprotein peroxidation a potential source of CML and the major source of CML formed during metal-catalyzed oxidation of low density lipoprotein *in vitro*. Since PUFAs, both in biological systems and *in vitro*, are, in general, more easily autoxidized in free-radical reactions than are carbohydrates, it is quite possible that most of the CML in tissue proteins comes from lipid peroxidation reactions, even during hyperglycemia, when concentrations of glucose and Amadori products on the proteins are increased [4, 7]. Glucose and other reducing sugars

react non-enzymatically with amino groups of free amino acids, peptides, and proteins, leading to AGE formation [32]. Surprisingly, most AGEs that have been detected *in vivo* are produced by the reaction of proteins with sugars, carbohydrate-related compounds such as ascorbate, or dicarbonyl intermediates that can be produced from glycoxidative and lipoxidative reactions [12].

The aim of this study was to determine whether AA is released and AGEs are formed in PRBCs at various periods of their storage and, possibly, to find correlation between these factors.

MATERIALS AND METHODS

Preparation of PRBCs

This study was approved by the Ethics Committee of the Wrocław Medical University. For the experiments, 20 units of PRBCs from randomly selected blood donors were supplied by the Regional Center of Blood Donation and Blood Therapy in Wrocław. The PRBCs were obtained after removing most of the plasma from full blood and adding a suitable volume of adenine, dextrose, sodium chloride, and mannitol (ADSOL) solution. Full blood from donors was collected in containers with 63–70 ml of citrate/phosphate/dextrose (CPD) solution, which was prepared with trisodium citrate, citric acid, glucose, and sodium dihydrogenphosphate. Then the blood with CPD was fractionated by centrifugation into fractions of red blood cells, thrombocyte concentrates, and plasma. After separating the red blood cells, 100 ml of ADSOL solution was added. The PRBCs were stored at 2–6°C. One unit of PRBCs consisted of red blood cells and different residual amounts of thrombocytes and leukocytes, depending on the centrifugation conditions. Samples of PRBCs for analyses were taken on days 0, 14, 28, and 42 of PRBC storage.

Determination of released AA in PRBCs

PRBC samples of 10 ml were centrifuged at 4000 rpm at temperature of 4°C for 10 min. The supernatant, containing extracellular AA, was collected and dried under a stream of nitrogen and then methanolized with 1 M methanolic HCl (1 ml) at 100°C for 1 h. The samples were extracted twice with a mixture of hexane and water (1:1, v/v) and the upper phase, containing methyl esters of fatty acids, was collected and dried under a stream of nitrogen. The internal standard (10 μg) of deuterated methyl ester of AA was added to the samples. Analysis of AA derivatives was performed using gas-liquid chromatography combined with a Hewlett-Packard 5971A mass spectrometer equipped with an HP-1 (0.22 mm×12 mm) glass capillary column, with helium as the carrier gas. The samples, dissolved in hexane, were applied (1 μg) to the column and measurements were made in the temperature range of 150–250°C in steps of 8°C/min.

Determination of total AGE level in PRBCs

The immunochemical assay used in this study involved the model protein AGEs and rabbit anti-AGE antibodies [31, 32]. The ELISA inhibition test for the determination of AGEs is based on an affinity-purified antibody and AGE antigen, where a serum sample treated with proteinase K is used as an inhibitor [32], and was carried out according to a modified procedure of Turk et al. [35]. Antigen AGE-BSA and anti-AGE-myoglobin antibody were prepared as previously described [31]. The specificity of the elaborated tests concerns epitopes distinct from those of routinely synthesized markers by using glucose, ribose, methylglyoxal, or glycolaldehyde (data not shown). The structure of the epitope recognized by the specific antibodies will be described elsewhere. The 96-well plates (Nunc, Denmark) were coated with AGE-BSA (0.7 µg/well) in 100 µl of coating carbonate buffer (0.2 M, pH 9.6), incubated for 4 h at room temperature, and then at 4°C overnight. On the following day, AGE-protein-coated plates were blocked with 0.05% casein and then washed three times with tris-buffered saline-Tween (TBS-T). Cells present in the PRBCs were lysed with phosphate buffer (0.005 M, pH 8.3) at room temperature for 10 min, then the samples were centrifuged (14000 rpm, 4°C, 10 min). To release AGEs, the samples were treated with proteinase K (Sigma, Germany). After 24 h the samples were added to the wells, then the plates were incubated with rabbit anti-AGE antibody (100 µl/well) at room temperature for 1 h. After that time, unbound antibodies were washed out with TBS-T. Then the plates were incubated with anti-rabbit IgG-HRP (DakoCytomation, Denmark) at room temperature for 1 h. After washing with TBS-T, o-phenylenediamine (Sigma, USA) diluted in methanol and citrate buffer (pH 4.5) and hydrogen peroxide was added to the wells. The reaction was stopped by the addition of 40% H_2SO_4. The absorbance at 492 nm was measured with an Opsys MR ELISA reader (Dynex Technologies, USA). The quantity of AGE in the samples was evaluated in U_{AGE} units, which are defined as the percentage of inhibition of the specific reaction. One U is considered equal to 1% inhibition under test conditions.

$$\%\ \text{inhibition} = [1-(A_s-A_{min})/(A_{max}-A_{min})]\times 100\% = U_{AGE}$$

where: A_s – mean value of absorption for serum samples, A_{min} – mean value of absorption for PBS only, A_{max} – mean value of absorption for rabbit anti-AGE antibodies only.

All measurements are expressed as mean ±SEM. Statistical analysis was performed with the U Mann-Whitney test (Statistica 5/97 software). A p value <0.05 was considered to represent a significant difference.

Synthesis of T2N adducts with proteins and analysis of their fluorescence properties

In order to examine the possibility of AGE formation with the participation of an aldehyde derived from

unsaturated fatty acids, namely T2N, two model compounds were synthesized as previously described [5]. Ethanol solvent was carefully removed at room temperature with a gentle stream of nitrogen from 3.85 mmoles of T2N solution (0.5 ml; Aldrich, Germany) and the sample was immediately dissolved in 0.5 ml of PBS, pH 7.3. Then 3 mg of protein (myoglobin and hemoglobin) was added to this solution and the reaction mixture was incubated at 36°C for 2 h. Then the material was lyophilized to remove any unreacted T2N. The lyophilized samples were dissolved in 300 µl of water. A small part of the undissolved material was separated by centrifugation at 10000 rpm at 4°C for 15 min. The obtained supernatants of synthetic model compounds (T2N-Hb – T2N--hemoglobin and T2N-Mb – T2N-myoglobin) were used for recording fluorescent spectra with a Luminescence Spectrophotometer LS50B. The samples were dissolved in 3 ml of PBS, pH 7.3, and their emission spectra were analyzed between 350–600 nm at three different excitation wavelengths (328, 336, and 370 nm).

RESULTS

In order to determine the level of released AA and the formation of AGEs in PRBCs at various periods of their storage, the experiments were performed on 20 units of PRBCs delivered from the Regional Center of Blood Donation and Blood Therapy in Wrocław. The material was stored at 2–6°C and analyzed from day 0 to day 42. AA was determined in the extracellular supernatant, whereas the total AGEs were measured in the whole PRBCs. A statistically significant increase in AA level could be observed from days 0 to 14 of the study, but after that period the level decreased. The highest mean value of AA was about 2 µg/ml (Fig. 1). In contrast, an increasing level of AGEs in the PRBC samples could be observed during the whole course of the experiment, with the highest increase in the last period of the study, reaching 44.2 U_{AGE}=44.2% inhibition under the test conditions compared with the initial value of 17.7% (Fig. 2). In order to test the assumption that the increase in AGEs might be related to the lowered level of released AA, T2N, a model aldehyde product, was subjected to reaction with hemoglobin, a major protein of erythrocytes, whereas myoglobin was used as a control protein. We tested T2N, a product also related to the degradation of AA, due to the fact that the role of HNE in forming protein adducts is already well established. The model high-molecular-mass AGEs, the T2N-protein conjugates (T2N-Hb and T2N-Mb), were synthesized and measurement of their fluorescence spectra revealed that these compounds did not express distinct fluorescence properties (data not shown). A further experiment was performed to assess the ability of the model T2N-Hb and T2N-Mb adducts to inhibit the polyclonal rabbit anti-AGE antibody. The ELISA inhibition test was used to measure the level of total AGEs in the synthesized T2N-Hb and T2N-Mb adducts. Results of

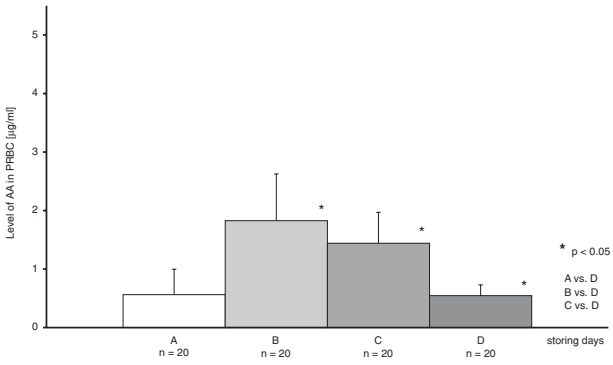

Fig. 1. Relationship between the level of released AA in PRBCs and the duration of PRBC storage. A, B, C, D – PRBC storage days: A – 0, B – 14, C – 28, D – 42, n – number of PRBC samples.

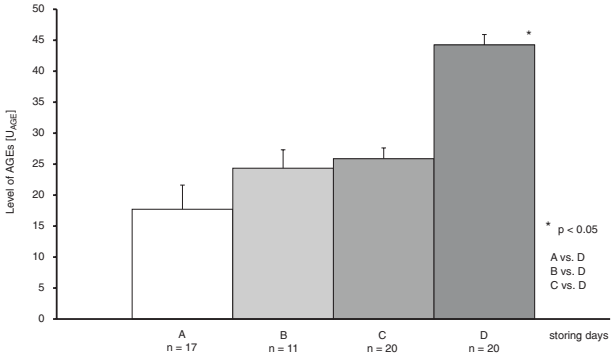

Fig. 2. Relationship between AGE level and the duration of PRBC storage. A, B, C, D – PRBC storage days: A – 0, B – 14, C – 28, D – 42, n – number of PRBC samples.

this experiment with the rabbit anti-AGE antibodies revealed the presence of AGEs in both T2N-protein adducts. The inhibition of anti-AGE antibodies by T2N--Hb adducts was 43.95%, similar to that of these antibodies by PRBC components, whereas the inhibition with T2N-Mb reached 86.32%.

DISCUSSION

The components of PRBC preparations and the storage time of PRBCs can be significant risk factors leading to complications of blood transfusion. Units of PRBCs also include residual platelets, plasma, and white blood cells (WBCs). The life spans of platelets and WBCs are short. In PRBCs, various proinflammatory cytokines and biological active lipids are formed, namely IL-1β, TNF-α, IL-8, and AA. They can lead to excessive PMN activation, which plays a central role in the pathogenesis of MOF [36].

The polyunsaturated fatty acyl groups of membrane phospholipids are highly susceptible to peroxidation by oxygen radicals, and the self-propagating chains of free--radical reactions produce various aldehydes, alkenals, and hydroxyalkenals, such as malondialdehyde and HNE. Many of these products are cytotoxic, probably

due to their reactivity with proteins. However, HNE does not only possess toxic properties. Structurally related compounds, such as T2N, trans-2-nonenoic acid, trans-retinal, and other molecules containing the reactive 2-alkenal group, have the same effect as HNE, suggesting that the alkenal group of these compounds reacts with a target protein in the mitochondria, leading to increased proton transport activity [6].

One of the essential PUFA, a constituent of the cell membrane phospholipids, is AA. AA is released from membranes by PLA_2 and its subsequent conversion into leukotrienes, prostaglandins, and other eicosanoids is an important catalytic event in the processes leading to inflammation [2]. AA can be also utilized in the non-enzymatic lipid peroxidation initiated by free radicals [26]. We believe that the decreasing AA level can be caused by the formation of peroxidation products, the highly reactive aldehydes T2N and HNE. HNE is able to produce a variety of powerful biological effects, such as enzyme inactivation, lysis of erythrocytes, chemotaxis of neutrophils [27], and also exhibits a number of hepatotoxic, mutagenic, and genotoxic effects [26, 27]. Similar biological effects have also been revealed for the protein adducts of T2N [5].

Most AGEs that have been detected *in vivo* are produced by the reaction of proteins with sugars, carbohydrate-related compounds such as ascorbate, or with dicarbonyl intermediates that can be produced from glycoxidative and lipoxidative reactions and from intracellular metabolic fluxes. At highly elevated sugar and protein concentrations in *in vitro* studies under decidedly unphysiological conditions, a number of AGE-compounds are formed. These reactions also engage lipid oxidation intermediates; thus most AGEs are formed by a combination of glycation and oxidation (glycoxidation) reactions, to which reactive oxygen species and free radicals may contribute and which require trace levels of catalytic redox-active transition metal ions [12].

It is very likely that the presence of glucose in the preservative solution may contribute to the production of AGEs in PRBCs. Protein and other membrane and cellular products are released as a result of partial but gradual cell lysis in PRBCs. These products, mainly hemoglobin, may participate in the glycation process in the presence of 2.6% glucose, resulting in increased AGE level. It is worth noting that the routine method of AGE synthesis relies on keeping a mixture of glucose with protein for over 30 days. In our experiments, the initial level of AGEs in PRBCs (with some percentage of hemolysis) was shown to increase gradually, but the level of released AA decreased. In further experiments we found that AGEs could be formed with the participation of hemoglobin and T2N, related to AA degradation products. Therefore it is highly probable that the observed gradual increase in AGE level is partially related to the decreasing AA level in PRBCs. AGEs are cytotoxic compounds [14] which may contribute to complications after PRBC transfusion. It was already shown in *in vitro* study that T2N-myoglobin conjugate is highly cytotoxic and genotoxic [5]. Due to the possible contribution of glucose present in preservative solution in AGE formation, this could be a reason to replace this reducing sugar with dextrane, sorbitol, or trehalose in this solution. The formation of AGEs, HNE, and T2N in PRBCs, their influence on PMNs *in vitro*, and confirmation of our assumption need further studies.

Our results can have essential practical significance. They provide a new view of the risk connected with the storage of blood products. We would like to draw the attention of clinicians to new aspects of post-transfusion complications. The results of our study show that it may be necessary to change the standard compositions of enriching and preservative solution for PRBCs as well as the blood banking procedures concerning PRBC storage time.

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