Red blood cell aggregation during cardiopulmonary bypass: a pathogenic cofactor in endothelial cell activation?

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Abstract

Objective: The bio-incompatibility of the cardiopulmonary bypass (CPB) circuit and the use of artificial colloids trigger massive defense reaction that involves endothelial cells and several blood cells: platelets, neutrophils, monocytes, red blood cells (RBC) and lymphocytes. Investigating the effects on RBC aggregation and endothelial cells activation, the present study addresses two different prime solutions commonly used in the clinical practice.

Methods: RBC aggregation was measured by means of Laser-assisted Optical Rotation Cell Analyzer, in an in vitro study designed to mimic the human blood–material interactions during extracorporeal circulation. A clinical study investigating endothelial activation was conducted in 20 patients undergoing elective coronary bypass surgery, randomly assigned for CPB using two different priming solutions: HAES-steril 6% (HES 200/0.5) and Voluven 6% (HES 130/0.4).

Results: Circulation through a Chandler loop of HES–blood mixes altered significantly RBC aggregability. The use of HES 130/0.4 resulted in marked decrease in RBC aggregation (aggregation index (AI) before and after circulation was 23.5 ± 3.8 and 18 ± 2.9, respectively), no significant differences being found when compared with Ringer’s lactate group. The use of HES 200/0.5 resulted in better maintained RBC aggregation (AI 39.7 ± 5.9 and 29.7 ± 4.7 before and after circulation, respectively). The AI measured for the whole blood (control) sample was 61.9 ± 4.9 before circulation, and 58.1 ± 4 after. Markers of endothelial activation (von Willebrand factor (vWF), thrombomodulin (TM), tissue plasminogen activator (tPA) and E-selectin) significantly increased during CPB. Differences between HES treatment groups were evident post-bypass. While the markers of endothelial activation returned to baseline in HES 200/0.5 group, HES 130/0.4 was associated on the first post-operative day with further increase of vWF and tPA.

Conclusion: RBC aggregation significantly dropped as consequence of blood dilution and blood–material interaction. We reason that low RBC aggregation added to plasma viscosity reduction and non-physiologic flow conditions during extracorporeal circulation are important factors contributing to loss of shear stress at the venous endothelial wall. The loss of shear stress triggers complex signaling leading to endothelial activation. Additional fundamental research is needed in order to verify the hypothesis introduced by the present study. Characterizing the impact of rheologic parameters on endothelial function could prove to be valuable in patients undergoing CPB.

Keywords: Red blood cell aggregation; Endothelial cell activation; HES 130/0.4; HES 200/0.5

1. Introduction

Cardiac surgery involving cardiopulmonary bypass (CPB) leads to activation of the haemostatic-inflammatory system associated with increased post-operative morbidity and prolonged hospital stay [1]. As documented in the literature, extracorporeal circulation triggers massive defense reaction that involves endothelial cells and at least five blood cells: platelets, neutrophils, monocytes, red blood cells (RBC) and lymphocytes. The intensity of the cellular and humoral activation is known to vary with patient factors, the surface area of biomaterials, duration of perfusion and exposure of blood to the wound [2]. As a consequence, a whole-body inflammatory response and microemboli are generated, leading to fever
and leukocytosis, compromised fluid balance, ischemia and microinfarctions [3].

The relations between a low hematocrit and the adverse outcomes in patients undergoing CPB is extensively discussed in the literature [4]. There are also reports addressing the mechanical trauma in RBC [5] and the decrease in RBC deformability during extracorporeal circulation [6]. In our opinion, a complete chapter has been excluded from the discussion around the pathogenesis of the ‘post-CPB syndrome’ modifications induced in RBC aggregation and potential consequences on microcirculation.

The aggregation property of RBCs is mainly considered to be pathophysiologic, since aggregation is elevated in many disease states such as diabetes mellitus [7] and hypertension [8]. However, red cell aggregation is normally present in humans and other ‘athletic’ species being most pronounced in those species having the highest capacity for oxygen consumption, while it is absent in sedentary animals [9]. This raised the possibility that normal levels of aggregation may serve homeostasis, having functional significance for normal physiology [10]. Earlier experiments conducted in our group conclusively showed that the physiological function of RBC to form aggregates is significantly affected in the presence of hydroxyethyl starch (HES) [11]. Since HES solutions are extensively used as volume substitutes and priming solutions, RBC aggregation is expected to suffer deviation from normal values during CPB. Additionally, the unavoidable hemodilution associated with the use of the heart–lung machine is also expected to result in a drop of plasma viscosity. These hemodynamic alterations could represent mechanical triggers of further endothelial cell activation already exposed to other insults occurring during CPB, such as hypoxia, inflammatory stimuli, and surgical manipulation. Endothelial activation is known to disrupt the barrier function, enhance vasoconstriction and increase the leukocyte adhesion [12].

The present study aims to test the potential effect of blood interactions with HES solutions and extracorporeal circuit on RBC aggregability and to document endothelial cell activation in the presence of two different prime solutions commonly used in the clinical practice, HAES-steril 6% and Voluven 6%. The clinical relevance and possible correlation between the pathophysiological mechanisms implicated are discussed.

2. Patients, materials and methods

2.1. Red blood cell aggregability, blood viscosity and plasma viscosity

RBC aggregability was investigated in vitro with a Laser-assisted Optical Rotation Cell Analyzer (LORCA R&R Mechatronics, Hoorn, The Netherlands). This instrument, based on the ektacytometric principle, is equipped with a video camera for detection of the laser diffraction-pattern, a thermostat unit and an ellipse-fit computer program calculating the aggregation index (AI) of the RBC [13]. For the determination of red cell aggregation, the blood is brought under a shear rate of 500 s⁻¹, after which the shear is stopped at \( t = 0 \). The backscattered intensity from the blood layer is measured during 120 s after shear stop. The intensity drops because of RBC aggregation. We considered the beginning point of the aggregation, the extrapolated value of the decay curve towards \( t = 0 \).

The RBC aggregation measurements were performed at 32 °C, in samples prepared by in vitro admixture of HES solutions (either HAES-steril 6% or Voluven 6%) to human fresh blood from healthy volunteers \( (n = 6) \), drawn from the antecubital vein, heparinized (4 U/ml) and oxygenated (10 min). The blood:prime mixture ratio was 5:2. In order to assess the effect of dilution alone, we also measured the RBC aggregation in Ringer’s lactate (RL)–blood mixtures (blood: RL ratio = 5:2). We considered AI values as controls measured in whole blood samples.

AI was measured before and after sample circulation trough a Chandler loop [14] of silicon tubing, mimicking the blood–material/device interactions during extracorporeal circulation. Silicon tubing with a total volume of 6.3 ml (inner diameter 4 mm, length 500 mm) was filled with 4.5 ml of sample leaving a gas volume of 1.8 ml. The tubing was closed into a loop using PVC connectors and then circulated vertically at 10 rpm, in a 32 °C water bath for 1 h.

The choice of using the Chandler loop as a model was based on results of numerous in vitro studies comparing the use of simpler or more complex in vitro models for characterization of blood–material/device interactions. Coagulation parameters, platelets activity and hemolysis were monitored in each model. In this regard, testing in the simple Chandler loop model produced findings, which overlapped with observations from the more complex CPB models [15].

Viscosity was measured by means of an automated dynamic shear rheometer with cone-plane geometry (AR1000 Rheometer, TA Instruments). Viscosity was measured both in blood–HES samples and plasma–HES samples. The blood–HES samples were prepared using the same method as used for the RBC aggregation measurements. The plasma–HES samples were prepared by in vitro admixture of HES solution to human fresh plasma in a mixture ratio of 5:2. In order to measure the modification that appear due to dilution alone, we also measured viscosity in plasma samples mixed with RL in the same ratio. During viscosity measurements the temperature was set at 32 °C. Viscosity was measured at four shear rates for blood samples (30, 60, 100 and 200 s⁻¹) and at three shear rates for plasma samples (60, 100 and 200 s⁻¹).

2.2. Endothelial activation during CPB

A prospective randomized single blind study, approved by the Medical Ethics Committee of Hospital de
Weezenlanden in Zwolle, Netherlands, was conducted in 20 patients, who underwent an elective coronary bypass surgery. The patients were randomly assigned for CPB with either HAES-steril 6% or Voluven 6%.

The patients were less than 75 years of age, had a body weight over 65 kg, underwent a CPB time of more than 30 min and had signed a written consent. Exclusion criteria were presence of severe heart failure, renal or liver dysfunction, bleeding diathesis, diabetes mellitus, and the use of platelet inhibiting drugs within 5 days before the operation.

Induction and maintenance of anesthesia, surgical techniques and CPB procedures including anticoagulation with heparin and its neutralization with protamine were performed in a standardized fashion [16].

The extracorporeal circuit consisted of an integrated microporous plate membrane oxygenator (Cobe—Duo, Cobe, CO, Lakewood, USA), polyvinyl chloride tubing and a centrifugal pump (Biomedicalic, Medtronic, Anaheim, CA, USA). The priming volume of the circuit was 2 l and the priming solution compositions were:

1. HAES-steril 6% (Fresenius AG, Oberursel, Germany) 1000 ml (6% HES 200/0.5, median molecular weight 200 kD, degree of substitution 0.5) supplemented with Ringer’s lactate to a final concentration of 3%.
2. Voluven 6% (Fresenius AG, Oberursel, Germany) 1000 ml (6% HES 130/0.4, median molecular weight 130 kD, degree of substitution 0.4) supplemented with Ringer’s lactate to a final concentration of 3%.

HES solutions served also as plasma substitutes, the dose limitation being 3 l in the pre-, peri-, and post-operative period. After reaching these defined study colloid dose limits postoperatively, isotonic pasteurized plasma was administered in case additional volume was needed. As standard practice in our clinic, 1500 IU heparin was added to all priming solutions.

During the operative day and on the first post-operative day (POD), three blood samples were taken for biochemical determinations: after induction of anesthesia (post-induction), at arrival on the intensive care unit (1 h ICU), and on the first POD. The post-CPB values (1 h ICU) were corrected for plasma dilution using hemoglobin values.

Blood samples were obtained from the radial artery catheter and were mixed with 3.06% sodium citrate, with a ratio of 9:1. The samples were put on ice during storage. The citrated blood was centrifuged at 1100 x g for 12 min to obtain platelet poor plasma, which was stored at −80 °C until further determinations of biochemical assays. Plasma concentrations of endothelial/platelet release products were investigated by means of ELISA: vWF (Gradipore, North Ride, Australia); tissue plasminogen activator (t-PA) (Coaliza, Innogenetics, Belgium); E- and P-Selectine (R&D Systems Inc., Abingdon, UK); thrombomodulin (TM) (Imubind, American Diagnostica Inc., Greenwich, CT, USA).

3. Statistics

Before data analysis, all the individual sample points were tested for distribution according to the Kolmogorov–Smirnov goodness of fit test. In case of not normally distributed data, Mann–Whitney test was used to quantify differences between groups. Within groups Wilcoxon Signed Ranks test was performed to show differences during treatment. Correlations between variables was tested using Spearman’s correlation test.

To detect possible differences in effect of each priming solution, for normal distributed data, one way analysis of variance (ANOVA) was used to compare groups. If differences between the groups were significant (P < 0.05), post-hoc multiple comparisons were performed to quantify any differences among groups using the Tukey HSD test with a level of significance P < 0.05. A Bonferroni correction was made for multiple testing. Within groups a paired t-test was performed to show differences during treatment.

The variables are expressed as mean and standard error of the mean, unless stated otherwise.

4. Results

4.1. Red blood cell aggregability, blood viscosity and plasma viscosity

The AI measured in control samples was 61.9 ± 4.9 before circulation, and 58.1 ± 4 after. Dilution of blood with Ringer’s lactate solution yielded a decrease of AI to 16.6 ± 2.6. Mixture with HES 130/0.4 resulted in low aggregation (AI before and after circulation 23.5 ± 3.8 and 18 ± 2.9, respectively). No significant differences were found between Ringer’s lactate group and HES 130/0.4 group. The use of HES 200/0.5 compensated by half the dilution effect on RBC aggregation; AI values in this group were 39.7 ± 5.9 and 29.7 ± 4.7 before and after circulation, respectively (Fig. 1). Circulation trough the closed silicone tubing system of blood: HES mixture significantly reduced RBC aggregability (paired Student Test P ≤ 0.01).

4.2. Method validation for AI measurements

Measurements of normal reference sample showed a mean value of 57.79, a SD of 0.88, a 95% CI of [56.86; 58.72] and a coefficient of variation of 14.66%. Measurements of low reference sample (blood: RL = 5:2) showed a mean value of 16.72, a SD of 1.49, a 95% CI of [15.15; 18.29] and a coefficient of variation of 24.83. The AI was measured six consecutive times at 32 °C.
Fig. 2a shows the viscosity curve of blood samples measured at 32°C and shear rates 30, 60, 100 and 200 s⁻¹. When measuring at shear rate 30 s⁻¹, the lowest values were registered after mixture with Ringer’s lactate solution (reduction to 59% of whole blood viscosity), followed by values given by HES 130/0.4 group (reduction to 64%) and HES 200/0.5 (reduction to 72%). ANOVA showed significant differences between groups (P < 0.001). Multiple comparison with post-hoc test detailed that the differences between the groups were significant at any tested shear rate (P < 0.001). The same ranking was observed when measuring plasma and plasma–HES mixes viscosity (Fig. 2b). When measuring at shear rate 100 s⁻¹, the addition of RL, HES 130/0.4 and HES 200/0.5 determined a decrease of plasma viscosity to 75, 85 and 92%, respectively, of the initial values. ANOVA showed significant differences between groups (P < 0.001). Multiple comparison with post-hoc test detailed that the differences between the groups were significant at any tested shear rate (P < 0.001). The values are represented as mean (symbols) and standard deviation of the mean (bars). Significant (P < 0.05) and highly significant differences (P < 0.01) within the groups and between the groups are indicated with * and **, respectively.

4.3. Endothelial activation during CPB

No significant differences were measured between the two groups with regard to age, sex, weight, body surface area, CPB and aortic cross clamp time, number and origin of grafts or volumes infused. Also, no relevant differences were found in concomitant diseases or medication between the treatment groups. On the ICU, 40% of the patients from both HES 130/0.4 and HES 200/0.5 group received additional isotonic pasteurized plasma, 178 ml in average, after the dose limit of 3 l colloid was reached. 40% of the patients in the HES 130/0.4 group and 50% patients in HES 200/0.5 group received allogenic blood products.

Von Willebrand factor (plasma) concentration did not change significantly during extracorporeal circulation.
although in both groups a trend to increase was observed. Between induction of anesthesia and the end of the surgical procedure vWF concentrations ranged between 60 and 260% of normal pooled plasma, being in average higher than normal. The values in HES 130/0.4 group started to increase in the reperfusion period, so the concentrations in the first post-operative day were significantly higher than baseline (Wilcoxon Sig. < 0.01). Significant differences were measured between groups, with higher plasma values in HES 130/0.4 group (Mann–Whitney Sig. < 0.01, Fig. 3).

Tissue-plasminogen activator concentrations were significantly higher at 1 h ICU in comparison with baseline values (Wilcoxon Sig. < 0.05). The values measured in HES 200/0.5 group were significantly higher than in the HES 130/0.4 group (Mann–Whitney Sig. < 0.01). During reperfusion time, the values in HES 200/0.5 group declined while the values in HES 130/0.4 group increased further (Fig. 4).

Thrombomodulin values significantly increased after the end of extracorporeal circulation (Wilcoxon Sig. < 0.05) for patients of both groups, with no difference between treatment groups. The values went down in the reperfusion period but remained significantly above the baseline (Wilcoxon Sig. < 0.01) (Fig. 5).

E-Selectin increased moderately but significant after CPB in both groups (Wilcoxon Sig. ≤ 0.01) with no

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Fig. 3. von Willebrand factor (vWF) plasma concentration (%) measured in two groups of patients undergoing cardiopulmonary bypass:
- group HES 130/0.4 (n = 10): HES 130/0.4 was used as a plasma volume expander;
- group HES 200/0.5 (n = 10): HES 200/0.5 was used as a plasma volume expander.

The measurements were efectuated at three time points: post-induction of anesthesia (post-induction), 1 h after transfer in intensive care unit (1 h ICU) and in the first post-operative day (first POD). The values are represented as mean (symbols) and standard error of the mean (bars).

+, Significant increase compared to baseline (P < 0.05).

Fig. 4. Tissue plasminogen activator (tPA) plasma concentration (ng/ml) measured in two groups of patients undergoing cardiopulmonary bypass:
- group HES 130/0.4 (n = 10): HES 130/0.4 was used as a plasma volume expander;
- group HES 200/0.5 (n = 10): HES 200/0.5 was used as a plasma volume expander.

The measurements were efectuated at three time points: post-induction of anesthesia (post-induction), 1 h after transfer in intensive care unit (1 h ICU) and in the first post-operative day (first POD). The values are represented as mean (symbols) and standard error of the mean (bars).

+, Significant increase compared to baseline (P < 0.05).

Fig. 5. Thrombomodulin (TM) plasma concentration (U/L) measured in two groups of patients undergoing cardiopulmonary bypass:
- group HES 130/0.4 (n = 10): HES 130/0.4 was used as a plasma volume expander;
- group HES 200/0.5 (n = 10): HES 200/0.5 was used as a plasma volume expander.

The measurements were efectuated at three time points: post-induction of anesthesia (post-induction), 1 h after transfer in intensive care unit (1 h ICU) and in the first post-operative day (first POD). The values are represented as mean (symbols) and standard error of the mean (bars).

+, Significant increase compared to baseline (P < 0.05).
Fig. 6. E-Selectin plasma concentration (U/l) measured in two groups of patients undergoing cardiopulmonary bypass: group HES 130/0.4 (n = 10): HES 130/0.4 was used as a plasma volume expander; group HES 200/0.5 (n = 10): HES 200/0.5 was used as a plasma volume expander. The measurements were effectuated at three time points: post-induction of anesthesia (post-induction), 1 h after transfer in intensive care unit (1 h ICU) and in the first post-operative day (first POD). The values are represented as mean (symbols) and standard error of the mean (bars).

+ Significant increase compared to baseline (P < 0.01).

4.4. Circulating platelet count

The mean platelet number was comparable between the treatment groups within the evaluation period. The platelet count remained within normal ranges except for the initial period after CPB (1 h ICU) when values decreased due to dilution effect. Corrected values showed no significant differences as compared to baseline values.

5. Discussion

The property of RBC to form aggregates at low shear rates was profoundly altered in our in vitro model mimicking the human blood–material interactions during extracorporeal circulation. In Ringer’s lactate: blood samples, the AI dropped to a quarter of the control AI values. Further decrease was registered as a consequence of blood circulation through silicon tubing.

The use of HES 200/0.5 compensated by half the dilution effect on RBC aggregation.

In parallel with the decrease in RBC aggregation, blood viscosity declined also. The highest viscosity was measured in HES 200/0.5: blood samples, followed by HES 130/0.4: blood and Ringer’s lactate: blood samples. The same ranking was observed when measuring viscosity in plasma samples.

Current understanding of the rheological effects of RBC aggregation suggests that blood shear stress at the venular wall increases when the RBC aggregability increases [10,17]. Accordingly, because of RBC aggregation drop, plasma viscosity reduction and non-physiologic flow conditions, it is expected a decrease in blood shear stress at the venous endothelial wall during extracorporeal circulation.

Endothelial cells are notorious for their ability to sense variations in mechanical forces, such as shear stress. Endothelial cells in vivo are normally exposed and presumably adapted to a normal level of shear stress in the range of 5–20 dyn/cm². Cells adapted to flow might be expected to respond to either an increase or decrease in shear stress from the normal level. Studies investigating the response of flow-adapted endothelial cells to an abrupt loss of shear stress, showed membrane depolarization, increased intracellular Ca²⁺, nitric oxide and reactive oxygen species generation [18]. In addition to synthesis and release on demand, several stored compounds are secreted during endothelial cell stimulation, in a Ca²⁺ dependent way. Elevation in intracellular Ca²⁺ triggers release of several vasoactive factors and factors involved in hemostasis and thrombolytic: nitric oxide, prostacyclins, vWF, tPA, tissue factor, adhesion molecules and chemotactrant proteins [19]. In our clinical study investigating the endothelial activation during CPB when either HES 200/0.5 or HES 130/0.4 were used, vWF and tPA recovered in HES 200/0.5 group while further increasing in the HES 130/0 group.

Because of lack of consensus in literature over the ‘gold standard’ for endothelial activation, our clinical study was designed to measure several markers related to endothelial activation: vWF, TM, t-PA, E-selectin.

vWF is a component of platelet α-granules and Weibel–Palade bodies in the endothelial cells. The majority of plasma vWF is derived from endothelial cells and an increase in plasma levels is generally considered to be mainly a marker of endothelial activation. However, vWF is also known to be an acute phase reactant affected by inflammatory cytokines, and as such, may be elevated even in the absence of definite endothelial damage [20,21]. TM is a surface protein of endothelial cells, which acts as a thrombin receptor and serves as an anticoagulation factor. Soluble fragments of TM, probably components of degradation, circulate in plasma. TM is not released in plasma constitutively or as a response to endothelial activation, but is released after acute endothelial cell injury.
As a drawback, TNF-α leads to a reduction in thrombomodulin expression by endothelial cells [21]. Endothelial release of t-PA initiates fibrinolysis. tPA may be used to evaluate endothelial stimulation induced by CPB, denoting a post-ischemic antithrombotic function of the endothelium [22]. E- and P-Selectins belong to the selectin family of adhesion molecules and both have been reported to increase in circulation or at lesion sites of several diseases reflecting endothelial activation. The disadvantage of using E-selectin as a marker is the fact that, E-selectin being an leukocyte adhesion molecule, some may be bound to its ligand in vivo, and be unavailable for measurement [21].

The findings of this study showed functional and/or structural alteration of vascular endothelial cell during extracorporeal circulation, as documented by elevated plasma concentrations of vWF, thrombomodulin, tPA and E-selectin. These markers have a proven endothelial origin, since platelet count was similar in both groups and did not vary extensively during CPB. In the HES 130/0.4 treatment group the increase in vWF correlated positively with the increase in tPA. In the HES 200/0.5 group a positive correlation was found between TM and tPA.

Differences between HES groups were evident post-bypass. While the markers of endothelial activation recovered in HES 200/0.5 group, HES 130/0.4 was associated on the first post-operative day with further increase of vWF and tPA. These reports may prove to represent additional help in the decision process of the clinician who is confronted with cardiac patients of different etiologies. Even if further investigation is needed, our results documenting the important rise in vWF suggest the necessity of a more careful selection of HES solutions. Hypertensive and atherosclerotic patients who have high basal levels of vWF might benefit from HES 200/0.5. HES 130/0.4 could represent a first choice for patients with bleeding tendencies and patients with acquired von Willbrand syndrome after aortic stenosis. In this respect, HES 130/0.4 was proved to be in various clinical settings at least comparable or better on coagulation parameters, blood loss or blood product consumption as compared to HES 200/0.5 [16].

Our observations made in vitro on RBC aggregability coupled to the observation made in vivo on endothelial cell activation suggest a hypothetical new pathophysiological mechanism implicated in the post-CBP syndrome. We hypothesize that the drop in RBC aggregation added to plasma viscosity reduction and non-physiologic flow conditions during extracorporeal circulation, are important factors contributing to loss of shear stress at the venous endothelial wall. The loss of shear is known to lead to a complex signaling response eventuating in membrane depolarization, intracellular Ca^{2+} accumulation with subsequent release of nitric oxide, prostacyclins, vWF, tPA, tissue factor, and generation of reactive oxygen species.

Additional fundamental research is needed in order to verify the hypothesis introduced by the present study.

Characterization of the interrelation between rheologic parameters and endothelial function could prove to be valuable in managing complications in CPB patients.

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