Impact of red blood cell transfusion on platelet activation and aggregation in healthy volunteers: results of the TRANSFUSION study†

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Aims
The underlying mechanisms leading to recurrent ischaemic events or mortality after red blood cell (RBC) transfusion in anaemic acute coronary syndrome patients are poorly understood. The aim of this paper is to determine whether RBC transfusion increases platelet activation and aggregation.

Methods and results
In vitro transfusions (n = 45) were performed by the addition of RBCs obtained from transfusion packs to fresh whole blood provided by healthy volunteers. Residual platelet aggregation (RPA) and maximal platelet aggregation (MPA) were assessed before and after in vitro transfusion using light transmission aggregometry performed with four different agonists. Flow cytometry was used for the measurement of P-selectin expression and vasodilator-stimulated phosphoprotein (VASP) platelet reactivity index (PRI). To control for the effect of haemoconcentration, the same experiments were repeated after hematocrit adjustment using volunteer’s platelet poor plasma. Transfusion increased platelet aggregation as measured by RPA with ADP 5 μM (57.7 ± 25 vs. 65.7 ± 24%; P = 0.03) or Collagen 2 μg/mL (59.4 ± 28 vs. 69.7 ± 24%; P = 0.03). There were no significant differences with Arachidonic Acid 1.25 mM or Epinephrine 20 μM and results were similar when MPA was considered. Platelet activation was also increased by transfusion as confirmed by an elevation of P-selectin expression induced by 20 μM ADP (12.2 ± 18 vs. 23.9 ± 18%; P = 0.002) or 50 μM ADP (15.4 ± 18 vs. 26.8 ± 21.2%; P = 0.004) and an increase in VASP PRI (77.8 ± 6 vs. 81.9 ± 3%; P = 0.03). These effects were all independent of hematocrit.

Conclusion
Red blood cell transfusion increases platelet activation and aggregation in vitro in healthy volunteers. This effect might be mediated through the P2Y12 activation pathway.

Keywords
Clopidogrel • Platelet response • Transfusion • ACS

Introduction
New aggressive antithrombotic regimens provide benefits in reduction of recurrent ischaemic events in acute coronary syndrome (ACS), but are offset by a consistent increase in bleeding complications with frequent utilization of red blood cell (RBC) transfusion.1 2 The true incidence of bleeding varies in reported studies depending on the populations studied and the management strategies employed (e.g. conservative vs. invasive; clinical trial vs. registry) as well as on the definition used to characterize bleeding events.3 Nevertheless, bleeding is consistently associated with an increased risk of adverse outcomes, including ischaemic complications, myocardial infarction, and death.4 5 6

The causal link between bleeding and mortality has not yet been demonstrated. This is because many confounding variables are involved, such as the development of hypotension, tachycardia, anaemia, and reduction in oxygen delivery all of which increase myocardial and coronary stress. Additionally, antiplatelet therapy
is frequently interrupted in these patients, leading to an increased risk of thrombosis.

Moreover, patients who bleed have more frequent comorbidities, are older, mostly female, have low body weight, renal insufficiency, and are treated more frequently with invasive procedures.\(^7,8\)

Importantly, transfusion by itself has been shown to be an independent risk factor for recurrent ischaemic events and mortality.\(^8–10\) Reported transfusion rates vary from 3.9% in the recent APEX-AMI study to 10.2% in the CRUSADE registry, and two studies suggest that a less liberal use in stable situations is favourable.\(^11–13\) Furthermore, there is a very similar association between cell-free haemoglobin-based blood substitutes and the risk of myocardial infarction and death.\(^14\) As red cell transfusion is a cornerstone in the management of bleeding events, concerns regarding the safety of this therapy have critical clinical importance.

The underlying mechanistic explanations for the excess risks due to transfusion are not well understood. We hypothesized that RBC transfusion could trigger platelet activation and facilitate aggregation. To test this hypothesis, we studied platelet aggregation and activation in an in vitro model of blood transfusion.

**Methods**

**In vitro model of transfusion**

The model of in vitro transfusion and the different experiment are presented in *Figure 1*. Red blood cells were obtained from different transfusion packs containing leucocyte-depleted blood. In order to mimic a RBC transfusion, we mixed in vitro blood from transfusion packs with whole blood provided by healthy volunteers. In vitro donor and recipient blood were matched with respect to ABO type and rhesus group as per standard in vivo transfusion compatibility criteria. Information on the date of collection and the date of thawing were obtained for each transfusion pack.

**Volunteers**

Healthy volunteers provided blood samples by venipuncture and blood was drawn into Becton-Dickinson 3.2% citrate vacuette tubes. All were at least 18 years old and were not receiving any antiplatelet agent, anti-inflammatory treatment, or any significant treatment. The platelet counts for all were in the normal range (between 150 000 and 350 000 per mm\(^3\)) and no volunteers had a history of platelet disease or transfusion. Written informed consent was provided before participation and this study was approved by the Pitie-Salpetriere University Hospital ethics committee (Comité de Protection des Personnes Participants à la Recherche Biomédicale).

**Sample preparation**

Platelet analyses were conducted under three different conditions as follows (*Figure 1*):

1. **Sample A**: Baseline fresh whole blood sample from healthy volunteer.
2. **Sample B**: in vitro transfusion of RBCs into fresh whole blood from healthy volunteers with a 1:4 ratio (3 mL of RBCs mixed with 9 mL of whole blood).
3. **Sample C**: Corrected in vitro transfusion, correspond to Sample B with additional dilution with platelet poor plasma (PPP) obtained from the same volunteer to lower the hematocrit (3 mL of RBCs mixed with 9 mL of whole blood and 3 mL of PPP).

**Platelet aggregation and activation measurements**

Forty-five in-vitro transfusions were performed and platelet reactivity was tested 30 min after addition of the RBC transfusion. We performed two successive sets of experiments as follows: (1) Evaluation of platelet aggregation was first performed at baseline (A) and after in vitro transfusion (B) using light transmission aggregometry (LTA) \((n = 25)\), (2) then platelet activation was evaluated by measurement of P-selectin expression \((n = 10)\) and the measure of the phosphorylation of vasodilatator-stimulated phosphoprotein (VASP) \((n = 10)\) were performed with flow cytometry with an additional condition (C) where hematocrit is corrected with PPP. All experiments were performed in duplicate.

**Light transmission aggregometry**

Light transmission aggregometry was performed in <2 h after venipuncture. Platelet-rich plasma (PRP) was obtained by centrifugation of citrated whole blood at 100 g for 10 min at room temperature. Platelet poor plasma was obtained by further centrifugation at...
4500 g for 15 min. In vitro platelet aggregation in PRP was measured at 37 ºC in an LTA (model 490-4D, Chrono-Log Corp, Kordia, the Netherlands) and was induced by four different agonists: (i) ADP 5 µM (Sigma-Aldrich, Saint Quentin Fallavier, France), (ii) Arachidonic Acid 1.25 mM (Sigma-Aldrich, Saint Quentin Fallavier, France), (iii) Collagen 2 µg/mL (Helena Biosciences, Tyde and Wear, UK), (iv) Epinephrine 20 µM (Helena Biosciences, Tyde and Wear, UK). Maximal platelet aggregation (MPA) and residual platelet aggregation (RPA) were measured 6 min after induction of aggregation by agonist were recorded. Pre-specified criteria used to define non-evaluable samples were the lack of sufficient signal, haemolysis, PRP platelet count <150 000 per mL and an unstable baseline.

Flow cytometry
Flow cytometry was performed in <2 h after venipuncture for P-selectin measurement. The phosphorylation of VASP was measured within 24–48 h using a Beckman Coulter FC500 cytometer (Beckman Coulter, Villepinte, France). To determine platelet P-selectin expression, blood samples previously activated by a dose of 20 µM of ADP were mixed with saturated concentrations of anti-CD62p-PE (Beckman Coulter, Villepinte, France) monoclonal antibody and anti-CD41a-FITC monoclonal antibody (Beckman Coulter, Villepinte, France). After staining with antibodies, samples were incubated for 30 min in the dark and diluted with 1 mL of FACs solution. Samples were immediately processed for flow cytometric analysis. To determine platelet CD62P expression, individual platelets were identified by size (forward and scatter) and anti-CD41a-FITC immunofluorescence using a logarithmic scaled dot plot. P-selectin expression on the surface of platelets was defined as positive for anti-CD62P-PE. Variation in activation corresponded to percent of gated platelets after activation by agonist minus percent of gated platelets at rest. Because of the lack of previous experiment on the effect of transfusion, we also used a higher dose of ADP (50 µM) for the same experiments.

The VASP phosphorylation was measured using Platelet VASP kits (Diagnostica Stago, Asnières, France) according to the manufacturer’s instructions. Briefly, blood samples were incubated in vitro with ADP and/or prostaglandin E1 (PGE1) before fixation. The platelet population was identified on its forward and side scatter distributions and 5000 platelet events were gated and analysed for mean fluorescence intensity (MFI). The MFI corresponding to each experimental condition (PGE1, ADP+PGE1) was determined to establish a ratio directly correlated with the VASP phosphorylation state. The VASP platelet reactivity index (PRI) was calculated from the MFI of each condition according to the formula: VASP PRI = ((MFI_PGE1)−MFI_PGE1+ADP)/MFI_PGE1×100.

All the measurements were done in the research laboratory on Thrombosys of our department and were validated by previous publications of our research group.15–18 The experiments were not blinded as we used different preparations in order to obtain three different conditions.

Statistical analyses
Means and SDs were used to describe the platelet function measures and measurements of cell counts in the three conditions. Data were analysed using a paired t-test. A confirmatory analysis by the Wilcoxon’s rank test was also performed to assess statistical significance in case of a possible non-Gaussian distribution. Correlations between length of conservation and platelet aggregation data were done with the Pearson’s test. The statistical tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA. All tests were two-sided with a statistical threshold for significance of 0.05. All the authors had full access to and take full responsibility for the integrity of the data.

Results

Description of transfusion packs and healthy volunteers
The median length of conservation of the transfusion packs used for the in vitro transfusion experiments before administration to patients was 14 days IQR6–20 before use (max 36 days to min 6 days). The repartition of ABO-rhesus blood type of transfusion packs was 40% (n = 17) of group A+, 33% (n = 15) of group B+, 16% (n = 6) of group O+, and 13% of group A− (n = 7). Healthy volunteers were medical students and residents recruited in the department, therefore, mean age was low with 32.2 ± 6.8 years old and 15% (n = 7) were women. None of them had prior history of coronary heart disease and they were free from major cardiovascular risk factors as from medication that could affect platelet function. The repartition of ABO-rhesus blood type of healthy volunteer was 38% (n = 17) of group A+, 22% (n = 10) of group O+, 13% (n = 6) of group B+, 13% (n = 6) of group A−, and 9% (n = 4) and 4% of group O− (n = 4).

Effect of red blood cell transfusion on platelet aggregation
Results of platelet aggregation measured by LTA before and after in vitro transfusion are detailed in Table 1. When compared with baseline values, transfusion resulted in a 7.2% increase in MPA and 8% increase in RPA after platelet activation by 5 µM of ADP and a 9.4% increase in MPA and 9.7% increase in RPA after 5 µM of ADP.

<table>
<thead>
<tr>
<th>Light transmittance aggregometry (n = 25)</th>
<th>Baseline</th>
<th>In vitro transfusion</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
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<table>
<thead>
<tr>
<th>MPA (%) ADP 5 µM</th>
<th>62.6 ± 22.7</th>
<th>69.8 ± 21.6</th>
<th>0.03*</th>
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</thead>
<tbody>
<tr>
<td>RPA (%) ADP 5 µM</td>
<td>57.7 ± 24.9</td>
<td>65.7 ± 24.1</td>
<td>0.03*</td>
</tr>
<tr>
<td>MPA (%) AA 1.25 mM</td>
<td>73.4 ± 24.1</td>
<td>77.1 ± 28.3</td>
<td>0.15</td>
</tr>
<tr>
<td>RPA (%) AA 1.25 mM</td>
<td>72.6 ± 23.1</td>
<td>77.2 ± 27.5</td>
<td>0.15</td>
</tr>
<tr>
<td>MPA (%) Collagen 2 µg/mL</td>
<td>60.5 ± 27.3</td>
<td>69.9 ± 23.8</td>
<td>0.03*</td>
</tr>
<tr>
<td>RPA (%) Collagen 2 µg/mL</td>
<td>59.4 ± 27.6</td>
<td>69.7 ± 24.2</td>
<td>0.03*</td>
</tr>
<tr>
<td>MPA (%) Epinephrine 20 µM</td>
<td>57.2 ± 25.2</td>
<td>57.2 ± 24.5</td>
<td>0.94</td>
</tr>
<tr>
<td>RPA (%) Epinephrine 20 µM</td>
<td>57 ± 25.3</td>
<td>57 ± 24.7</td>
<td>0.90</td>
</tr>
</tbody>
</table>

*Denotes P-value < 0.05 for comparison to baseline.
activation with 2 \( \mu g/mL \) of collagen. No significant differences were found in MPA or RPA after activation by 1.25 mM of arachidonic acid or by 20 \( \mu M \) of epinephrine.

**Effect of red blood cell transfusion on platelet activation (flow cytometry)**

Values for P-selectin expression for non-stimulated platelets measured before activation by ADP were similar under the three conditions (\( P = 0.24 \)). The level of P-selectin expression at rest was subtracted from the raw value of P-selectin expression after activation by ADP in order to compare the variation in delta of P-selection expression. Results of the variation in delta of P-selection expression measure by flow cytometry at baseline and after *in vitro* transfusion are represented in Figure 2. The effect of transfusion on platelet activation was confirmed by a significant two-fold increase in the variation of P-selectin expression after activation with either 20 \( \mu M \) ADP (12.2 \( \pm \) 18 vs. 23.9 \( \pm \) 18%; \( P = 0.002 \)) or 50 \( \mu M \) of ADP (15.4 \( \pm \) 18.6 vs. 26.8 \( \pm \) 21.2%; \( P = 0.004 \)) when compared with baseline values.

The VASP PRI was also significantly increased when compared with baseline (77.8 \( \pm \) 6 vs. 81.9 \( \pm \) 3%; \( P = 0.03 \)) (Figure 3).

**An effect independent from hematocrit and haemoglobin level**

As shown in Table 2, *in vitro* transfusion resulted in an increase in the levels of both haemoglobin and hematocrit while there was a decrease in platelet count due to dilution.

The 1.6 \( \pm \) 0.8 g/dL (mean \( \pm \) SD) increase in the haemoglobin level with our mixing ratio of RBC to whole blood is comparable to that of two RBC packs transfusion in patients *in vivo*. To assess whether the effect of *in vitro* transfusion on platelet activation was independent of the above changes, we corrected the level of haemoglobin and hematocrit with the addition of PPP. The resulting adjustment resulted in levels of haemoglobin and hematocrit comparable to baseline. The effects of *in vitro* transfusion on both platelet activation and platelet aggregation were similar in these experiments (Figures 2 and 3).

**Effect of transfusion packs conservation length and blood type on platelet activation**

With the exception of arachidonic acid (\( r = 0.5, P = 0.01 \)), we found no correlation between the length of conservation of transfusion pack and the increase of platelet aggregation or platelet activation with the different agonist and techniques (correlation coefficient were, respectively, \( r = 0.01 \) for ADP; \( r = 0.08 \) for collagen, \( r = 0.04 \) for epinephrine, \( r = 0.12 \) for P-selectin, and \( r = -0.18 \) for VASP; all \( P \)-value were non-significant). There was no clear impact of the ABO /rhesus blood type on platelet reactivity, but these results are subject to caution due to the multiple possible blood type combination (\( n = 15 \)) between donors and receivers.

**Discussion**

Red blood cell transfusion is the most readily available method to increase hematocrit in anemic patients and therefore theoretically increases oxygen delivery, but by itself has been associated with recurrent ischaemic events and increased mortality.\(^8\)\(^-\)\(^10\),\(^19\) Unfortunately, there are limited scientific data to understand its potential hazard when used in non-lethal situations. Red blood cell transfusion raises haemoglobin levels and may lead to an absence of change or even a paradoxical decrease in tissue oxygenation.\(^20\) In addition, prolonged storage of blood cells may lead to cellular depletion of 2,3 disphosphoglycerate and nitric oxide (NO)\(^21\) favouring vasoconstriction and platelet aggregation.\(^22\) Furthermore, it has been hypothesized that stored RBCs could induce microvascular obstruction through loss of mechanical distensibility, an
inflammatory reaction with increases in mediators such as CRP and IL6,23 or conversely immunosuppression, increasing the likelihood of infection in patients with acute myocardial damage.24 However, to date, there have been few plausible biological models developed to investigate how transfusion may be harmful.25,26

We demonstrate in this report that in vitro transfusion enhances platelet aggregation in healthy volunteers. This effect was consistent and confirmed by three different techniques commonly used in clinical research. Increases in platelet reactivity were most evident when measured with P-selectin expression (activated by ADP) and also with LTA when using ADP and collagen as agonists. This effect was persistent even with a lower platelet count due to dilution after the in vitro transfusion.

The increased expression of P-selectin after in vitro transfusion provides a potential explanation to the detrimental effect of transfusion. This platelet membrane receptor mediates platelet–endothelium interactions and is a global marker of platelet activation.27 Our results suggest a possible direct activation by agonist or mediators contained in RBC transfusion packs. A direct role of an increased viscosity due to higher hematocrit which can theoretically increase platelet aggregation and activation is another plausible mechanism. However, our finding that platelet activation persisted after adjusting for hematocrit suggests that this mechanism is unlikely.

Increased platelet aggregation in LTA by ADP or collagen further confirmed the hypothesis that transfusion activates platelets through pathways involving the P2Y_{12} receptors. Additionally, the modest but significant increase in VASP PRI due to its high specificity suggests partial activation of the P2Y_{12} receptors, which are stimulated by ADP. The liberal quantities of ADP contained in RBCs have been shown to play a role in platelet adhesion.28 ADP may be readily liberated in the context of blood storage thus representing a potential stimulus for platelet activation and aggregation. These considerations need to be studied by additional experiments. Finally, we did not find any relationship between storage and the level of platelet aggregation after in vitro transfusion in our experimental model.29 This latter finding may reflect the fact that the transfusion packs available for these experiments were relatively old, having been prepared a median time of 14 days IQR^{8–20} before use.

We confirmed that in vitro transfusion of RBC packs, which produces a similar change in haemoglobin level as transfusion of two RBC packs in vivo increases platelet aggregation. The magnitude of the effect of transfusion on platelet aggregation is within the threshold of a clinically relevant low responsiveness to clopidogrel previously reported in ACS studies indicating this observation to be potentially clinically meaningful.29–31 The potential role of the P2Y_{12} pathway in this activation also suggests that interruption of clopidogrel, in particular, may exacerbate the detrimental effects of transfusion, while aspirin interruption may be less of a concern considering the data of our study on the AA pathway.

This study has several limitations. First, the impact of transfusion on platelet aggregation was performed in a pure in vitro model using blood from non-anaemic healthy volunteers free of any antiplatelet agents. However, the observation made in these conditions may be relevant to ACS patients with a bleeding complication when antiplatelet agents are interrupted despite a high degree of platelet activation. Secondly, whether this effect persists in coronary patients remains to be demonstrated, but is challenging because measures would not permit to the distinction between an effects due to treatment change or a potential biological effect of transfusion. Finally, whether the effect of transfusion on platelets persists or is just present in the hours following transfusion is unknown.

A systematic overview of 10 randomized trials of transfusion strategies that found that a restrictive transfusion strategy is associated with non-significant trends towards decreased mortality, myocardial infarction, and congestive heart failure.32 More recent studies have shown an increased risk of RBC transfusion in stable patients with mild anaemia (hematocrit level decrease to 30%) suggesting a cut-off value of haemoglobin above which the harm of transfusion overcomes its benefits.33

Our data indicate that platelet activation by transfusion may be one of the contributor to the detrimental effect of in vivo blood transfusion in patients. This plausible explanation needs now to be confirmed in clinical studies to acknowledge the transferability of these results to anaemic ACS patients treated with anti-platelet therapy and receiving transfusion.

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