Author's response to reviews

Title: Association between red blood cell storage duration and clinical outcome in patients undergoing off-pump coronary artery bypass surgery: A retrospective study

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Author's response to reviews: see over
Response to Editor and Reviewers for MS: 7227281961315799

Dear Editor:

We would like to convey our sincere thanks for the in-depth review of our manuscript.

We have made several changes as recommended by the reviewers, and these are described below. We believe that these changes have improved our manuscript and hope that these alterations satisfy both the editor and the reviewers.

Reviewer 1 (Michael Andrawes)

Major compulsory revisions:
1. None

Minor essential revisions:
1. There is some variability in how the data is presented in terms of odds ratios with confidence intervals vs r values. It would be good to have consistency here.

Thank you for your comments. The statistical methods were preplanned prior to receiving IRB approval and analyzing the results. The binary and continuous variables were designed as follows:

We used binary logistic regression analysis for the risk of various factors on the occurrence of any postoperative outcome (occurrence: yes or no) and used correlation analysis for the continuous variables (e.g., Red blood cell (RBCs) age vs. postoperative laboratory value or length of hospital stay in Table 4).

Following your advice, we generated logistic regression models, including categorical variables, and found that the oldest age of the transfused RBCs was not significantly associated with the risk of the lower base excess (lower than -2.4, which was the median value of the population) or the prolonged hospital stay (longer than 7 days, which was the median value). The amount of transfused RBCs remained a significant predictive factor for a lower base excess or a prolonged stay.

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base excess &lt;-2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount of transfused RBCs</td>
<td>1.09</td>
<td>1.05-1.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oldest age of transfused RBCs</td>
<td>1.02</td>
<td>0.99-1.05</td>
<td>0.12</td>
</tr>
<tr>
<td>Hospital stay &gt;7 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Value 1</td>
<td>Value 2</td>
<td>p-value</td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Amount of transfused RBCs</td>
<td>1.1</td>
<td>1.06-1.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oldest age of transfused RBCs</td>
<td>1.01</td>
<td>0.98-1.04</td>
<td>0.27</td>
</tr>
</tbody>
</table>

It seems that changing the statistical methods to a binary method could produce different results from the submitted results, especially by cut-off values. Statistical experts at our institution recommend that such changes in statistical methods can induce false-positive or negative and suggest using pre-planned statistical methods. Therefore, if you allow, we would like to retain the previous results in the manuscript.

2. The wording of this sentence should be changed to clarify that the last part of the sentence is referring to this study's population. "Moreover, the study population in this study was relatively more homogeneous than those of previous studies on cardiac surgery patients, which contained only coronary disease patients undergoing OPCAB by a single surgeon in a single tertiary center."

Thank you so much for your advice. We changed the sentence to clarify its meaning, as follows (p.13, line 4):

Our study population contained only coronary disease patients who underwent OPCAB by a single surgeon in a single tertiary center. Therefore, it was relatively more homogeneous than populations in previous studies of cardiac surgery patients. However, the retrospective design means that uncontrolled biases could have affected our analyses.

**Discretionary revisions:**

1. Consider referencing the Red Cell Storage Duration Study (RECESS) (clinicaltrials.gov #NCT00991341), another RCT looking at this topic in cardiac surgical patient which has also recently completed enrollment.

Thank you for your suggestion. The RECESS trial (clinicaltrials.gov #NCT00991341) you mention is thought to be referenced in reference number 36 in our manuscript (Addressing the question of the effect of RBC storage on clinical outcomes: the Red Cell Storage Duration Study (RECESS) (Section 7). Transfus Apher Sci. 2010,43(1):107-116). Considering that RECESS was a multicenter randomized controlled trial, which included a broad range of populations, its results have important implications for general clinical outcomes, including many of the postoperative outcomes retrospectively analyzed in our study. We have added this information in the Discussion section of the revised version of the manuscript, as follows (p.13, line 4):
Our study population contained only coronary disease patients undergoing OPCAB by a single surgeon in a single tertiary center. Therefore, it was relatively more homogeneous than populations in previous studies of cardiac surgery patients. However, the retrospective design means that uncontrolled biases could have affected our analyses. As it is difficult to randomize patients to receive older blood transfusions due to ethical concerns and technical problems, the results of some randomized controlled trials of cardiac surgery patients (ClinicalTrials.gov No.: NCT00458783 and NCT00991341) [28] awaiting publication will be important in drawing conclusions about the effects of old stored RBCs on various clinical outcomes.

2. Consider commenting on the transfusion thresholds used in this study. The transfusion rate (both percentage of patients as well as the number of units per patient is quite high).

Thank you for your insightful comment. Although we counted all RBC units transfused during the perioperative hospital stay, we agree that the transfusion rate of our study was much higher than that reported in previous studies (50.7% in off-pump CABG and 63.3% in on-pump CABG; Ref: N Engl J Med 2012;366:1489-1497). We think that several possible factors may explain the increased RBC transfusion in this study.

First, a major factor was the perioperative coagulation status. Many surgeons and anesthesiologists in Korea, especially if they have experience in both western countries and Korea, believe that Koreans are more susceptible to bleeding and that there are racial differences (Refs: Relation of race to activated clotting time after weight-adjusted bolus of heparin during percutaneous coronary intervention. Am J Cardiol 2010;105:629-632, Racial differences in venous thromboembolism. J Thromb Haemost 2011;9:1877-1882). We often find that some surgeons trained in western countries have difficulty in controlling bleeding in Korean patients. Second, preoperative aspirin was maintained until the morning of the surgery, and intraoperative heparin was given to maintain the activated clotting time (ACT) above 300 sec, as per the protocol of our institution in the study period. Third, most of the enrolled Korean patients were smaller than patients in western countries. Thus, even a relatively small amount of bleeding can easily cause hypovolemia and anemia. Thus, a higher rate of transfusion was required to prevent anemia.

We have added the information in the Methods and Discussion sections of the revised version, as follows (p.5, line 21):

The perioperative coagulation management strategy was as follows: all patients took aspirin until the day of the surgery and resumed it as soon as possible after the surgery, usually one day
postoperatively. During the surgery, the patients were given an initial dose of heparin (1.5 mg/kg) and periodic supplemental doses to maintain an ACT >300 sec. Heparin was neutralized at the end of the surgery to only one-third of the required protamine dose. The perioperative target hemoglobin level was 10 g/dl.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Reviewer 2 (Yasuko Nagasaka)

The current article by Jeong Jin Min et al. highlights the pitfalls of stored blood transfusion. Despite of this study being retrospective, the authors elegantly present the risks of stored blood that increases wound infection, negative base excess and longer hospitalization.

Here are my comments that may help improve this article.

Major Compulsory Revisions:
A. Overall comments
1. Unclear rationale: The use of off-pump CABG vs. on-pump significantly reduced risk of blood transfusion (50.7% vs. 63.3%, relative risk 0.80, CI 0.75-0.85, p<0.001) (Ref 1). Since the patient population already has reduced risk for blood transfusion, please specify why this study is important.

Thank you for your valuable comments.

The purpose of this study was to evaluate the clinical effect of old stored blood, not the amount of blood transfusion. As we proved in our study, the mechanism of this effect was related to hypoperfusion and the base excess could be a surrogate marker. In cases of on-pump surgery, the stronger effect of cardiopulmonary bypass (duration, technique, etc) may overwhelm these effects. Thus, we believed that the use of off-pump CABG in our study would more clearly evaluate and reflect the effect of an old blood transfusion.

Moreover, previous studies that investigated the clinical effect of old blood transfusions in patients undergoing cardiac surgery had a mixed population of CABG or various valve surgeries. Even in studies with only CABG patients, most included on-pump CABG patients in their study population. As the presence of various valvular heart diseases or the use of cardiopulmonary bypass itself could affect the postoperative outcome, we thought that analyzing only patients undergoing off-pump CABG would make it possible to interpret more clearly the vascular effect of stored RBCs.

Finally, we reviewed the article you recommended (Ref. 1). Although the transfusion rate was relatively lower in off-pump CABG, more than half of the OPCAB patients in that study still needed an RBC transfusion (50.7%). In our study population, about 96% of patients had an RBC transfusion during the perioperative hospital stay. In patients with coronary arterial disease, an RBC transfusion is essential for adequate oxygen delivery. Meanwhile, the detrimental effect of transfusions of old stored blood is a concern in such patients.

We have added this information in the Background section of the revised version, as follows: (p.3, line 22).
Although the transfusion rate of RBCs in off-pump CABG surgery was lower than that in on-pump surgery, more than half of the OPCAB patients still needed RBC transfusion.[12] In patients with coronary arterial disease, RBC transfusion is essential for adequate oxygen delivery. Meanwhile, old blood transfusion is a concern in such patients because of the possible harmful vascular effects.[2, 3] (Refs: [2] Effect of processing and storage on red blood cell function in vivo. Semin Perinatol 2012;36:248-259, [3] Vascular effects of the red blood cell storage lesion. The Education Program of the American Society of Hematology American Society of Hematology Education Program 2011, Hematology 2011:475-479.)

2. Overlapping endpoints and contradiction:
(Primary endpoints)

a. Four primary endpoints (death from cardiac causes, myocardial infarction, coronary revascularization and stroke) have been studied. I see the first three points are cardiac; which include death from cardiac causes, MI, coronary revascularization. I wonder what exactly was the diagnosis of cardiac death in each patient.

Thank you for your comments. There were 15 cardiac deaths during the long-term follow-up, and we reviewed their medical records at the time of death. The table below shows the individual diagnosis of cardiac death in each patient.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Postoperative survival time</th>
<th>Diagnosis and management</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 months</td>
<td>Sudden syncope, initial rhythm: Vfib; CPR done but no ROSC</td>
</tr>
<tr>
<td>2</td>
<td>13 months</td>
<td>Sudden cardiac arrest, initial rhythm and asystole; CPR done but no ROSC</td>
</tr>
<tr>
<td>3</td>
<td>55 months</td>
<td>Chest discomfort and a response to nitroglycerine (+). Sudden cardiac arrest, with fatal arrhythmia 2 h later; CPR done but no ROSC</td>
</tr>
<tr>
<td>4</td>
<td>4 months</td>
<td>Sudden cardiac arrest (r/o lethal arrhythmia attack in medical record); Management details not known (expired at another center).</td>
</tr>
<tr>
<td>5</td>
<td>4 months</td>
<td>Suspected congestive heart failure patient, admitted due to progressive hypotension with bradycardia. Taking dobutamine (20 µg/kg/min) and isoproterenol (2 µg/min). During the hospital stay, sudden cyatic change occurred with no pulse; CPR administered for 130 min but no ROSC</td>
</tr>
<tr>
<td>6</td>
<td>18 months</td>
<td>New onset LBBB, with elevated cardiac enzymes; acute myocardial infarction diagnosed; a percutaneous coronary intervention performed Sustained VT with narrow QRS → wide QRS pulseless VT, Vfib; CPR performed for 30 min but no ROSC</td>
</tr>
<tr>
<td>7</td>
<td>13 months</td>
<td>Semicomatose state due to bacterial meningitis, on antibiotics therapy (admitted to the Department of Neurology). Occurrence of NSTEMI, progressive hypotension, bradycardia, and sudden cardiac arrest (asystole) during the hospital stay On inotropics (dobutamine and dopamine), DNR patient, no CPR</td>
</tr>
</tbody>
</table>
Patient admitted due to pneumonia. Occurrence of atrial fibrillation, with a rapid ventricular response (>170 bpm) and hypotension during the hospital stay, followed by fatal ventricular arrhythmia; CPR done but no ROSC.

Heart failure due to ischemic cardiomyopathy, aggravated with combined pneumonia. Occurrence of sudden cardiac arrest (initial rhythm: asystole) during the hospital stay; CPR done for more than 30 min but no ROSC.

Aggravated cardiogenic shock due to stress-induced cardiomyopathy; written DNR and no CPR performed.

STEMI (ECG change, elevated troponin I) in a septic shock state, No CPR performed due to DNR patient.

Sudden syncope, rhythm: Vfib → PEA → ROSC. Finally, multiorgan failure, with cardiovascular collapse; DNR patient and no CPR.

Sudden cardiac arrest; CPR done but no ROSC.

Epigastic pain → sudden cardiac arrest (Vfib → asystole); CPR done but no ROSC.

Sudden cardiac arrest (initial rhythm: PEA); CPR done for 45 min but no ROSC.

Vfib, ventricular fibrillation; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation; VT, ventricular tachycardia; NSTEMI, non-ST elevation myocardial infarction; DNR, do-not-resuscitate; PEA, pulseless electrical activity.

We have added this information as an additional file 1.

b. Did death from cardiac causes include low cardiac output, ventricular tachycardia, ventricular fibrillation or other fatal arrhythmias? How many of them required immediate intervention, IABP or re-open heart surgery. Please describe in detail.

Among the 15 cardiac deaths, 12 patients expired due to cardiac arrest, with fatal arrhythmia, one had fatal STEMI, and two died from heart failure. Two patients with fatal cardiac arrhythmia also had MI (Case no. 6 and 7 in the table above). We have added this information in the additional file 1.

c. What is the definition of MI? What are the troponin levels in each patient? Are the MIs all STEMI? What are the results from TTE/TEE, radionuclear imaging or electrophysiological studies? What other treatments? Did they receive anything besides of revascularization?

The diagnosis of MI was based on the universal definition of MI (Ref: Universal definition of myocardial infarction. Eur Heart J 2007;28:2525-2538).

Among 48 MI patients, 27 were diagnosed with STEMI. Twenty-two patients had a regional
wall motion abnormality on echocardiography, two patients had perfusion defects on cardiac SPECT, 35 patients had an abnormal electrocardiogram (e.g., new Q wave, new LBBB, abnormal ST, etc.), and all MI patients showed a rise and fall in cardiac enzymes (CK-MB, troponin). For the management and treatment strategy, 11 had coronary revascularization (one died), two had an intra-aortic balloon pump (IABP) inserted, and the others had an intravenous infusion of heparin and nitroglycerine. Two patients who had combined pulmonary edema required intubation and mechanical ventilation support. There were four deaths with three of them were cardiac deaths due to MI. One patient died from hypoxemia-induced multiorgan failure due to ARDS. We have added this information in an additional file 1.

d. Are stroke hemorrhagic or thromboembolic stroke?
Did you see any association between each stroke and old blood transfusion?
What were the outcomes of stroke and did they correlate with old blood transfusion?
Thank you so much for your comments. We reviewed the medical records of the patients with postoperative stroke.
There were 44 postoperative strokes. The detailed descriptions of each stroke are presented in the table below.

<table>
<thead>
<tr>
<th>Stroke type</th>
<th>Number of cases</th>
<th>Description of the event and the prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemorrhagic</td>
<td>2</td>
<td>One patient with a known cerebral arteriovenous malformation died from cerebral hemorrhage, and another who had previously undergone MCA-STA bypass surgery died following an acute intracranial and intraventricular hemorrhage during the follow-up period.</td>
</tr>
<tr>
<td>Embolic</td>
<td>19</td>
<td>Among 19 embolic strokes, 15 occurred during the early postoperative period (within months) and four in the late postoperative period (1 or 2 yrs after the surgery). Ten patients had postoperative onset atrial fibrillation. One patient had a left ICA stent insertion, and most of the patients were discharged from the hospital after a few days of care.</td>
</tr>
<tr>
<td>Undetermined</td>
<td>23</td>
<td>Twenty-three strokes of undetermined type were classified as either ischemic or embolic infarction, without intracranial hemorrhage (from a review of their brain imaging). One patient expired from stroke, and two were comatous after the stroke. However, there were limitations in obtaining exact prognostic details from the retrospective chart review because of the limited information.</td>
</tr>
</tbody>
</table>

MCA-STA, middle cerebral artery to superficial temporal artery; ICA, internal carotid artery
In accordance with your recommendation, we analyzed the association between the stroke type and the RBC age (oldest or mean age of transfused RBCs and any RBC unit older than 14 days) by Cox proportional hazards analysis, adjusting for the same confounders used in Table 2. The results revealed no significant relationship between each stroke type and the RBC age. However, we could not include the 23 strokes of undetermined type in the analysis, and there was only limited information on stroke outcomes in the medical records. In the five patients who died or became comatous, there was no significant correlation between old RBCs and a poor stroke outcome.

<table>
<thead>
<tr>
<th></th>
<th>Embolic stroke</th>
<th>Hemorrhagic stroke</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR 95% CI</td>
<td>P value</td>
</tr>
<tr>
<td>Mean RBC age</td>
<td>1.02 0.92-1.12</td>
<td>0.75</td>
</tr>
<tr>
<td>Maximum RBC age</td>
<td>1.01 0.93-1.10</td>
<td>0.8</td>
</tr>
<tr>
<td>Any RBCs &gt;14 days</td>
<td>1.37 0.52-3.50</td>
<td>0.52</td>
</tr>
</tbody>
</table>

We have added this information in an additional file 1.

(Other endpoints)

a. The authors have defined respiratory complications as ‘postoperative pneumonia or over 48 hrs of ventilatory support’. However, ‘prolonged intubation’ is listed as a separate endpoint in a different section. Typically prolonged intubation is defined as ‘longer than 48 hours’ (Ref 2) and is identical to the former endpoint. Please describe in details.

Thank you so much for your comments. Previously, we analyzed the correlation between the age of the RBCs and the “postoperative intubated time” as a continuous variable. However, we considered it more appropriate to use a standardized definition of prolonged intubation. Thus, in the revised version of the paper, we have removed the earlier results where we used intubated time as a continuous variable.

b. What are the inclusion criteria for the postoperative pneumonia?

Postoperative pneumonia was suspected on the basis of clinical presentation (e.g., fever, leukocytosis, increase in purulent respiratory secretions, or worsening hypoxemia) and a chest x-ray (appearance of a new infiltrate). The diagnosis was confirmed by culture of tracheal aspirate or blood. We have added this information in the Methods section of the revised version, as follows (p.6, line 9):

Respiratory complications included prolonged ventilator support (>48 h) or postoperative pneumonia. The diagnosis of pneumonia was based on a combination of physical signs and a chest X-ray and
often confirmed by microbiological tests.

c. Postoperative wound complication and mediastinitis may co-exist. However, the authors listed as separate endpoints at the beginning. Nevertheless, both were included in later paragraph: ‘superficial and deep sternal wound including mediastinitis’. It is very confusing. These contradictions need to be sorted out.

Thank you for your comments. Postoperative wound complication covers a wide number of complications, including superficial and deep sternal wounds, as described in the definition of the study endpoints. We intended to analyze mediastinitis as a separate endpoint because it is potentially a serious and more severe form of wound complication. However, all cases of mediastinitis were included in the deep sternal wound problem. As you recommended, to avoid duplication of the results and to clarify the meaning, we have removed the description of the separate analysis of mediastinitis from the Methods and Results sections.

It may also be helpful if you could utilize the scoring system to summarize the severity of your postoperative patients, i.e. SOFA, MODS, etc.

Thank you for your advice. We calculated SOFA scores retrospectively and analyzed the relationship between the age of the RBCs and the postoperative highest SOFA score. The amount of transfused RBCs and the oldest age of the transfused RBCs showed a significant correlation with the postoperative highest SOFA scores, as below. We have added this information in the Results (p.10, line 12) section and an additional file 4.

<table>
<thead>
<tr>
<th></th>
<th>( r )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of transfused RBCs</td>
<td>0.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oldest age of transfused RBCs</td>
<td>0.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>after removing the effect of the transfusion amount</td>
<td>0.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Mean age of transfused RBCs</td>
<td>0.003</td>
<td>0.933</td>
</tr>
<tr>
<td>after removing the effect of the transfused amount</td>
<td>0.03</td>
<td>0.39</td>
</tr>
</tbody>
</table>

We also calculated the postoperative Sepsis related Organ Failure (SOFA) scores[13] and analyzed the relationship with RBCs ages. The total number of transfused RBCs and the oldest age of the transfused RBCs showed significant correlations with the postoperative highest SOFA scores (see additional file 4) (p.10, line 12 in Results).

B. Major comments
1. Background
a. The authors state cell free hemoglobin decreases bioavailability of nitrous oxide. The two references (Ref 1 and 2) are written based upon the observations on nitric oxide. Same statement (nitrous oxide) is found in the discussion section as well. Please comment on this.

Thank you for your insightful comments. Actually, we meant nitric oxide. We apologize for the confusion caused by the unintentional error. We have corrected the error in the Background and Discussion sections of the revised version.

b. Several statements in the background are made without references. Please insert each reference that applies.

Thank you so much for your comments. The references for several consecutive sentences in the Background section were the same, and we marked those references only after the last sentence. To correct any misunderstanding, we have inserted the relevant reference numbers after each statement in the revised version.

2. Methods

a. Results were adjusted for confounders (Table 3). What confounding factors did you adjust and how? Please explain in detail.

The variables presented in the tables in the additional file number 2, are the confounders that we adjusted in the multivariate logistic regression analyses for the various postoperative outcomes. They were the total number of transfused RBCs; the patient’s age, sex, and body mass index; the presence of diabetes mellitus, hypertension, dyslipidemia, renal failure, left ventricular dysfunction (LV ejection fraction less than 35%), chronic obstructive pulmonary disease, or cardiac reoperation; a previous history of MI or stroke; perioperative IABP insertion; emergency operation; and the duration of the surgery.

We assessed the impact of each factor on the postoperative outcome in a univariable analysis. After each univariable analysis, we generated multivariate logistic regression models, including variables that were significantly associated with the analyzed postoperative outcome in the univariate analysis ($P<0.1$). Then, we analyzed significant predictors for each postoperative complication by the forward variable selection method. After selection of the variables, as the effect of old stored blood was the topic of interest in this study, the age of the RBCs was included in the final multivariate models as a covariate in one of three different models: oldest age of transfused RBCs in adjusted model 1, mean age of transfused RBCs in adjusted model 2, and any RBC unit older than 14 days in adjusted model 3. We have added this information in the Methods section (statistical
b. Unclear time frame. How did you define ‘long-term’ for the postoperative major adverse cardiovascular and cerebral events (MACCEs)?

Thank you for your comment. We defined ‘long-term’ MACCE as MACCE that occurred during the follow-up period after hospital discharge. The event-free survival time until a new MACCE diagnosis was variable among the patients (from weeks to years). We have added the following description in the Methods section of the revised version. (p.6, line 1)

: The long-term follow-up was initiated after hospital discharge and concluded in September 2012. The mean follow-up period was 31 months, with a range of 0 to 80 months (median 29, interquartile range [IQR], 11–51 months).

c. Factors that affect outcome of cardiac events are not analyzed yet.
i. Preoperative medications (beta-blocker, statins, aspirin, metformin, ace inhibitors in patients with heart failure, etc)

ii. Intra and peri-operative factors (anesthesia methods i.e. volatile anesthetics vs. intravenous, hemodynamics i.e. lowest MAP and/or HR, lowest hemoglobin, events of cardiac arrest, etc)

iii. Postoperative factors (pain control, medications, etc).

Thank you for your valuable comments. Following your recommendation, we re-generated the Cox regression models for long-term MACCEs, including possible additional variables as covariates. As the presence of hypertension or diabetes was included in the previous models, we could not add the antihypertensive or hypoglycemic medications in the models because of possible interactions between the covariates. All patients took aspirin until the day of the surgery and resumed it as soon as possible after the surgery, usually one day postoperatively. Postoperative pain was managed with a standard regimen of intravenous PCA in all patients. The additional perioperative factors analyzed were as follows: pre- and postoperative use of statins, anesthetic agent (sevoflurane or propofol) used, lowest values of intraoperative heart rate, mean arterial pressure, mixed venous saturation, cardiac index, and hematocrit level.

In the multivariate Cox regression model, the amount of transfused RBCs (OR 1.03, CI 1.02–1.04), $P<0.001$ and postoperative use of statins (OR 0.56, CI 0.37–0.86, $P=0.007$) were the significant predictive factors for MACCEs. Any of analyzed RBCs age was not a significant predictive factor for MACCEs. We have added this information in the Methods (Statistical analysis) and Results sections and in Table 2 in the manuscript.
In the Cox regression model for MACCEs, the perioperative use of statins, the anesthetic agent used, the lowest values of intraoperative hemodynamic variables, and the lowest hematocrit level were included as covariates (p.7, line 2 in Methods).

After adjusting for the variables with \( P < 0.1 \) in the univariate analyses, the total number of transfused RBCs (HR, 1.03; 95% CI, 1.02–1.04; \( P < 0.001 \)) and the use of postoperative statins (HR, 0.56; 95% CI, 0.37–0.86; \( P = 0.007 \)) were significantly associated with MACCEs. We constructed separate adjusted models, including the two significant factors and the age of RBCs as covariates. These models revealed no significant association between the age of the RBCs and long-term MACCEs (Table 2, p.8, line 16 in Results section).

3. Results and discussion

a. Please comment on the impacts of male dominancy on the outcome of this study (Table 1: 71.5% of the studied patients were male).

We adjusted sex as a confounding factor in the multivariable regression analysis, and there was no significant association between sex and any postoperative outcomes. Due to possible sex-related differences in blood volume or initial hemoglobin level, we analyzed whether there was a sex-related difference in the amounts of transfused RBCs. We found no sex-related differences in the amounts of transfused RBCs, oldest age of the RBCs, or mean age of the transfused RBCs, as shown below.

<table>
<thead>
<tr>
<th></th>
<th>Male (n=766)</th>
<th>Female (n=306)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfused amounts of RBCs</td>
<td>7.1 ± 9.3</td>
<td>6.7 ± 5.9</td>
<td>0.503</td>
</tr>
<tr>
<td>Oldest age of transfused RBCs</td>
<td>14.5 ± 5.8</td>
<td>14.0 ± 5.2</td>
<td>0.208</td>
</tr>
<tr>
<td>Mean age of transfused RBCs</td>
<td>12.1 ± 4.7</td>
<td>11.8 ± 4.5</td>
<td>0.285</td>
</tr>
</tbody>
</table>

We have added this information in the Results section in the revised version, as follows; (p.8, line 2)

Although the present study had male dominance (71.6%), there was no sex-related difference in the transfused amounts of RBCs.

b. The sole positive result associated with old blood transfusion was wound infection, which has already been reported previously. In the author’s institution, leukoreduction was not routinely performed. Recent study shows prestorage leukoreduction reduces bacterial contaminations (Ref 3). Please comment on this.
Thank you for your suggestion. We reviewed Ref. 3 as you recommended. In their prospective cohort study, Phelan et al. reported that prestorage leukoreduction abrogated detrimental effects of old stored blood on multiple-organ dysfunction and infectious complications, including wound infections. They proposed that this might be due to the removal of harmful immune-modulatory effects of donor leukocytes. However, several factors in their study differ from ours, making it difficult to compare the findings. For example, the patients in their study cohort were much younger than ours (40 ± 16 vs. 65 ± 9 yr), and they did not have coronary arterial disease. Moreover, as they were all trauma patients, only their initial lactate values were included. Furthermore, RBC processing has been reported to cause cellular changes (e.g., activation of inflammatory mediators). Thus, comparison with a nonprocessed RBC group may be required to conclude (Refs: Red blood cell hemolysis during processing. Transfusion Medicine Reviews 2002;16:46-60; Effect of processing and storage on red blood cell function in vivo. Semin Perinatol 2012;36:248-259). As you recommended, we have added the following information in the Discussion section of the revised manuscript (p.12, line 26):

A previous prospective cohort study with trauma patients reported that prestorage leukoreduction abrogated the detrimental effect of old stored blood. Although the different characteristics of the study population make it difficult to compare the findings of that study with our results, leukoreduction might have reduced the wound infection in our study.

c. Was there any other infections? Please state presence/absence of other infections, i.e. urinary tract infection, cavitary abscess, empyema, blood culture positive bacteremia, etc. What are the results from microbiology or radiology?
Thank you for your comments. We reviewed the patients’ medical records to check for the presence of other infections. Two patients were diagnosed with catheter-related infections, confirmed by blood culture. Both had diabetic end-stage renal disease (ESRD) and were undergoing periodic hemodialysis. One patient was discharged after antibiotics therapy for enterococcal bacteremia (E. faecalis was isolated from four blood samples: two peripheral and two central veins, Case 1). Another patient who had gram-positive bacteremia, postoperative pneumonia, and a diabetic foot infection died from septic shock, despite receiving antibiotics therapy (Case 2). Information on the RBCs transfused into the two patients are presented in the table below. None of the patient records listed diagnoses of a urinary tract infection, cavitary abscess, or empyema.

<table>
<thead>
<tr>
<th>Case 1</th>
<th>Case 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
We have added this information in the revised version, as follows: (p.10, line 11)

There were two patients with postoperative complications other than wound infections. The details of those patients are presented in an additional file 3.

d. Long-term MACCEs: The authors have included prevalence of MACCE= 181 patients. If we add the numbers of patients, i.e. 15 deaths from cardiac causes, 48 MIs, 88 coronary revascularizations, 44 strokes = 195 patients. Please comment on the overlapping numbers of patients.

Thank you for your comments. We agree that a more detailed description is required to clarify the numbers of patients. Among 15 deaths, two patients had MI. One patient who had revascularization due to MI died. One patient who had MI also had a stroke. Ten patients had coronary revascularization following MIs: 195-(2+1+1+10) = 181. We have added this information in the additional file 1 in the revised version.

e. In the discussion the authors state tissue hypoperfusion predisposes wound infection: ‘transfusion-related immunomodulation in the blood recipients, ..., and the persistent inflammation at the wound site due to reduced bioavailability of nitrous oxide’. Please comment if this is about nitric oxide (NO).

Is there any literature that suggests reduced NO availability triggers wound infection?

Thank you for your comments. We meant nitric oxide and have corrected the error in the revised version. We have searched and reviewed several previous studies on nitric oxide and wound healing. Following your advice, we have added the following information in the Discussion section in the revised version: (p.12, line 21)

Moreover, reduced bioavailability of nitric oxide in older RBCs units might have delayed the wound healing,[3] A number of studies have investigated the role of nitric oxide in wound healing,[30-33] Synthesis of nitric oxide occurs during wound healing, especially in the early stages of healing,[30] and the released nitric oxide improved wound repair by angiogenesis, collagen formation, cell proliferation, and fibroblast migration in damaged tissue.[31-33]


f. Did you look at the indicators for hemolysis in patients received old/new blood? i.e. haptoglobin, plasma Hb level, bilirubin, or Hb level after transfusion?

Thank you for your comments. As this was a retrospective study, we were unable to obtain data on haptoglobin and plasma Hb because they were not included in the routine blood laboratory tests in the study period. Instead, we analyzed the highest postoperative total bilirubin as an indicator of hemolysis to determine the association with old RBC transfusions. Total bilirubin showed a non-normal distribution and we analyzed its correlation with the age of RBCs using Spearman’s correlation coefficient, with and without the effect of the amount of transfused RBCs (as a controlled variable).

<table>
<thead>
<tr>
<th>Total bilirubin</th>
<th>r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfused RBCs amounts</td>
<td>0.188</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oldest age of transfused RBCs</td>
<td>0.124</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>after removal the effect of transfused amounts</td>
<td>0.058</td>
<td>0.056</td>
</tr>
<tr>
<td>Mean age of transfused RBCs</td>
<td>0.054</td>
<td>0.076</td>
</tr>
<tr>
<td>after removal the effect of transfused amounts</td>
<td>0.055</td>
<td>0.073</td>
</tr>
</tbody>
</table>

We have added this information in the Methods (p.6, line 9) and Results (p.9, line 23) sections in the revised version.

Pearson’s correlation or Spearman’s rank correlation coefficients were used as appropriate to analyze the relationships between RBC transfusion amount and some continuous variables such as postoperative base excess, total bilirubin, length of ICU and hospital stay. Partial correlation analyses were used to remove the effects of the number of transfused RBCs in the relationships between the three ages of RBCs and those continuous variables (p.6, line 9 in Methods).

We evaluated the relationship between RBCs age and the postoperative highest total bilirubin as an indicator of hemolysis. Postoperative highest total bilirubin was significantly correlated with the total number of transfused RBCs ($r = 0.19, P < 0.001$) and the oldest age of transfused RBCs ($r = 0.12, P < 0.001$). However, after adjusting for the transfusion amount, the oldest age of transfused RBCs was not significantly related with the postoperative highest total bilirubin ($r = 0.06, P = 0.056$) (p.9, line 23 in Results).
Minor Essential Revisions:
The native English speaker should correct grammatical errors.
The English has been checked by at least two professional editors, both native speakers of English.

Again, thank you so much for all your advice and help with our paper.

Best regards,
Yunseok Jeon, M.D. on behalf of the authors