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The effect of temperature and shear rate on platelet aggregation

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Samples of whole blood were obtained from male volunteers and exposed to combinations of shear rates and temperatures representative of cardiopulmonary bypass (CPB) in a modified computer-controlled concentric cylinder rotational viscometer for a period of 100 s. Blood sampled from the chamber was fixed in paraformaldehyde, stained with CD41 and analysed by flow cytometry. Only platelet-positive particles were acquired, each individual cell, or aggregate of cells, identified by analysis of its fluorescence and forward light scatter characteristics. Little platelet aggregation was observed at shear rates of less than 4000 s^{-1} for temperatures of greater than 24 °C, but large numbers of aggregates were formed at all temperatures at 4000 s^{-1} ($p < 0.05$), with more aggregates forming at 24 and 30 °C than at 37 and 42 °C ($p < 0.05$). We conclude that the process of aggregation is dependent on both temperature and shear rate. We note that a large number of platelets become involved in aggregates under conditions of temperature and shear-rate typical of CPB.

1. Introduction

In order to maintain vital organ function during open-heart procedures, a patient's own blood must be mechanically pumped through an extracorporeal circuit in a procedure known as cardiopulmonary bypass (CPB), both to oxygenate the patient's blood and to maintain normal cardiac output. Although membrane oxygenators suitable for CPB in humans were introduced as early as the mid 1950s [1], such procedures are still associated with an increased risk of adverse cerebral outcomes (memory loss, stupor, seizure or coma). Indeed, a recent article in the *New England Journal of Medicine* stated that acute changes in cerebral function after elective coronary-artery bypass surgery are so common and serious that "new diagnostic and therapeutic strategies must be developed to lessen such injuries" [2]. The incidence of in-hospital neuropsychological dysfunction was reported as ranging from 25%–79% in selected studies of CPB [3–6]. Additional serious complications have also been reported, including gastrointestinal bleeding [7], retinopathy [8], pulmonary dysfunction syndrome [9] and choreal manifestations in infants [10].

There is unlikely to be one single cause for this, but it is generally agreed that haemodynamic changes during CPB are of major importance. Elevated shear rates are often encountered within extracorporeal circulations, for example in blood pumps, filters and within the oxygenator element itself where mixing is often used to improve further the efficiency of the gas-exchange process. The interaction of blood with a large surface area of synthetic material, coupled with the exposure of blood cells to high shear stresses, can result in gross haematological alteration. Indeed, platelet depletion [11, 12] caused by adhesion [13] and aggregation [9, 14], complement activation [15], acute leukopaenia [16] and haemolysis [17], have all been reported following CPB. As a result, patients may suffer from abnormalities in platelet function that contribute to bleeding disorders, whilst circulating debris, platelet and leukocyte aggregates may block the microcirculation of the pulmonary and cerebral systems. The picture is further complicated by the need to lower the body temperature of patients undergoing cardiac surgical procedures in order to reduce their metabolic rate and thereby reduce the demand for oxygen. Previous studies have alluded to the possibility that platelets aggregate to a larger extent at lower temperatures [18] and comparisons of hypothermic and normothermic CPB in animals have demonstrated compromised platelet function during "cold" bypass [19]. Currently, the potential effects of CPB are, to some extent, mitigated by the use of therapies during surgery, including aprotinin [19–21], nafamostat mesilate [22] and a number of protease inhibitors including plasminogen activator inhibitor-1 [23].

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In this paper we describe an *in vitro* experiment on whole blood, in which we quantified platelet aggregation at a series of temperatures and shear rates, using multiple subjects. We wished to establish the conditions under which platelets could be adversely affected by the perfusion of blood through an extracorporeal device.

2. Materials and methods

2.1. Collection of blood

Human blood was taken from healthy, male volunteers who had been free of medication for at least 14 days, by insertion of a 19-gauge needle into a median cubital vein. After collection into a syringe, the blood was carefully mixed with 3.8% (wt/vol) tri-sodium citrate at a volume ratio of 9 parts blood to 1 part citrate. The sample was incubated at 37 °C for 30 min prior to exposure to experimental shear rates.

2.2. Exposure of blood samples

Samples (2 ml) of blood were transferred to a temperature-controlled environmental cabinet 7 min prior to the start of each experiment. The air temperature in the cabinet was controlled by a 200 W solid-state heating element with integral fan (RS Components, Corby, Northamptonshire, UK) coupled to a proportional temperature controller. A concentric cylinder rotational viscometer (Contraves LS2, Zurich, Switzerland), having a couette geometry as shown in Fig. 1, was modified by isolating the torque-measuring apparatus, fitting an uprated motor and controlling by a computer to provide constant acceleration and deceleration to and from a steady angular velocity. The modified viscometer was situated within the cabinet and was used to subject 310 µl blood to known shear rates (0, 1000, 2000, 4000 s^{-1}) for a period of 100 s in addition to 15 s ramping periods. Experiments were performed at 42, 37, 30 and 24 °C. A non-exposed control sample was taken at each temperature. Within each experiment the sequence of shear-rate exposure was randomized for any given temperature. The full range of exposure treatments was performed on each portion of exsanguinated blood in order to obviate day to day variations. A variety of subjects was used as blood sources in order to reduce individual biological variation. The sample size, n, was 6 for each shear rate and temperature combination.

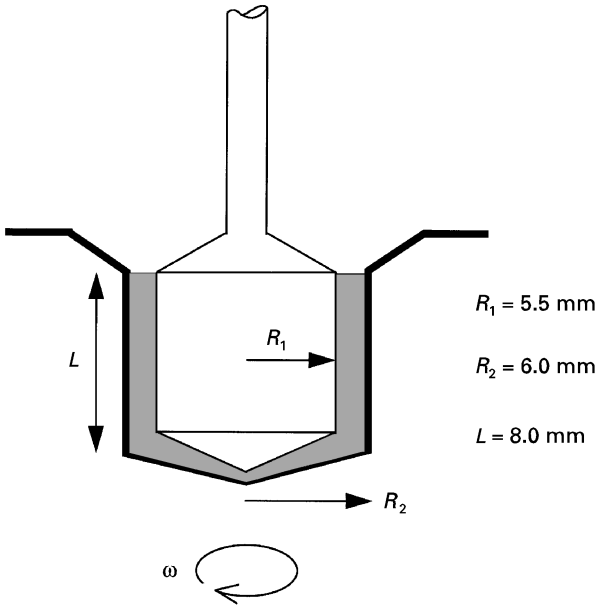

Figure 1 Schematic diagram indicating the dimensions and shape of concentric cylinder arrangement used to generate shear-rates between 0 and 4000 s^{-1}.

2.3. Analysis of platelet aggregation

From each sample, 40 ml were added to an equal volume of paraformaldehyde (2% in PBS) and left to stand for 10 min at room temperature. Platelets were stained by incubating 10 µl fixed blood with 5 µl anti-human CD41 conjugated with phycoerythrin (Pharmingen, San Diego, CA, USA) for 20 min at 4 °C. The sample was then diluted with isotonic saline and analysed using a Becton Dickinson FACSort flow cytometer (San Jose, CA, USA), counting 30 000 platelet-positive events per sample.

2.4. Data analysis

Particles were quantified in terms of their forward scatter (FSC) and marker fluorescent intensity (FL). For each subject, a two-dimensional dot-plot (FSC versus FL) of the 37 °C control sample was constructed (Fig. 2a). The dot-plot was separated into five polygonal regions to identify: (I) normal platelets; (II) platelet–platelet aggregates; (III) platelet–red blood cell (RBC) aggregates; (IV) microparticle (MP)–RBC aggregates, and (V) MPs. These regions were then applied to dot-plots generated from the treatment samples and platelet aggregation was quantified (Fig. 2b). The mean number of platelets per aggregate (MPA) was estimated by dividing the mean value of FL for the platelet aggregate group (II) by the mean value of FL for the normal platelet group (I). This assumes that the intensity of the fluorescent platelet marker, CD41, was proportional to the number of platelets involved in an individual platelet–positive particle. A platelet aggregation index (PAI) could then be calculated by the following formula

$$\mathrm{PAI}(\%) = \frac{N(\mathrm{II})\,\mathrm{MPA}}{N(\mathrm{I}) + (N(\mathrm{II})\,\mathrm{MPA})}\,100 \qquad (1)$$

where N(I) and N(II) are the number of particles within regions I and II.

3. Results

A two-way analysis of variance with replication was performed on the dataset depicted in Fig. 3. There was significant variation of platelet aggregation with both shear rate and temperature ($p < 0.005$).

Values of PAI at each combination of shear rate and temperature were compared to a control sample at 37 °C by a one-tailed paired t-test. Table I indicates

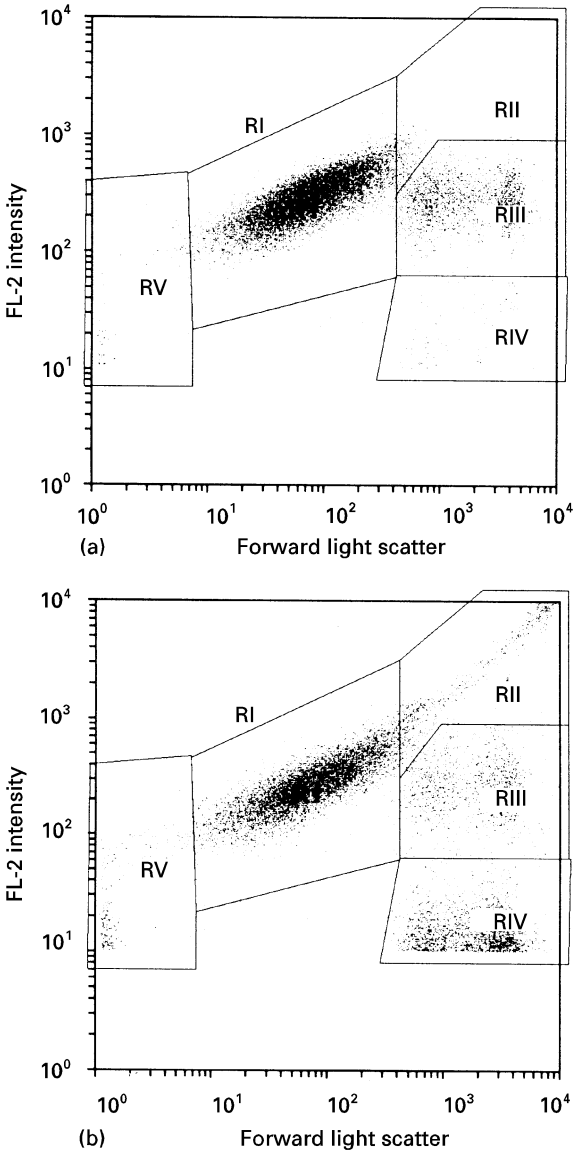

Figure 2 Scattergram of fluorescence intensity against forward light scatter for 30 000 platelet-positive particles in whole blood for (a) 37 °C control sample; (b) exposure for 100 s to 4000 s^{-1} at 24 °C. Region consist of (I) normal platelets, (II) platelet–platelet aggregates, (III) platelet–red blood cell aggregates, (IV) microparticle–red blood cell aggregates, (V) microparticles.

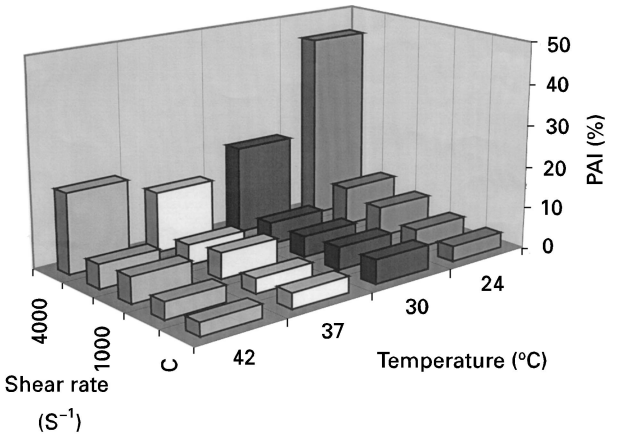

Figure 3 Three dimensional graph of mean values of platelet aggregation index (%) defined in Section 2.4 as a function of temperature and shear rate. C is control.

TABLE I Mean values ($n = 6$) of platelet aggregation index (%), as a function of temperature and shear rate. The level of significance of the results when compared to control samples taken at 37 °C is indicated

Temperature (°C)	Shear rate (s^{-1})			
	0	1000	2000	4000
24	4.6	7.2	9.4[a]	44.9[c]
30	5.6	5.1	5.3	21.2[b]
37	3.6	6.3[a]	5.0	14.7[a]
42	4.1	6.3[a]	5.8[a]	19.4[a]

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.005$.

that aggregation was higher at 24 °C and 4000 s^{-1} than at any other combination of temperature and shear rate. Also, aggregation was significantly greater than the control at 4000 s^{-1} for any temperature. Platelet aggregation, as quantified by PAI, was greater at lower temperatures (24 and 30 °C) for a shear rate of 4000 s^{-1} ($p < 0.05$).

4. Discussion

In experiments performed *in vitro*, we have shown that platelet aggregation is a function of temperature and shear rate. Particularly, a combination of a low temperature and a relatively high shear rate may give rise to a dramatic increase in platelet aggregation.

4.1. Mechanisms of platelet aggregation

There are a large number of biochemical factors which govern the tendency of platelets to form aggregates in the fluid phase, but in simple terms, platelet aggregation may be thought of as a function of binding affinity between platelets and the frequency of their collisions [24]. From Fig. 3, it is clear that as shear rates increase between 0 and 2000 s^{-1}, there is a moderate increase in aggregation, consistent perhaps with a proportionate increase in collision frequency. At these shear rates, there is little effect of temperature on aggregation, indicating that platelet–platelet binding affinity is largely unchanged, whereas at a shear rate of 4000 s^{-1} an order of magnitude increase in aggregation is observed and we associate this with an increase in binding affinity. The increase in aggregation above shear rates of 3000 s^{-1} has been attributed to a platelet release of procoagulant from α-granules [25].

Another result arising from this study is the significant variation of aggregation with temperature at 4000 s^{-1}. The activity of enzymes such as ADPase is a function of temperature. Higher levels of enzyme activity could be responsible for a decrease in the extracellular concentration of the agonist, ADP, reducing aggregate formation at physiological temperatures. However, plasma consists of a complex system of inhibitors and proactivators which make up networks of negative and positive feedback systems. Additionally, platelets can release a large number of

different agonists from one of four different internal granules, making a hypothesis on the exact cause of differential platelet response to a variety of environmental conditions quite difficult to form.

4.2. Clinical relevance

There is considerable debate as to whether hypothermic or normothermic CPB should be used in patients undergoing lengthy cardiac surgery. Each of these practices has advantages that have been widely reported in the clinical literature. During hypothermic CPB, the temperature of the blood is reduced to about 25 °C. Lowering the body temperature reduces the patient's metabolic rate and consequently oxygen consumption. This technique has the benefit that shear rates in the oxygenator do not have to be so high to maintain an adequate oxygen supply. A greater platelet loss is associated with "cold" CPB due to the formation of aggregates, and some workers have observed a temporary loss of function in circulating platelets. Significant levels of platelet adhesion to the synthetic surfaces in the extracorporeal circuit may also occur. Together, these factors may result in excessive bleeding times.

Adverse cerebral incidents often occur after open-heart procedures. While most of the more serious events may be attributed to risk factors, such as atherosclerosis, in the major vessels, old age and a history of cerebrovascular problems, the presence of large platelet aggregates after CPB may be responsible for the incidence of many minor perioperative neurological episodes.

Oxygenators and filters in the extracorporeal circuit may expose blood to large shear rates for considerable lengths of time. Local warming of blood before passage through these elements may reduce the number of aggregates formed and consequently the incidence of minor cerebrovascular events and platelet loss. To validate this hypothesis further, we intend to conduct investigations of the effect of rapid temperature changes on platelet function in a more realistic experimental model of CPB.

5. Conclusion

In a series of *in vitro* experiments, we have demonstrated that platelet aggregation is markedly higher at the low temperatures and shear rates experienced in blood oxygenators and filters. We believe that careful design of extracorporeal circuits with particular regard to shear rates and temperature, could serve to decrease the platelet loss observed with patients undergoing procedures involving a lengthy period of "cold" cardiopulmonary bypass.

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