Shape change and hypotonic shock response measurements to assess functional efficacy of platelets

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Several variables such as anticoagulant, plastic bag composition, gas transport, residual plasma volume, agitation and storage temperature influence the viability and function of platelets during storage. Measurements of pH, pO₂ and pCO₂ are good enough to predict acid–base balance but not for detecting functional stability of platelets. A quick and simple screening method is required to establish the functional response of stored platelets to agonists. Minimal activation of platelets may be a reversible change, but if they are stimulated to change shape and secrete their granular contents, they turn non-functional and refractory to agonists. So refractoriness of platelets to agonist response can be a reliable method to detect the functional loss of platelets. Here it is demonstrated that refractoriness can be measured by a simple spectrophotometric shape change measurement. Platelets that were deliberately stimulated were used as a model for refractory platelets. The data obtained for shape change response was double-checked using conventional aggregometric analysis to detect loss of function. Measurement of hypotonic shock response to assess the post-transfusion recovery of stored platelets is also studied in unstimred suspension, using a double beam spectrophotometer. Thus a simple spectrophotometric analysis will enable the transfusion centres to check quality of the stored platelets and predict their post-transfusion recovery and hemostatic efficacy.

CANCER patients under chemotherapy are the major victims of thrombocytopenia and they require transfusion of platelet concentrates (PC) to prevent haemorrhagic complications. Theoretical requirements on quality assurance of platelet concentrates, prepared for transfusion, are that they should be uniformly resuspended, their biological activity should be preserved and the transfused platelets should in vivo behave like a healthy person’s own cells. All criteria and standards required for the collection and processing of whole blood also apply to PC preparation.

While assuring the quality of stored platelets, it is required that there is sufficient platelet count present in the concentrate as single platelets. Swirling movement of platelets during gentle shaking is considered to be a quick visual test for preservance of platelet morphology¹. Aerobic metabolism in stored platelets requires that sufficient oxygen levels be maintained within the storage container. If the oxygen level is insufficient, the platelets must resort to anaerobic metabolism resulting in decline of pH². Therefore, a primary consideration in

Figure 1. Normal and agonist-treated platelets viewed under phase contrast microscope. Platelet suspensions before treating with ADP and after treating with different concentrations of ADP were fixed with 0.2% glutaraldehyde, for 2 h, washed with PBS and then a smear on glass slide was dried with 30%, 50%, 70%, 90% and dehydrated acetone. The smears were examined under phase contrast microscope. a, Normal platelets. b, 2.0 μM ADP-treated and c, 4.0 μM ADP-treated.
selecting a plastic container is that it provides maximum
gas exchange across the bag, allowing carbon dioxide to
e scape and oxygen to enter.

Platelet packs require gentle agitation to improve dif
cus ion of gases and horizontal mode is preferred over tumbling or rotary movements so that the viability of the cells are maintained better. Exposure of platelets to temperatures as low as 4°C results in their activation and they turn refractive to physiological stimulators and hence storage at 22°C is recommended. Kunicki et al. and Gottschall et al. have suggested that it is not only during storage but that the temperature of the processing laboratories also should be maintained between 20 and 22°C to keep the viability of stored platelets in terms of recovery and survival.

The platelet response to hypotonic shock may provide a measure of platelet viability, which has been corre
lated to 51Cr-labelled platelet recovery in vivo, but not to hemostatic effectiveness. Ultimately, their response to agonists is the criterion to predict hemostatic efficacy of the stored cells. We demonstrate that a simple spectro
got photometric shape change measurement will enable assessment of response to agonist, which eventually decides hemostatic potential. The platelets which showed depolished shape change response in spectropho
tometric measurements, seemed to show reduced aggregatory response as measured in the aggregometer. Hypotonic shock response can also be measured in un
tered suspension using a double beam spectrophotome
ter with thermostated cuvette holders.

Thirty ml blood was collected in polypropylene test tubes with 4.5 ml ACD, from normal volunteers who had not taken aspirin, at least for a week, before the collection day. Blood was centrifuged at 400 g for 3 min in a Hitachi SCR20BA high-speed centrifuge (Hitachi, Japan) using RPR-18-3 rotor. The platelet-rich plasma (PRP) was aspirated and the blood was centrifuged at 1,500 g for 5 min to get platelet-poor plasma (PPP). Adenosine diphosphate (ADP) was obtained from Sigma Chemicals, USA and the concentration of ADP solution was determined using a molar extinction coefficient of 1.54 x 10^4 at A269.

Ten ml PRP was diluted with Tyrode’s buffer to get an optical density (OD) of 0.8 at 540 nm, measured against
Table 1. Shape change data and aggregation data obtained with preactivated platelets

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Extent (Maximum A_{S40})</th>
<th>Spectrophotometric analysis</th>
<th>Aggregometric analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extent, decrease in A_{S40}/min</td>
<td>Extent, increase in T/min</td>
</tr>
<tr>
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<td>0.029</td>
<td>38</td>
</tr>
<tr>
<td>0.5</td>
<td>0.036</td>
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<td>43</td>
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<td>1.0</td>
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<tr>
<td>2.0</td>
<td>0.019</td>
<td>0.021</td>
<td>15</td>
</tr>
<tr>
<td>3.0</td>
<td>0.011</td>
<td>0.017</td>
<td>14</td>
</tr>
<tr>
<td>4.0</td>
<td>0.014</td>
<td>0.014</td>
<td>8</td>
</tr>
<tr>
<td>5.0</td>
<td>-0.004</td>
<td>0.00</td>
<td>2</td>
</tr>
</tbody>
</table>

Shape change was measured using a double beam spectrophotometer and aggregation measured using a conventional aggregometer. For both studies PRP from same aliquots, treated with concentrations of ADP as mentioned in the table were used. So the platelet number, and extent of activation and the threshold concentration of ADP used to challenge were same. (A, absorbance; T, transmission).

PPP diluted in the same proportion with buffer. The required diluent volume varied from batch to batch depending on the platelet count. Five ml aliquots of dilute PRP were used for each concentration of ADP and incubated at 32°C to stimulate platelets. After such mild activation, for 2 min, the OD was redetermined against diluted PPP.

After controlled stimulation with ADP, 1 ml PRP was pipetted into each of the reference and sample cuvettes and challenged with aggregatory dose of ADP (16 μM) to measure shape change, using the method of Jamaluddin and Krishnan. The increase in absorbance at 540 nm from a base line value of zero was recorded on a Shimadzu UV-Vis 240 spectrophotometer.

The aggregatory response of preactivated platelets was also studied, using Chronolog Lumi aggregometer (Chronolog, USA). For aggregatory measurements, 0.45 ml of PRP from preactivated platelets were taken and challenged with 16.0 μM ADP. The extent and rate of aggregation was calculated by the IBM compatible software and integrator obtained from Chronolog, USA.

Samples from the control PRP and from activated platelets were fixed with 0.1% glutaraldehyde for 2 h. The suspension was washed with PBS and a smear was made on microscope slide. The smear was dehydrated using graded concentrations of acetone. The dried smears were viewed under a Nikon biphot phase contrast microscope.

Addition of ADP in concentrations ranging from 0.5 μM, to 3.0 μM to PRP having an initial OD of 0.86 at 540 nm, resulted in an increase of A_{S40} to 0.91, while 4.0 μM and 5.0 μM ADP when added, A_{S40} obtained was 0.90 and 0.89 respectively. Thus even in the absence of continuous stirring, platelets changed shape, and the increased A_{S40} remained steady. With stronger stimulus (5.0 μM), other than shape change, microaggregates also formed to result in a reduced A_{S40}. The formation of doublet or higher order aggregates due to the added ADP can be seen under phase contrast microscope as shown in Figure 1 a–c. Apparently, platelet activation could be produced in vitro to different levels, by addition of subthreshold concentration of ADP. Such activations were also observed in stored platelet concentrates. In this study, the platelets were deliberately activated and were used as a model to demonstrate how the cells turn nonfunctional and to measure their refractoriness by challenging them with threshold concentrations of agonist.

Agonist response, which is measured as the extent of increased A_{S40} due to the shape transformation from discs to spheres, of normal and refractory platelets, is shown in Figure 2. The increase in A_{S40} from base line due to shape change and the rate of reduction in A_{S40} (slope) due to platelet clumping, on addition of 16 μM ADP into PRP, preactivated to various levels, are given in Table 1. The rate of decrease in OD (slope of the curve) was smaller, if ADP used for preactivation was higher. Thus, if PRP pretreated with 5.0 μM ADP was challenged with 16 μM ADP, there was no further shape change response.

The refractoriness was also measured using an aggregometer. The concentration used for challenging was the same as that used for the spectrophotometric measurements. Both the extent and rate of aggregation were affected by the pretreatment of PRP with small doses of ADP. The tracings are seen in Figure 3 (refs 1–7). The differences in amplitude and slope of aggregation due to preactivation of platelets are also shown in Table 1. Platelets preactivated with 0.5 μM ADP showed an increased extent of aggregation compared to the control. It is known that stimulated platelets have proaggregatory effect as long as they have not turned refractory. The extent of refractoriness was proportional to the dose of ADP used to preactivate platelets.

Conventionally, agonist response is measured using aggregometry, as suggested by Born in stirred platelet
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Figure 4. Hypotonic shock response of platelets measured on a double beam spectrophotometer. Normal platelets (Δ) and 4.0 μM ADP-treated platelets (■) were diluted at a ratio of 3:1 with 0.25 ml saline and the As40 measured is the first data point. Second data point is the absorbance at 0.2 min after mixing 0.75 ml PRP with 0.25 ml distilled water, then the absorbance increase with time (1 min to 4 min) was also noted.

suspensions. However, when the platelet suspension is stirred, the shape change and aggregation occur simultaneously and hence shape change cannot be analysed accurately. As the collision between platelets is negligible during spectrophotometric measurements in unstirred suspension, no large aggregates are formed. Hence it allows shape change to continue and reach the extent without any significant reduction in platelet number to cause reduction in As40. It has been shown before that the shape change response at 37°C and at 32°C were similar10 and from our observations the shape change response was insensitive to temperature variations between 25°C and 37°C (unpublished data). Hence the measurements were done at ambient temperatures.

The results of hypotonic shock response measurement are shown in Figure 4. The first point indicates initial As40 of platelet when diluted with normal saline. Mixing distilled water with PRP in the same proportion gives the least absorbency at 0.2 min. Later on, readings were recorded at one min interval and the increased OD indicates the recovery of platelets from the hypotonic shock. The curve no. 1 (Δ) represents HSR of normal platelets; and curve 2 (■), of platelets that were pretreated with 4.0 μM ADP. In normal platelets there was 3.4% reduction in As40, from which 4.0% was recovered. While in ADP-treated platelets there was a reduction by 14.8% and only 3.0% was recovered. So the net shock response of normal platelets was 9.4% decrease in As40, while that of preactivated platelets was 11.8%. Hypotonic shock response is accepted as a measure of platelet recovery after transfusion3. Conventionally, HSR has been detected by reduction of As40 in stirred platelet suspension when mixed with a third volume of distilled water and the recovery measured in few min. Here it is demonstrated that HSR can also be determined using a double beam spectrophotometer and continuous stirring is not required. Though there is no drastic difference between normal and activated platelets in terms of their hypotonic shock response, there is a significant difference in the functional response between the normal and activated platelets.

Different stages of platelet activation, similar to that seen in storage bags11, could be generated in vitro by controlled additions of ADP to PRP. These preactivated platelets were then used as a model to demonstrate the use of a spectrophotometer to detect refractoriness on shape change response to higher agonist dose. The agonist response measured using conventional aggregometer also showed similar refractory effects on the model platelets. The spectrophotometric method, due to its simplicity, can be chosen to monitor haemostatic efficacy and post-transfusion recovery, by measuring agonist response and hypotonic shock response, respectively, of platelets stored for transfusion.


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