Experimental Investigation of Pulsatility Effect on the Deformability and Hemolysis of Blood Cells

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Abstract: In this study, we investigated the differences between pulsatile cardiopulmonary bypass (CPB) procedure and nonpulsatile CPB procedure in terms of their effects on hemolysis and deformability of red blood cells (RBCs) under various shear stress conditions. In order to research the effects on hemolysis and deformability, four parameters—free hemoglobin (fHb) concentration, normalized index of hemolysis (NIH), deformability index (DI) of RBCs, and elongation index of RBCs—have been deeply investigated. For these investigations, two randomly assigned adult mongrel dog groups—nonpulsatile group (NP, n = 6) and pulsatile group (P, n = 6)—were examined. According to our results, both types of perfusion did not show any statistical differences in terms of the concentrations of fHb as well as NIH. In addition, there were no significant differences in RBC deformability between perfusion types within an operation time of 3 h. Therefore, our studies suggest that pulsatile perfusion has no significant difference from nonpulsatile perfusion in terms of hemolysis and deformability of RBCs. Key Words: Pulsatility—Plasma free hemoglobin—Normalized index of hemolysis—Hemolysis—Deformability index—Elongation index.

Cardiac surgical procedures require a more precise and stable extracorporeal circulation technique, which includes a cardiopulmonary bypass (CPB) device for cardiac surgery as well as an extracorporeal circulation device in emergency situations such as acute myocardial infarction and respiratory failures (1). There are two types of CPB devices depending on their perfusion methods: continuous and pulsatile perfusion. Pulsatile perfusion utilizes a pulsatile pump, which is preferred in clinical practice because the characteristics of pulsatile perfusion are similar to physiological flow in human heart (2).

It has been reported that pulsatile CPB procedure provides more beneficial effects on organ functions such as microcirculation, myocardial perfusion, and oxygen exchangeability. It also reduces systemic vascular resistance compared with nonpulsatile CPB procedures (1–6). From these advantages of pulsatile perfusion, many researchers propose that pulsatile blood perfusion is better than nonpulsatile perfusion in a physiological and hemodynamic point of view (1–6).

Several research groups, however, have suggested that a pulsatile pump incurs more blood trauma than a nonpulsatile pump because the higher pressure in a membrane oxygenator during the CPB procedure causes higher concentrations of free hemoglobin (fHb) and higher viscoelasticity (7–9). Orime et al. (10) reported that fHb is found to be increased under both CPB procedures with no difference statistically. However, pulsatile perfusion was found to be more effective in reducing endothelial damage because it activated fewer amounts of cytokine and endothelin than nonpulsatile perfusion did. Ündar et al. (11) examined the effects of pump pulsatility on blood viscoelasticity before and after deep hypothermic circulation arrest in a neonatal piglet model. This group
reported that the pulsatile pump incurred less blood trauma than a nonpulsatile roller pump did because of its lower viscoelasticity under CPB procedures. In addition, because the concentrations of fHb alone do not provide information on sublethal red blood cell (RBC) trauma or damage (12), various methods including normalized index of hemolysis (NIH) as index of blood hemolysis (13,14) as well as deformability tests via narrow pore filters (12), erythrocyte fragility (EF) (15), cyclic reverse shear stress (16), and flow cytometric method (17) were applied to quantitatively evaluate sublethal RBC trauma or damage.

Deformability of RBCs evaluated by the methods mentioned here was found to be constant, while the fHb concentration increased under CPB procedures (12,15–17). Korin et al. (18) studied motion and deformation of RBCs in a microchannel via rheoscope measurement technique. This group found that the deformability of RBCs had higher sensitivity on the shear modulus of the RBC membrane as well as shear stress. Thus, they utilized the deformability of the RBCs as a quantitative tool to evaluate the mechanical properties of blood cells.

In this article, we hypothesized that a pulsatile CPB procedure has no significant differences with a nonpulsatile CPB procedure in terms of its effects on hemolysis and deformability of RBCs under various shear stress conditions. In order to verify the hypothesis, four parameters (fHb, NIH, deformability index [DI], and elongation index [EI]) were investigated with respect to pump pulsatility because concentrations of fHb and NIH could be correlated with hemolysis of RBCs, and DI as well as EI could be correlated with deformability of RBCs.

**MATERIALS AND METHODS**

In this study, all experiments were performed according to the “Guide for Care and Use of Laboratory Animals” issued by the School of Medicine, Korea University. Twelve adult mongrel dogs weighing between 20 and 30 kg (pulsatile: 25.7 ± 2.3; nonpulsatile: 26 ± 3.1) were prepared and randomly assigned into two groups: a nonpulsatile group (NP, n = 6) and a pulsatile group (P, n = 6). All animals were premedicated with intramuscular ketamine (10 mg/kg) and placed on the surgery table after weighing. Electrocardiography electrodes were attached.

An intravenous fluid route was then established at the upper foreleg. After general anesthesia was induced with thiopental sodium (5–10 mg/kg) and vecuronium bromide (0.1 mg/kg), endotracheal intubation was performed with an endotracheal tube (7 mm inner diameter).

Anesthesia was maintained with a N2O/O2 gas mixture (2 L/min for each gas) and 1–1.5% isoflurane. Mechanical ventilation was then maintained at a tidal volume of 10 to 15 mL/kg and a respiratory rate of 25 to 30 breaths/min. After vertical median sternotomy, the pericardium was dissected and secured by suturing to the sternum. Heparin (300 U/kg) was injected and infused at the rate of 100 U/kg/hr. The heparin injection rate was adjusted to maintain an activated clotting time of 400 to 500 s.

CPB circuit was constructed by inserting an 18 Fr arterial cannula into the ascending aorta and two 22 Fr venous cannulas were inserted at superior vena cava and inferior vena cava through the right atrium, which was then connected to CPB circuit operated either by a centrifugal pump (Bio-Pump, Medtronic, Minneapolis, MN, USA) or a pulsatile pump (Twin Pulse Life Support, T-PLS, SL-1000, Newheartbio Co., Seoul, Korea). Then, the CPB circuit was primed with lactated Ringer’s solution, which was required to fill a reservoir. The reservoir level was maintained to insure sufficient blood flow during pump operation. In both groups, a conventional membrane oxygenator (CAPIOX SX10R, Terumo Co., Tokyo, Japan) was used. The total prime volume was approximately 1300 mL. Gas flow to the membrane oxygenator was fixed at 2 L/min (FiO2 0.6) during the CPB procedure.

Ventricular fibrillation was induced by placing a DC 9V battery on the right ventricular outflow. CPB was operated for 3 h and the blood pump flow was maintained at 80 mL/kg/min with the body temperature of the dogs kept constant at 36°C. At the end of the experiment, the animals were sacrificed under an anesthetic state according to the Korea University guidelines.

**Sample preparation**

Five milliliters of blood was first drawn from the carotid artery to establish a baseline (i.e., just prior to CPB operation). Next, another 5 mL of blood was extracted from the right femoral artery after 1, 2, and 3 h of CPB, respectively. These samples were kept in a bucket of ice water before being placed in a centrifugal separator. fHb was then measured in a clinical laboratory. In order to measure and analyze DIs and EIs, blood samples were diluted to a hematocrit of 3% using a phosphate buffered saline solution (GIBCO, pH 7.4, 1X).

**NIH**

A NIH is generally used to evaluate blood hemolysis because fHb would not provide sufficient information on blood damage caused by various factors. The
NIH could be calculated using the following formula (19–21),

\[
NIH (mg/100 \text{ L}) = \left( \frac{\Delta fHb}{\Delta T} \right) \times \left( \frac{100 - H_{ct}}{100} \right) \times \left( \frac{V}{Q} \right)
\]

where \( \Delta fHb \) is the increase in plasma fHb concentration (mg/dL) over sampling time interval (\( \Delta T \), min), \( H_{ct} \) is hematocrit (%), \( V \) is the circuit volume (L), and \( Q \) is the flow rate (L/min).

**Deformability**

The deformability of each RBC was evaluated by measuring DI and EI. DI is defined as the ratio of a major length to a minor length of a RBC assuming that each RBC has an elliptical shape under shear stress. EI was then calculated using a predefined mathematical relationship between DI and EI. A simple and disposable microfluidic device was prepared to measure DI and EI under various flow rates, as shown in Fig. 1.

The test setup was composed of a microscope, a cooled CCD camera DP-71 (Olympus, Tokyo, Japan), a syringe pump (NEMESYS, Centoni GmbH, Germany), and commercially available image analysis software (SPIP, Image Metrology ApS, Lyngby, Denmark, version 4.7). Images were acquired and analyzed to obtain DI values for each RBC. In order to verify the analysis capability of SPIP for the arbitrary orientation of RBCs, known elliptical shapes having a DI ranging from 1 to 7 were used at oblique angles between 0° and 45°. These elliptical shapes were drawn using AUTOCAD (Version 2002, Autodesk, San Rafael, CA, USA) and then analyzed with SPIP, as shown in Fig. 1b. As a result of a regression analysis, the correlation between identified DIs and known DIs had higher value of \( R^2 = 0.95 \) and its linear slope also was calculated as 1.23, which implies that SPIP overestimated 23% for known DIs without respect to oblique angles. This result indicates that SPIP is a suitable tool for image analysis because it could accurately analyze DI for arbitrary-positioned RBCs. However, it would be necessary to adjust for the 23% overestimation when analyzing the captured images.

In order to estimate the shear stress acting on RBCs in a microfluidic device, which has a 30 \( \mu \text{m} \) width and a 9 \( \mu \text{m} \) height, the mathematical relationship between the flow rate \( Q \) and average shear stress \( <\tau> \) can be simply expressed as

**FIG. 1.** Schematic drawing of experimental setup and image analysis (a), image calibration results (b), snapshots of deformed RBCs with a high speed camera (c), deformability indices (d) under different flow rates.
where $Q$ is a flow rate, $w$ is a width of a microfluidic channel, $h$ is a height of a microfluidic channel, $\mu$ is a fluid viscosity, and $\langle \tau \rangle$ is an average shear stress. Based on this relationship, the flow rates were determined to be 10, 50, 100, 150, and 200 $\mu$L/h, corresponding to average shear stresses of 48, 240, 480, 720, and 960 dyne/cm$^2$, respectively.

Figure 1c presents the RBC images captured at the flow rates of 10, 50, 100, 150, and 200 $\mu$L/h, respectively. Figure 1d shows the relationship between the DI and the flow rates, where the DI also tended to increase with respect to the flow rate. From the results of analysis of variance test as a statistical method, it was found that the DI strongly depended upon the flow rate and had a linear relationship from 50 to 200 $\mu$L/h ($P < 0.05$). However, at the flow rate of 200 $\mu$L/h, the captured images were indistinctive due to too fast movement of the RBCs. Therefore, the flow rates for investigating the deformability of RBCs were limited at 50, 100, and 150 $\mu$L/h. Using the measured DI of RBCs, the EI of RBCs was calculated by applying the following mathematical relationship.

$$EI = \left( \frac{DI - 1}{DI + 1} \right)$$

where $EI$ is an elongation index and $DI$ is a deformability index. Then, based on a linear regression model for identifying the deformability of RBCs under three flow rates (50, 100, and 150 $\mu$L/h), the DI and the EI were modeled as $DI = D_0 + D_1 \cdot Q$ and $EI = E_0 + E_1 \cdot Q$, respectively. Here, four constants, $(D_0, D_1)$ for DI and $(E_0, E_1)$ for EI, were utilized to determine the statistical differences between perfusion type within the perfusion times.

### Statistical analysis

A commercially available software, Minitab (Version 14, Minitab, Inc., State College, PA, USA), was used for the statistical analysis of all data, with all results being expressed as mean ± SD. In addition, the Mann-Whitney $U$-test was applied to statistically determine the equality of two group medians. Also, the Kruskal-Wallis test was utilized to verify the equality of medians for perfusion times with respect to both groups. For all tests, if the $P$ value is less than 0.05, the result is considered to be statistically significant within the 95% confidence interval.

### RESULTS

#### Plasma fHb

As shown in Fig. 2, the concentration of the fHb significantly increased with the time course of the CPB operation for each group (all $P$ values <0.05). However, the Mann-Whitney $U$-test with a 5% significance level returns $P$ values greater than 0.05 indicating that the median of each group at the specific operation time cannot be distinguished.

#### NIH

In order to quantitatively determine a difference of blood hemolysis for each group, NIH was compared using NIH formula as shown in Fig. 3. As a result, the NIH values of pulsatile and nonpulsatile perfusions were $13.2 \pm 4.0$ and $15.8 \pm 17.7$ mg/100 L, respectively. The $P$ value obtained by the Mann-Whitney $U$-test is greater than 0.05. Thus, there is no statistically significant difference for both groups.

#### Deformability

The DIs and the EIs measured and analyzed for three kinds of flow rates as well as CPB operation...
times are summarized in Table 1. Based on these results, a linear regression analysis was conducted to identify four constants: D0, D1 for DIs, and E0, E1 for EIs. Figure 4 represents two constants (D0, D1) estimated from the linear regression model for the DI. As a result of the statistical analysis for the two constants, no significant difference was found for both groups at specific operation times and the DI increased with the time course of the operation times (all \( P \) values > 0.05). Thus, it could be concluded that there is no statistically significant differences in the medians of the DI with respect to the perfusion types.

Similarly, Fig. 5 shows two constants (E0, E1) that were identified from a linear regression model of the EI for different perfusion types and operational times. Again, no significant difference in the medians of the EIs was found (all \( P \) values > 0.05), excepting that the intercept (E0) at 3 h of perfusion had a significant difference in the median for both groups (all \( P \) values < 0.05).

**DISCUSSION**

During CPB procedure, blood hemolysis occurs when blood cells contact a nonphysical surface and are exposed to higher shear stress. This hemolysis tends to severely increase under higher flow rate and larger gas–fluid interfaces. In addition, blood damage increases under higher dP/dt (pressure/time) developed in CPB circuit, which is influenced by the membrane oxygenator as well as pump types (22,23). In this study, blood hemolysis caused by pulsatile and nonpulsatile pumps was only taken into account because flow rate was fixed and the same membrane oxygenator was applied.
From the results of fHb measurements, there was no statistical difference found between two groups \((P > 0.05)\). However, the concentrations of the fHb significantly increased within the perfusion times under CPB procedures for both groups \((P < 0.05)\). These results were consistent with previous studies. In other words, Orime et al. (10) reported that the concentration of the fHb increased under CPB and there was no significant difference between pulsatile and nonpulsatile (roller) CPB. Svenmarker et al. (12), Ding et al. (15), and Watanabe et al. (16) showed that the concentration of the fHb increased under CPB with a roller pump and a centrifugal pump usage. Valeri et al. (24) reported that there was no statistical difference for nonpulsatile (roller) pump in terms of the concentrations of the fHb.

In addition, as a result of NIH, there was no statistical difference between two groups \((P > 0.05)\). The results of fHb and NIH demonstrated that pulsatile perfusion has no significant difference from nonpulsatile perfusion in terms of hemolysis of RBCs.

The deformability of RBCs determines their ability to adapt and change their shapes for minimizing their resistances in dynamic flow conditions. Also the deformability of RBCs is related to their capability of passing through narrow capillaries and depends on factors such as the surface area-to-volume ratio (S/V ratio), intracellular viscosity, and viscoelastic properties of the cell membrane (18,25,26). When the deformability of RBCs is reduced, it would be highly possible to directly block a capillary vessel. Thus, reduced deformability could be strongly related to impaired perfusion and oxygen delivery in peripheral tissues. As a result of statistical analysis on the DIIs, no significant differences have been found between the perfusion groups as well as the perfusion times. Thus, there was no remarkable difference between the groups for the EI values within the perfusion times \((P > 0.05)\) other than the intercept \((E_0)\) at the 3 h of CPB, which had a significant difference for both groups \((P < 0.05)\).

Furthermore, neither of the two constants \((D_1, E_1)\), which are sensitive for shear stress, showed significant differences between the groups at the specific operation time and different operation times for each group during CPB operation, which suggests that RBC deformability in terms of shear stress was independent of perfusion type. These results were compatible with previous studies. Svenmarker et al. (12) reported that the initial filtration rate and clogging slope—used to indicate RBC deformability—did not indicate a statistical difference within 60 min under CPB, although fHb increased. Similarly, Ding et al. (15) reported that the change of EF was negligible because the S/V ratio was maintained even though fHb linearly increased. Watanabe et al. (16) stated that the RBC deformability could be maintained for 8 h using a centrifugal pump, although fHb increased.

Thus, from the results of hemolysis and RBC deformability analyses, it should be considered that both perfusion types have a potentially equivalent effect on the sublethal damage of RBCs. This sublethal damage could impair mechanical properties, and thereby adversely affect microcirculation, and could also potentially promote the activation of platelets and white blood cells as well as cause anemia (27). Quantitative analysis for echinocytes as a direct indicator of sublethal RBC damage was not performed due to limited function of our microfluidic device. For more quantitative echinocyte analyses, it is necessary to design a new microfluidic device that is able to analyze cell morphology. It is known that cytoskeletons play an important role in the deformability of RBCs (26). However, we did not directly examine cytoskeletons for sublethal damage. Nevertheless,
this study is meaningful because it provides information about the difference in not only the concentrations of fHb and NIH but also the deformability for two types of perfusion under precisely controlled hematocrit level.

CONCLUSION

In order to verify that pulsatile CPB procedures have no significant differences with nonpulsatile CPB procedures in terms of effects on hemolysis and deformability of RBCs under various shear stress conditions, four parameters such as fHb, NIH, DI, and EI have been investigated for two perfusion types in CPB procedure. According to our results, there were no statistically significant differences in the concentrations of the fHb and the NIH. In addition, there was no significant difference in RBC deformability between the perfusion types within an operation time of 3 h. Therefore, it could be concluded that pulsatile perfusion has no significant difference to nonpulsatile perfusion in terms of hemolysis and deformability of RBCs within 3 h of CPB procedures.

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