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CORRELATION OF FREE HEMOGLOBIN LEVEL AND PLASMA NITRIC OXIDEIN PACKED RED CELL DURING BLOOD BANK STORAGE PERIOD

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ABSTRACT

Stored red blood cells undergo morphological and biochemical changes with increased storage time, commonly referred to as the storage lesion, reduced integrity of erythrocyte membrane, causing hemolysis and increased free plasma hemoglobin level. Nitric Oxide (NO) is an endogenous vasodilator with the major role in vascular and blood flow regulation. Accumulation of free hemoglobin (Hb) during storage RBC hemolysis is thought to be correlated with elevated NO consuming causes low NO level that significantly impair endothelial function. This study aimed to was to analyze the correlation of free Hb and plasma nitric oxide level during PRC storage period in the blood bank. This research was an analytic study with a cross-sectional design to 14 unit PRC those were stored in the Dr. M. Djamil Padang Hospital Blood Bank. The study was conducted from May 2016 to August 2017. The assay was performed for 28 days of storage with one week intervals. Free plasma hemoglobin and NO level were analyzed by cyanmethemoglobin and colorimetry method respectively. Spearman test was used to access the correlation between free Hb level and nitric oxide plasma with p<0.05 means significant. Most donors were male (85.7%) with age range were 33(9) years. Free Hb and nitric oxide level were significantly differentiated in each week storage periods (p<0.05). Two parameters was significantly had moderate negative correlation during storage periods (r=-0.56; p=0.01).

Key words: Free Hb, hemolysis, nitric oxide, storage lesion

INTRODUCTION

Transfusion of blood component represents one of the most common medical therapies, with more than 14.5 million Packed Red Cell (PRC) units administered in the United States per year in 2011. It is remarkable that approximately 40% of all critically ill adult patients receive at least one unit of PRC in the intensive care unit, with a mean of 5 units per patient.2,3 Packed red cells, the most common using blood component, are derived by the collection of 450–500 mL of whole blood collected in plastic bags, preferably in double or multiple plastic bag system. Plasma is separated from red blood cells following either centrifugation or undisturbed sedimentation at any time before the expiry date of blood. The product consist of large quantities of hemoglobin-containing erythrocyte and hematocrit adjusted to 55-65%.4,5 The Food Drug Administration and American Association of Blood Banks currently permit blood preserved in ADSOL solution to be stored for 42 days.5,6

Stored red blood cells undergo some morphological and biochemical changes with increased storage time, commonly referred to as the storage lesion. Morphological changes include reduced deformability, increased osmotic fragility, and spheroechinocyte formation, which are associated with a progressive reduction in intracellular levels of ATP and 2,3-diphosphoglycerate and dropping pH values. Also, prolonged red cell storage favors oxidant stress conditions, which lead to lipid peroxidation, oxidation of membrane and cytoskeletal proteins, and reduced integrity of the erythrocyte membrane.10,12 There is increasing hemolysis during packed red blood cell storage that increases cell-free plasma hemoglobin over time. Fragmentation and formation of 50- to 100-nm microparticles, which contain a substantial amount of hemoglobin, also occur as red blood cells age.13,14 According to current guidelines, at the end of the storage period, only 75% of erythrocytes are required to be recoverable 24 hours after transfusion, which suggests that further hemolysis and microparticle formation occur in-vivo after transfusion. These findings are concerning because it is known that free hemoglobin reacts with nitric oxide.13,14

Nitric Oxide (NO) was an endogenous vasodilator with the major role in vascular and blood flow regulation.15 Endothelial Nitric Oxide Synthase (eNOS) was thought to be the major source of NO
for regulating vasoactivity. Nitric oxide acts by diffusing into the smooth muscle cells and activating soluble guanylyl cyclases resulting in smooth muscle relaxation and vasodilation. Reduce of NO concentration contributes to adverse effects of vascular dilatation response.\textsuperscript{15,19}

Free hemoglobin is the potent scavenger of NO. It reacts with NO through a classic dioxygenation reaction which formed methemoglobin and nitrate, 1000 times faster than an intact erythrocyte. Accumulation of free hemoglobin during storage RBC hemolysis was thought to be correlated with elevated NO consuming causing low NO level. Hemolysis in stored blood also decreases NO production by releasing red cell arginase-1, an enzyme that converts arginine to ornithine, thereby reducing arginine, a substrate for NO synthase. Reduced NO production and elevated NO consuming by free hemoglobin will decrease NO bioavailability that significantly impaired endothelial function.\textsuperscript{15,15,24,25}

The number of studies reporting on the potential adverse effects of PRC transfusion on patient morbidity and mortality, has increased significantly in recent years, which has initiated an intense debate on the benefits and risks of PRC. The release of free hemoglobin and its influence on the intravascular NO metabolism after transfusion has been attributed an important role underlying the adverse effects of PRC administration.\textsuperscript{17,22} Researchers initiated a study of stored blood to analyze the correlation of free hemoglobin and NO level during PRC storage period in the blood bank.

METHODS

This research was an analytic study with a cross-sectional design, conducted from May 2016 to August 2017. The study protocol was approved by the Ethical Review Board of The Andalas University Faculty of Medicine. Fourteen selected PRC units from the donor with informed consent were obtained from the Dr. M. Djamil Central Hospital Blood Bank, Padang. These units were nonleukoreduced and nonpreserved. The units were just for this research only and did not use for any transfusion. The units were stored in a temperature-controlled refrigerator at 2°C to 6°C and sampled weekly over 28 days starting at the age of 0 days old. Samples collected at weekly intervals were measured for free plasma hemoglobin by conversion to cyanmethemoglobin with Drabkin’s reagent and then by spectrophotometric measurement of absorbance at 540 nm.\textsuperscript{24} Samples than centrifuged at room temperature at 2700 g for 20 minutes. The supernatant was stored at -80°C until the last day of research and later thawed for analysis of NO consumption with an NO colorimetry analyzer.\textsuperscript{24} Sample with noticeable hemolysis, bacterial contamination, an indication of clot and leakage by researcher observation during storage were excluded.

Statistical analysis was performed using computer programme. The difference each timecourse experiments of banked blood were analyzed by General Linear Model (GLM) Repeated Measures and Wilcoxon test for normal data distribution of NO and free hemoglobin with unnormal data, respectively. The Spearman correlation test was used to access the correlation between these two continuous variables with p < 0.05 means significant.

RESULTS AND DISCUSSION

Fourteen selected PRC units from informed consent donor were studied weekly over 28 days starting at then age of 0 days old. Most of the donors were male (85.7%) with age average was 33(9) years. The mean of hemoglobin level were 14.7(0.9) g/dL, and the majority of the donor blood type were O (42.9%). The sample units were nonleukoreduced and nonpreserved with CPDA anticoagulants. The average of hemoglobin level dan hematocrit of the PRC units were 24.9(1.4) g/dL and 77.1(4.8)%, respectively (Table 1).

Prolonged RBC storage results in elevated free plasma hemoglobin levels during PRC storage period in the blood bank. In total 14 units we studied, free plasma hemoglobin level was increased with time. The mean of free hemoglobin level was 0.03(0.05) g/dL in 0 days old of storage and rose to 0.26(0.11) g/dL at the end of the study. Free hemoglobin level in each time-course experiments was significantly differed in each week storage periods (p < 0.05) (Figure 1). The enhancement of free hemoglobin level proves occurrence of hemolysis during PRC storage. Although the exact mechanisms have not yet been fully elucidated, the storage lesion occurring as a result of morphological and biochemical change during RBC storage has been attributed an important role causing fragmentation and rupture of erythrocyte, which releases hemoglobin. In line with this study, Spinelli et al. also obtained that free hemoglobin level rose significantly in linear with PRC storage duration, from 0.03 g/dL in day 5 to 0.11 g/dL in day 33 of storage.\textsuperscript{25}

Nitric oxide concentration was subsequently
measured in all plasma samples. In contrary with free hemoglobin data, the NO level decline significantly over time of PRC storage from baseline 33.92(4.75) μmol/L to 9.09(3.57) μmol/L at the end of storage. Statistical analysis results indicate the mean differences of NO content which is significant for each storage period (p<0.05) (Figure 2). Considerable decrease in NO level during PRC storage demonstrate NO consuming by hemolysis product as a consequence of storage lesion. Corresponding to previous reports, the concentration of NO consumed increased dramatically with RBC storage time. This reports obtained NO consuming in reaction stoichiometry by measuring NO catabolism through PRC supernatant injection into an aerobic solution.13 Another study also documented significant NO decline caused by NO consuming, which is correlated with storage duration (r=0.62 dan p=0.02).26

The examined correlation of free plasma hemoglobin and nitric oxide level during PRC storage period in the blood bank. The Spearman correlation test which was conducted to access the correlation between two parameters found a significant moderate negative correlation (r=-0.556;

**Table 1. Donor and packed red cell unit characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean(SD)</th>
<th>F</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>33 (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>12</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>14.3</td>
</tr>
<tr>
<td>Hemoglobin level (g/dL)</td>
<td>14.7 (0.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td></td>
<td>21.4</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td></td>
<td>28.6</td>
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<tr>
<td>O</td>
<td>6</td>
<td></td>
<td>42.9</td>
</tr>
<tr>
<td>AB</td>
<td>1</td>
<td></td>
<td>7.1</td>
</tr>
<tr>
<td><strong>Packed Red Cell</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin level (g/dL)</td>
<td>24.9 (1.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>77.1 (4.8)</td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 1.** The differences between free hemoglobin mean level in packed red cell during the storage period

**Figure 2.** The differences between nitric oxide plasma mean level in packed red cell during the storage period
p<0.05) (Figure 3). Previous studies have documented substantial hemolysis that increases free plasma hemoglobin as a function of time and a significant correlation with NO plasma level during blood storage ($\beta=0.98$, $p<0.001$). The concentration of NO consumed rose to a similar extent as a concentration of free plasma hemoglobin which suggest a 1:1 reaction stoichiometry. In accordance with this study, Windsant et al. study on 52 units storage PRC and 30 patients receiving transfusion also reported similar results ($r=0.61$ and $p=0.002$) and found that transfusion at least two units PRC significantly increased free circulating hemoglobin level and recipient NO consumption ($p<0.001$ and $p<0.005$).

This study was conducted in proving the hypothesis that NO bioavailability would disrupted in corresponding with increased of free hemoglobin as a result of hemolysis during the storage. Free circulating hemoglobin scavenges endothelium-derived NO primarily through a classic dioxygenation reaction in which NO reacts with oxyhemoglobin to form methemoglobin and nitrate. The major mechanism contributes to reduced NO scavenging is erythrocyte-encapsulated hemoglobin. Efficiently compartmentalized in the lumen and does not extravasate into the endothelium and interstitium. This mechanism surely make hemoglobin will never have a direct contact with NO. Other mechanisms are the presence of a cell-free zone in the vascular space where the erythrocytes are pushed away from endothelium and circulate in the middle of the bloodstream. In this circumstance, the rate of the reaction is largely limited by external diffusion of NO to the erythrocyte and NO diffusion is partially blocked by a physical barrier across the erythrocyte membrane. All of these mechanisms responsible for reduced NO scavenging by encapsulated hemoglobin in intact erythrocyte pertain to either free hemoglobin in plasma or hemoglobin in microparticles and all of these mechanisms will break down on hemolysis.

The enhanced ability of cell-free plasma hemoglobin to scavenge NO has been widely attributed to hypertension, increased systemic and pulmonary vascular resistance, morbidity and mortality. Previous study demonstrated that intravascular hemolysis led to vasoconstriction and impairs renal function in a canine model. Koch et al. reported that PRC transfusion with storage duration for more than two weeks correlated with postoperative complication. Another study also obtained that transfusion at least two units PRC significantly increased NO consumption that might contribute to the underlying pathologic mechanisms of adverse patient outcome.

CONCLUSION AND SUGGESTION

This study showed that free plasma hemoglobin had a significantly negative correlation with plasma nitric oxide level during storage periods of PRC. These two parameters level were significantly differed in each week storage periods.

Although we were able to demonstrate significant result, this study was only conducted in PRC storage unit (in-vitro). Future research is essential to prove the direct impact of RBC transfusion in a critically ill patient (in-vivo) to provide additional insight into the relationship between transfusion of stored blood, increased free plasma hemoglobin, NO consumption, and clinical outcome of the recipient. Furthermore, this study did not consider the contribution of RBC microparticles, known to be released during hemolysis and contain high concentrations of free hemoglobin.

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