Preliminary study of the effect of hemolysis on platelet aggregation through microscopic observation under physiological shear flow

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Abstract Hemolysis during mechanical circulatory support has been suggested as a possible trigger for non-hemorrhagic stroke. The purpose of this study was to investigate the hypothesis that slight hemolysis enhances thrombus formation through platelet aggregation under physiological shear stress in humans. Using sodium citrate-treated porcine whole blood from a slaughterhouse, platelet suspensions with a constant platelet density of 20.5 ± 1.3 × 10^3 cells/µL with three plasma free hemoglobin (pfHb) concentrations (44.9 ± 15.0, 74.3 ± 18.3, and 130.6 ± 20.3 mg/dL) were prepared through centrifugation. These suspensions were exposed to a physiological shear rate up to 200 (1/s) using our custom-built shear chamber for 0, 5, 10, and 15 min. After exposure to each shear load, microscopic image acquisition was performed and the images were analyzed to count the number of aggregated platelets. It was observed that platelet aggregation increased in an exposure time-dependent manner in all suspension fluids. In addition, the samples with the highest mean pfHb concentration of 130.6 mg/dL showed 1.23- and 1.28-fold numerically greater aggregation than those with a pfHb concentration of 74.3 and 44.9 mg/dL, respectively. The Wilcoxon signed-rank sum test showed p-values of 0.028 and 0.047 between 5 vs. 10 min and 10 vs. 15 min under the lowest pfHb concentration, respectively, and a p-value of 0.028 between 0 vs. 5 min under the medium pfHb concentration. However, there was no significant difference in aggregation according to pfHb concentration at the same exposure time. From these results, our data suggest that hemolysis might enhance platelet aggregation under physiological shear conditions.

Keywords platelet aggregation, hemolysis, physiological shear stress, shear chamber

1. Introduction

Mechanical circulatory support devices [1], such as extracorporeal membrane oxygenators and left ventricular assist devices, are now increasingly used along with advances in medical technology. However, these devices are associated with the occurrence of slight hemolysis [2] as well as platelet activation and further thrombus formation [3–5]. These secondary undesirable effects are understood to be induced by the supraphysiological shear stress [6–9] generated by the pump incorporated within such devices.

In addition, it has been recently reported that high shear stress-induced hemolysis is associated with thrombus formation. Specifically, clinical blood monitoring data after extracorporeal membrane oxygenator application showed a significantly higher incidence of non-hemorrhagic stroke in the presence of hemolysis, even at plasma free hemoglobin (pfHb) concentrations of 11–50 mg/dL [10]. From such evidence, it is important to determine whether hemolysis increases the incidence of thrombus formation in the presence or absence of physiological shear stress. Therefore, the purpose of this study was to investigate the platelet aggregation response at several levels of hemolysis under physiological shear stress.

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2. Materials and Methods

2.1 Platelet-rich plasma preparation

We prepared platelet-rich plasma having its density of 200,000 platelets/μL and three levels of plasma-free-hemoglobin increase of 0, 25, and 100 mg/dL from default plasma condition. In followings, detailed sample preparation procedure is described.

As anticoagulant treatment, 3.24 wt% sodium citrate solution was added in porcine whole blood with 10% volume ratio within 10–20 seconds right after blood acquisition at slaughterhouse to avoid blood clotting. Then, it was delivered to our laboratory at the Shibaura Institute of Technology 1 day after animal sacrifice. During such delivery process, the blood temperature was kept under 0–10 degrees. In our Laboratory, the blood (50 mL) was firstly centrifuged at $1,000 \times g$ for 2 min using a tabletop centrifuge (Model 4000; Kubota), and the supernatant (2–3 mL) was collected as platelet-rich plasma (PRP). The remaining blood sample was centrifuged at $2,000 \times g$ for 10 min, and the supernatant (4–5 mL) was collected as platelet-poor plasma (PPP) and used to determine platelet density.

The sedimented red blood cells were frozen once and then thawed for hemolysis. Phosphate-buffered saline was added to the hemolyzed sample and centrifuged at $2,000 \times g$ for 10 min. Next, the supernatant (~3 mL) was collected as a hemoglobin-rich isotonic solution (Hb solution). Then platelet density of PRP, PPP, and Hb solution [cells/microliter] was measured using blood cell counter (Celltac alpha; Nihon Kohden Corporation). At this measurement, hemoglobin concentration in Hb solution [mg/dL] was also derived.

Test sample was prepared by mixing PRP, PPP and Hb solution to satisfy following equations, those are representing platelet amount [cells] (Equation 1) and hemoglobin amount [mg] (Equation 2), respectively.

$$px + qy + rz = n(x + y + z)$$  \hspace{1cm} \text{Equation 1}  \\
$$Az = m(x + y + z)$$  \hspace{1cm} \text{Equation 2}

, where n is target platelet density 200000 [cells/microliter] within test sample, p, q, r was measured platelet density [cells/microliter] of PRP, PPP, and Hb solution through the blood cell counter, and x, y, z was volume [microliters] of PRP, PPP, and Hb solutions those are contained in test sample, respectively.

The pfHb concentration of those final test samples was measured using the Harboe method [13] with a spectrometer (UVmini-1240; SHIMAZU). Detailed sample preparation procedure is shown in Figure 1.

**Figure 1** Sample preparation and experimental procedure.
2.2 Microscopic observation of the platelet-rich suspension after shear flow generation

Our custom-built counter-rotational shear chamber was used to produce shear stress on platelets. This device has been described previously [11–13]. In the shear chamber, 100 μL platelet suspension fluid was pipetted into the narrow space between the upper bob and bottom plate, with an angle of 0.4°. A rotational speed of 100 rpm was set for the bob and plate to generate a physiological shear rate of up to 200 (1/s) in the central clearance region, which is the observation target. In such a narrow gap (10 μm), the flow field is sandwiched between counter-rotating parallel plates formed by the flat surface of the cut bob tip and the bottom plate. Therefore, Couette flow is generated in this region along the circumferential direction, but it is not homogeneous, and the shear rate changes linearly according to radial location because of the counter-rotational mechanism of the parallel plates. However, the same method was used in all experiments for the comparison of platelet rheological behavior. After exposure to this shear flow condition for 0, 5, 10, and 15 min, the rotational motion of the chamber was stopped and microscopic observation was performed immediately through a ×40 objective lens (LUCPLFLN; Olympus Japan) mounted on an inverted microscope (IX-74; Olympus Japan) and an additional ×3 expanding lens (SV-3.0XV; VS Technology) into a 512 × 512-pixel CMOS video camera (GX-1; Nac Japan). Each experiment was repeated for six times using different porcine blood on different days to evaluate biological replicates. All experiments using the same blood samples were performed within the day when blood was delivered to Laboratory. Because we purchased the blood, this study did not require approval from an animal experimental committee.

2.3 Image analysis to quantify platelet count and platelet aggregation

By using the recorded image data, platelet count and the number of aggregated platelets were quantified manually. The platelet aggregation rate (PAR) expressed by the following equation 3 was derived to evaluate aggregation levels:

\[
\text{PAR} (%) = \left( \frac{\text{number of platelets clumping together}}{\text{number of all analyzed platelets}} \right) \times 100
\]

Equation 3

Statistical analysis with the Mann–Whitney U test was performed for platelet counts and platelet aggregation after each exposure time among the three pfHb concentrations. In addition, the Wilcoxon signed-rank sum test was used to compare platelet aggregation at various exposure times (i.e., 0 vs. 5 min, 5 vs. 10 min, 0 vs. 15 min, and 5 vs. 15 min). Then, we examined whether pfHb concentration affected platelet aggregation.

3. Results

As shown in Table 1, a platelet density level of approximately \(2.0 \times 10^5\) cells/μL was provided in each experiment (mean platelet density: \(2.05 \pm 0.12 \times 10^5\) cells/μL). As shown in Table 2, the three actual pfHb concentrations were 44.9 ± 15.0, 74.3 ± 18.3, and 130.6 ± 20.3 mg/dL (mean value ± standard deviation).

Under each hemolytic condition, platelet aggregation was observed, as shown in Figure 2, which contains an image taken immediately after shear flow was stopped. Figure 3 shows the PAR (%) calculated by dividing the number of platelets in aggregates by the total number of platelets according to manual image analyses. Table 3 shows the results of time series statistical analysis of platelet aggregation levels according to the Wilcoxon signed-rank sum test. PARs of

| Assumed pfHb concentration | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Mean | Standard Deviation |
|----------------------------|----------|----------|----------|----------|----------|----------|------|-------------------|
| 0 mg/dL                    | 192      | 183      | 203      | 212      | 216      | 214      | 203  | 12                |
| 25 mg/dL                   | 187      | 191      | 204      | 205      | 197      | 199      | 197  | 6                 |
| 100 mg/dL                  | 214      | 201      | 204      | 233      | 227      | 213      | 215  | 11                |

Table 1 Platelet counts in the sample solutions (\(10^3\) cells/μL).

| Assumed pfHb concentration | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Mean | Standard deviation |
|----------------------------|----------|----------|----------|----------|----------|----------|------|-------------------|
| 0 mg/dL                    | 39.9     | 63.1     | 68.3     | 32.9     | 32.7     | 32.6     | 44.9 | 15.0              |
| 25 mg/dL                   | 74.7     | 95.1     | 102.1    | 57.4     | 59.2     | 57.1     | 74.3 | 18.3              |
| 100 mg/dL                  | 150.7    | 147.9    | 153.8    | 108.0    | 112.5    | 110.7    | 130.6| 20.3              |

Table 2 Measured plasma free hemoglobin (pfHb) concentration in each test blood sample (mg/dL).
22.3%, 21.2%, and 27.6% at 0 min increased over time, for example, to 60.1%, 53.0%, and 67.4% at 15 min under pfHb concentrations of 44.9, 74.3, and 130.6 mg/dL, respectively, in an exposure time-dependent manner in all pfHb concentrations. In all hemolysis concentrations and at shear load for 15 min, the platelet suspensions showed higher platelet aggregation compared with the control non-shear loaded samples. Platelet aggregation levels were numerically greater in the higher hemolysis conditions. Statistical analysis with the Mann–Whitney U test suggested a significant increase of aggregation compared with the non-shear condition; however, at each exposure time, there was no significant difference among the three pfHb concentrations (Table 4).

4. Discussion

In this study, we performed an experimental comparison of the degree of platelet aggregation under different hemolysis levels within physiological shear rate conditions up to 200 (1/s) using our original rotational shear chamber. In the experimental platelet suspension fluid, platelet count was maintained at approximately 200,000 cells/μL (Table 1). Against assumed hemolysis levels of 0, 25, and 100 mg/dL, actual hemolysis conditions were measured as 44.9 ± 15.0, 74.3 ± 18.3, and 130.6 ± 20.3 mg/dL, respectively (Table 2). We speculated that this difference in pfHb concentration occurred because of default hemolysis in the blood that happened during the 1-day delivery period from the slaughterhouse. Therefore, fresher blood with lower hemolysis should be used in future studies. Although the pfHb concentrations in this study were much higher than we expected, we observed a 2.2- and 1.3-fold numerical increase in platelet aggregation as exposure time increased from 0 to 5 min and from 10 to 15 min, respectively (Figure 3 and Table 3) as normal platelet behavior. In addition, numerically greater platelet aggregation occurred in higher hemoglobin concentrations (Figure 3 and Table 4), for example, the PAR under a pfHb concentration of 130.6 mg/dL was 1.28- and 1.23-fold higher than under pfHb concentrations of 44.9 and 74.3 mg/dL, respectively (Table 4). These findings support our hypothesis. However, according to analysis with the Mann–Whitney U test, there was no significant difference in platelet aggregation between the three pfHb levels (Table 4). A possible reason for this discrepancy was the limited amount of platelet data analyzed from the images. In our experiment, in order to count the number of platelets contained within aggregates, we used a ×40 objective lens and a ×3 lens.
between the microscope and camera; therefore, the number of platelets in each image was very limited. In addition, our visualization target was a narrow gap of only 10 μm, further limiting platelet numbers for quantification.

Furthermore, the observed flow position is at the central location between the parallel plates formed by the cut topbob tip and the bottom plate; therefore, flow at this point is not assumed to be homogeneous. Although, because all of the experiments were performed with the same rotational speed, the data are comparable. However, investigations with

Table 3 Comparison among time series platelet aggregation rates (PARs) under each plasma free hemoglobin (pfHb) concentration, PAR ratios, and p-values from analysis of platelet aggregation using the Wilcoxon signed-rank sum test.

| Mean pfHb concentration (mg/dL) | 44.9 | 74.3 | 130.6 |
|--------------------------------|------|------|-------|
| Comparison of PAR (%) between 0 vs. 5 min | PAR (%) at 0 min (PAR0) | 22.3 | 21.2 | 27.6 |
| | PAR (%) at 5 min (PAR5) | 45.7 | 49.1 | 62.4 |
| | Ratio of PAR5/PAR0 | 2.0 | 2.3 | 2.3 |
| | p-value between PAR0 and PAR5 | 0.028* | 0.028* | 0.116 |
| Comparison of PAR (%) between 5 vs. 10 min | PAR (%) at 5 min (PAR5) | 45.7 | 49.1 | 62.4 |
| | PAR (%) at 10 min (PAR10) | 36.5 | 50.5 | 52.0 |
| | Ratio of PAR10/PAR5 | 0.8 | 1.0 | 0.8 |
| | p-value between PAR5 and PAR10 | 0.346 | 0.917 | 0.173 |
| Comparison of PAR (%) between 10 vs. 15 min | PAR (%) at 10 min (PAR10) | 36.5 | 50.5 | 52.0 |
| | PAR (%) at 15 min (PAR15) | 60.1 | 53.0 | 67.4 |
| | Ratio of PAR15/PAR10 | 1.6 | 1.0 | 1.3 |
| | p-value between PAR10 and PAR15 | 0.047* | 0.917 | 0.249 |

*Significant difference.

Table 4 Comparison of platelet aggregation rates (PARs) among different plasma free hemoglobin (pfHb) concentrations at the same exposure time, PAR ratio, and p-values from analysis of pfHb concentration levels after each exposure time to shear flow using the Mann–Whitney U test. In all comparisons, no significant difference was obtained.

| Comparison of PAR between assumed Plasma Free Hemoglobin Concentrations (PFHC) 0 vs. 25 mg/dL |
| Exposure time (min) | PAR at PFHC of 0 mg/dL (A) | PAR at PFHC of 25 mg/dL (B) | Ratio of platelet aggregation rate (B/A) | p-value as result of Mann Whitney-U test |
|---------------------|-----------------------------|-----------------------------|----------------------------------------|-----------------------------------------|
| 0                   | 22.3                        | 21.2                        | 1.0                                    | 0.465                                   |
| 5                   | 45.7                        | 49.1                        | 1.1                                    | 0.749                                   |
| 10                  | 36.5                        | 50.5                        | 1.4                                    | 0.200                                   |
| 15                  | 60.1                        | 53.0                        | 0.9                                    | 0.262                                   |

Comparison of PAR between assumed Plasma Free Hemoglobin Concentrations (PFHC) 0 vs. 100 mg/dL

| Exposure time (min) | PAR at PFHC of 0 mg/dL (A) | PAR at PFHC of 100 mg/dL (C) | Ratio of platelet aggregation rate (C/A) | p-value as result of Mann Whitney-U test |
|---------------------|-----------------------------|------------------------------|-----------------------------------------|-----------------------------------------|
| 0                   | 22.3                        | 27.6                         | 1.2                                    | 0.810                                   |
| 5                   | 45.7                        | 62.4                         | 1.4                                    | 0.078                                   |
| 10                  | 36.5                        | 52.0                         | 1.4                                    | 0.423                                   |
| 15                  | 60.1                        | 67.4                         | 1.1                                    | 0.749                                   |

Comparison of PAR between assumed Plasma Free Hemoglobin Concentrations (PFHC) 25 vs. 100 mg/dL

| Exposure time (min) | PAR at PFHC of 25 mg/dL (B) | PAR at PFHC of 100 mg/dL (C) | Ratio of platelet aggregation rate (C/B) | p-value as result of Mann Whitney-U test |
|---------------------|-----------------------------|------------------------------|-----------------------------------------|-----------------------------------------|
| 0                   | 21.2                        | 27.6                         | 1.3                                    | 0.689                                   |
| 5                   | 49.1                        | 62.4                         | 1.3                                    | 0.200                                   |
| 10                  | 50.5                        | 52.0                         | 1.0                                    | 0.749                                   |
| 15                  | 53.0                        | 67.4                         | 1.3                                    | 0.200                                   |
a constant shear condition would also be of value. Further study incorporating a fluorescent method with a more scaled-down view would enable us to accumulate more data and perform validation through statistical quantification. In addition, the short examination time is a limiting factor, and a much longer exposure time and investigations using human blood are desirable for future work.

Previous analysis of conventional platelet aggregation behavior under low shear stress of less than 1,000 (1/s) showed no aggregation [14]. In the present study, the maximal shear rate within our microscopic shear flow system was estimated at approximately 200 (1/s); however, platelet aggregation was observed in an exposure time-dependent manner. Additionally, our experimental platelet counts indicated hemolysis greater than 44.9 mg/dL. From such facts, we speculate that the level of hemolysis would have resulted in a slight pro-coagulant effect and platelet aggregation occurred under the higher hemolytic conditions.

Concerning the possible mechanism, it has been reported that increased pHb concentrations limit the bioavailability of nitric oxide, which is known to inhibit the adhesion of white blood cells on endothelial cells and platelet aggregation in vivo [15]. Therefore, increased pHb concentrations can be expected to exert a pro-coagulant effect in the circulatory system. In addition, when erythrocytes lyse, they release not only hemoglobin but also adenosine diphosphate, which activates platelets and induces platelet aggregation [16]. From these interpretations, the results of this study partially support the hypothesis that hemolysis is a trigger for platelet aggregation under conditions of physiological shear flow.

5. Conclusion

In the present study, a physiological shear rate of up to 200 (1/s) in platelet-rich plasma with the highest pHb concentration of 130.6 mg/dL resulted in a 1.28- and 1.23-fold numerically greater PAR than under pHb concentrations of 44.9 and 74.3 mg/dL, respectively, in comparisons of exposure time up to 15 min. The findings of this study suggest that platelet aggregation might be enhanced by hemolysis under a physiological shear rate of up to 200 (1/s).

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