Correlation of *in vitro* platelet quality measurements with *in vivo* platelet viability in human subjects

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**Background and Objectives** Changes in *in vitro* platelet quality parameters during platelet storage are associated with a decrease of *in vivo* platelet viability after platelet transfusion. Many attempts have been made to identify the most predictable *in vitro* parameters for *in vivo* performance. We used a riboflavin-based ultraviolet (UV) light treatment process designed to inactivate pathogens and white blood cell (WBC) contaminants in blood products as a model system in which to study the correlation of *in vitro* cell quality with *in vivo* viability.

**Materials and Methods** Platelet products (*n* = 18) were collected by a standard Trima apheresis procedure and treated with one of three dose levels of UV light (0, 7·2 or 12·4 J/ml) in the presence of 50 µM riboflavin. Lactate production, glucose consumption and P-selectin expression, pH, pCO₂, pO₂, hypotonic shock response and swirl were measured during 5 days of platelet storage post-UV/RB treatment. Aliquots of these products were radiolabelled on day 5 of storage and were subsequently used to determine platelet recovery and survival time in autologous subjects.

**Results** The responses of *in vitro* cell quality were observed to occur in a UV dose-dependent manner. Lactate production and pH were identified as the parameters most strongly correlated with platelet *in vivo* recovery, which ranged from 5 to 82%. The correlation coefficients (*r*) for lactate production and pH with *in vivo* recovery in human subjects were 0·9090 and 0·8831 with *P*-values of 0·007 and 0·031, respectively. Lactate production and pH were also found to be correlated with platelet survival time, with correlation coefficients of 0·8063 and 0·8384 (the *P* values were 0·01 and 0·001, respectively).

**Conclusions** Using conditions of riboflavin-based UV light treatment, lactate production and pH were identified as having the highest correlations with recovery and survival of radiolabelled platelets in healthy subjects.

**Key words:** *in vitro* platelet quality, *in vivo* platelet recovery, platelet survival, platelet viability prediction, UV light treatment.

**Introduction**

Platelet transfusion is a mainstream therapy for preventing or treating bleeding episodes in thrombocytopenic patients or patients with a high risk of bleeding [1, 2]. Success in platelet transfusion depends on the cellular viability and haemostatic activity of the transfused platelets and on the physiological status of the recipient. While the physiological status of the recipient is reflected by the ability of the recipient to tolerate the transfused platelets and the propensity to clear them from the circulation through the reticuloendothelial system, platelet viability is often measured in autologous donors by the *in vitro* recovery and survival post-transfusion...
of radiolabelled platelets. Because of the development of a platelet storage lesion, platelet products stored under current blood banking conditions show a storage time-dependent reduction in their in vivo viability. Proposed changes in platelet handling or storage methods can have the outcome of changing the rate or the nature of this storage lesion. Thus, determination of in vivo cell viability becomes a critical step in developing any new technology for platelet production, processing and storage, as well as for quality control of currently used platelet products.

Evaluation of cell viability in vitro using a method of radiolabelling test platelets is a standard method for determining the potential clinical utility of platelets [3,4]. As the reduction of in vivo cell viability is always associated with significant changes in in vitro cell quality tests, the possibility of using in vitro tests to predict in vivo viability has been extensively explored [5–7]. In early studies on platelet storage at low temperature [8], in first-generation containers [9,10] and upon freezing [11], it was observed that a platelet morphology change from a discoid shape to a spherical form during platelet storage was accompanied with low platelet recovery. This observation provided a basis for using platelet swirl to predict platelet viability. Although scoring swirl is one of the simplest laboratory methods available, it is a qualitative test and lacks sensitivity and reproducibility. Assays on the extent of shape change and response to hypotonic shock are quantitative and showed much better correlations with the in vivo recovery, with correlation coefficients (r) of 0·71 and 0·57, respectively [12]. The degree of correlation relied on the variability of the recovery values and sample numbers [4]. Metabolic parameters for platelets, such as lactate production and pH change, were also shown to have a significant correlation with platelet recovery and survival [13]. Measurement of the correlation of P-selectin expression with in vivo recovery has yielded inconsistent results. Holme et al. reported a poor correlation with platelet recovery [13], while others found significant correlations [14–16]. The clinical utility of P-selectin expression as a reliable predictor has been questioned by the findings that neither mouse platelets genetically lacking P-selectin [17] nor human thrombin-activated platelets fully expressing P-selectin [18], had different in vivo life spans from normal and resting platelets. Platelet apoptosis has also been shown to contribute to the development of the platelet storage lesion, but direct relevance to in vivo cell viability has not been established [19,20].

All the studies mentioned above analysed the correlations of various in vitro platelet quality parameters with in vivo viability and concluded that some of the parameters may be possible predictors of in vivo recovery. We have been developing a pathogen-reduction process, based on a combined treatment of platelet products with riboflavin and ultraviolet (UV) light, intended for potential use in the routine blood bank setting. It has been observed previously that exposure of platelets to UV light results in changes in a number of in vitro platelet quality measurements [21,22]. The impact of these changes on in vivo performance, however, has not been fully established. In this study, in vitro measurements for each of the platelet products were performed during storage for 5 days post-UV exposure. Results from the in vivo measurements were correlated with the observed in vivo recovery and survival of the platelet products in autologous subjects. This approach allowed us to identify in vitro cell quality parameters that had the best correlations with recovery and survival time for platelets treated with a combination of riboflavin and UV light and to establish an appropriate UV dose range in which platelet quality was not too adversely affected by the treatment process.

Materials and methods

Trima®-collected apheresis platelet concentrate preparation

All platelet products in the studies were apheresis platelet concentrates collected in 1·1 citrated poly(vinyl chloride) (PVC) ELP™ bags by the local blood centre using a Trima® Automated Blood Component Collection System (Gambro BCT, Lakewood, CO, USA).

Clinical study design

The clinical study was designed to identify the correlations of in vitro cell–quality parameters with in vivo recovery for platelets treated with various doses of UV light in the presence of riboflavin. The study was conducted at the Department of Haematology & Cell Biology, Faculty of Health Sciences, University of the Orange Free State (Bloemfontein, South Africa) under reviews and approvals of the Ethics Committee of the University of the Orange Free State and the South African Medicine Control Council (MCC). Upon completing informed consent forms and passing eligibility screening based on the local criteria and standard blood banking requirements for platelet donation, a total of 11 normal eligible volunteers, 18–65 years of age, were enrolled in the study and provided a total of 18 platelet products.

The platelet concentrates collected on day 0, with a volume of 250 ml, were transferred into 3·1 polyolefin bags (Senge, Rohrdorf, Germany), followed by the addition of 27 ml of sterile 500 μM riboflavin, resulting in a final concentration of ≈ 50 μM riboflavin. The platelet products were then exposed to UV light at wavelengths of 265–370 nm, at either a medium dose (7·2 J/ml) or a high dose (12·4 J/ml). Total illumination time varied from 5–10 min, with agitation at 25–30 °C, controlled by the illuminator. After exposure to UV light, platelet products were transferred into a citrated PVC ELP™ bag (Gambro BCT) for storage. Control platelet
products were prepared in the same manner as the treated counterparts except that they were neither exposed to riboflavin nor to UV light. The UV-treated and control platelet concentrates were stored for an additional 5 days at 20–24 ºC under standard blood bank conditions.

Samples from platelets were taken for laboratory tests on days 0 (pretreatment), 3 and 5 of platelet storage using an aseptic technique, and analysis was completed within 2 h. The in vitro cell-quality tests for platelet count, swirl score, pH, pO2, pCO2, lactate and glucose were performed per standard operating procedures (SOPs) of the trial site. Hypotonic shock response (HSR) and P-selectin expression were measured as described by Ruane et al. [23].

At the end of the 5-day storage period, a 10-ml aliquot of the platelets was radiolabelled with 111In-tropolonate, according to the SOP of the study site (in agreement with local and international standards for radiolabelling of human platelets). The labelling procedure was performed after the formation of 111In-tropolonate through mixing of 111InCl (Amersham, Buckinghamshire, UK) with tropolone [24]. The total radioactivity of radiolabelled platelets given to a subject was < 8 MBq. Blood samples for radioactivity counting were collected 15 min, 1 h and 2–3 h after infusion, twice (at and 24 h) on day 1 and once on days 2–6 postinfusion. After correcting for 2 h of radioelution, as described by Holme et al. [25], the recovery and survival values of in vitro radiolabelled platelets were calculated by the cost computer program using the multiple-hit model [26]. This correction generally resulted in an average of 2% changes in reported recovery levels for treated and control samples.

Statistics
For all in vitro cell-quality parameters, means and standard deviations were calculated. Statistical comparisons were performed using analysis of covariance (ANCOVA) for repeated measurements, where applicable. This analysis was performed using ‘PROC MIXED’ in SAS v8.1 (SAS Institute, Inc., Cary, NC, USA). Sequence effects were initially included in the model, but dropped if non-significant.

Results
UV treatment accelerates cellular glycolytic metabolism
A total of 18 platelet products were collected from healthy volunteers, with yields ranging from 2.9 to 3.8 × 1011 platelets. Products in a polyolein Sengewald bag were exposed to UV light, at a dose of 7.2 J/ml (n = 5) or 12.4 J/ml (n = 6) in the presence of 50 µM riboflavin, on the day of collection. Seven products served as controls. Table 1 summarizes the results of the cell metabolic and quality measurements. UV-treated platelets, when compared with untreated control platelets, showed increases in lactate production and glucose consumption accompanied with a decrease in sample pH during storage, indicating that exposure to UV light increased cellular glycolytic metabolism. UV treatment also accelerated an increase in P-selectin expression and decreases in HSR and swirl score. It appears that these changes during platelet storage were directly dependent on the levels of UV dose exposure.

<table>
<thead>
<tr>
<th>Table 1 In vitro cell-quality parameters of the platelets treated with various doses of ultraviolet (UV) light and stored for 5 days</th>
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</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
</tr>
<tr>
<td>Day 0</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Total cell no. (1011)</td>
</tr>
<tr>
<td>Plt count (109/µl)</td>
</tr>
<tr>
<td>Lactate (µM)</td>
</tr>
<tr>
<td>Glucose (µM)</td>
</tr>
<tr>
<td>pH @22 ºC</td>
</tr>
<tr>
<td>pO2 (mmHg)</td>
</tr>
<tr>
<td>pCO2 (mmHg)</td>
</tr>
<tr>
<td>P-selectin (%)</td>
</tr>
<tr>
<td>HSR (%)</td>
</tr>
<tr>
<td>Swirl</td>
</tr>
<tr>
<td>Lactate rate</td>
</tr>
<tr>
<td>Glucose rate</td>
</tr>
</tbody>
</table>

HSR, hypotonic shock response; Plt, platelet.
Correlation of in vitro platelet quality with in vivo cell recovery and survival

On day 5, all UV light-treated and control platelets were radiolabelled with $^{111}$In, followed by infusion of the radiolabelled platelets into autologous subjects. The radioactivities of infused platelets in the circulation were measured for up to 7 days, and the in vivo recovery and survival time of infused platelets were calculated using a multiple-hit model analysis. The average in vivo recoveries were 61 ± 16%, 30 ± 8% and 14 ± 7% for the platelets exposed to 0, 7.2 J/ml and 12.4 J/ml of UV light, respectively. Similarly, the average survival times were 157 ± 40 h, 89 ± 46 h and 57 ± 28 h for these platelets, respectively. All UV-treated platelets had a lower recovery and shorter survival time than control platelets (P-values < 0.05). In Fig. 1, each metabolic or cell quality parameter measured during storage or on day 5 was plotted against the platelet recovery, which ranged from 5 to 82% for individual platelet products, and survival time, which ranged from 20 to 220 h. A clear correlation of the parameters with the in vivo recovery, as well as survival time, was observed in several cases. Among these parameters, lactate production, pH, glucose consumption and P-selectin appeared to have good correlation with the measured in vivo recovery. The degree of these correlations was quantified by linear regression analysis and is summarized in Table 2. Both correlation coefficient r-values and F-values identified lactate production rate and pH to be most significantly correlated with in vivo recovery and P-values of 0.007 and 0.031, respectively. The remaining parameters, in order of their extent of correlation to in vivo recovery, were glucose consumption rate, P-selectin expression, HSR, swirl, pCO$_2$ and pO$_2$. The P-values for each of these latter parameters was greater than 0.05.

A very similar pattern for correlation of each cell-quality parameter with platelet survival time was observed using the same linear regression analysis approach (Table 2). Again, lactate production and pH were identified to possess the strongest correlation with platelet survival. The P-value for pH was 10-fold smaller than that for lactate production rate, indicating better correlations of pH with survival. F-values for both of these determinations indicated significantly higher levels of variation than those observed for recovery correlations. For this reason, algorithms used for platelet survival were deemed to be less reliable predictors of platelet survival.

Discussion

Many attempts have been made to predict platelet in vivo viability from in vitro cell-quality measurements [6]. Success has been only sparsely reported, probably owing to the limitation of relatively poor correlations between these in vitro tests and in vivo measurements of recovery and survival time. The challenge stems from considerably large biological variations in in vivo measurement and in the in vitro tests [4]. The extent of correlations also depends on the range and distribution of each variable. The wider the range and more even the distribution of values for cell quality that are obtained, the better the correlations that can be observed. To broaden the range of cell-quality values, platelets were treated with different doses of UV light and stored for 5 days, accompanied by parallel measurements of in vitro cell quality and cell viability, as described in this study. The results showed that all measured variables in cell quality responded to UV light treatment in a dose-dependent manner. All of these changes in cell quality were correlated with platelet in vivo recovery and survival time to varying degrees. Among them, lactate production and pH were identified, by linear regression analysis, as the parameters most strongly correlated to platelet in vivo recovery. The correlation coefficients for lactate production and pH were 0.909 and 0.883, with P-values of 0.007 and 0.031, respectively. A similar pattern for correlations of lactate production and pH with platelet survival time was also observed, although the P-value for pH was 10-fold lower than that for the lactate-production rate.

The reliability of using lactate rate and pH parameters for predicting in vivo recovery and survival was verified in a subsequent human clinical trial for the Mirasol® PRT process [27]. The Mirasol PRT process utilizes 6.2 J/ml UV light delivered to platelets in a standard ELP bag containing 50 µm riboflavin. The treated platelets demonstrated an average recovery of 50.0 ± 18.9% and a survival time of 104 ± 26 h [27]. The measured value agrees extremely well with an average predicted recovery of 49.2 ± 5.6% (see Table 3), which was calculated from all individual values for each product using the lactate rate equation developed from this study, as lactate rate had the best correlation with observed in vivo recovery (P = 0.007). Similarly, using pH to predict survival times (P = 0.001) for treated products in the Mirasol PRT-treated platelet recovery and survival study, we arrived at a value of 112 ± 15 h. These results demonstrate that platelet in vivo recovery and survival is predictable from in vitro cell-quality measurements, as previously suggested [4–6,28], and that under the conditions utilized here, lactate production and pH are the most relevant in vitro indicators for Mirasol PRT-treated platelet viability. It is also important to note that the observations reported in this study, relative to the correlations of in vitro parameters with in vivo recovery and survival, are consistent with those previously reported for products treated at a single UV energy dose by AuBuchon et al. using the same general treatment method described here. This suggests an inherently consistent underlying mechanism of damage as a result of UV treatment, which is directly dependent on both the level of UV dose delivered and donor-dependent variables.

Our observations, reported here with regard to lactate-production rate effects, are also consistent with previous work [6,28,29]. Lactate is a final metabolic product in the glycolytic pathway. Lactate accumulation directly reflects the status of platelet glycolytic flux, while UV light exposure stimulates the lactate-production rate in a dose-dependent manner, indicating that high-energy UV light accelerates platelet glycolytic flux. Accumulation of lactic acid attributes to a decrease in plasma pH during storage. As fresh plasma has buffering capacity, the pH would not be expected to have...
the same degree of correlation with in vivo recovery as does lactate production. Our linear regression analysis confirmed this. Interestingly, glucose consumption demonstrated a relatively lower correlation coefficient with in vivo recovery than lactate-production rate, with no statistical significance ($P > 0.05$). One possible explanation for this observation is that glucose consumption may not be completely linked to the glycolytic pathway alone, leading exclusively to the end product of glycolysis. Indeed, many of the glucose-derived intermediates in glycolysis and the TCA cycle could also be used to synthesize fatty acids, lipids, amino acids and proteins [30].

It is also important to emphasize that the in vivo viability prediction from the linear regression analysis of in vitro platelet quality for one specific condition of platelet process or storage system may not necessarily extrapolate to other platelet process or treatment systems. Because the mechanism underlying development of the platelet storage lesion is not fully understood, and the responses of platelets to various treatment factors could be different, the predictions available from one system may not apply to other systems. For example, lactate production and pH, demonstrated to be the best predictors for in vivo recovery in this report, may not necessarily be useful in determining the recovery and survival of frozen or cold-storage platelets. Nevertheless, the results reported here suggest that changes in platelet storage lesion development induced by this treatment are adequately and accurately predicted by changes in these parameters and may serve as a means for guiding future development work with this methodology as well as providing insight into the potential reasons for acceleration of platelet storage lesions in UV-treated platelet products in general.

Recovery and survival studies are the first clinical step in evaluating the viability of platelets for clinical use prior to larger-scale clinical studies of efficacy in preventing or stopping bleeding in thrombocytopenic or thrombopathic patients when new storage methods and processes for platelet products are developed. This general approach has been outlined for industry by the Food and Drug Administration [3]. A general requirement before proceeding to these human trials is the evaluation of platelet in vitro characteristics using a number of measurements. The value of in vitro measures has been questioned owing to a lack of apparent correlations in a number of settings. The results presented here support the utility of this approach in predicting in vivo outcomes.

We are presently undertaking studies in which the riboflavin and UV light-treatment process is adjusted in a manner which changes the sample pH and lactate-production rates

Table 2 Correlation of in vitro cell quality and in vivo platelet recovery and survival time

<table>
<thead>
<tr>
<th>Regression equation</th>
<th>Correlation coefficient ($r$ value)</th>
<th>$F$-value</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate rate</td>
<td>$y = -372.08x + 75.964$</td>
<td>0.909</td>
<td>79.83</td>
</tr>
<tr>
<td>pH</td>
<td>$y = 50.041x - 312.4$</td>
<td>0.8381</td>
<td>64.74</td>
</tr>
<tr>
<td>Glucose rate</td>
<td>$y = -710.32x + 73.359$</td>
<td>0.8398</td>
<td>58.39</td>
</tr>
<tr>
<td>P-selectin</td>
<td>$y = -0.8561x + 72.57$</td>
<td>0.839</td>
<td>53.43</td>
</tr>
<tr>
<td>HSR</td>
<td>$y = 0.8167x - 21.145$</td>
<td>0.6492</td>
<td>33.05</td>
</tr>
<tr>
<td>Swirl</td>
<td>$y = 16.701x + 0.4253$</td>
<td>0.6586</td>
<td>20.62</td>
</tr>
<tr>
<td>$pCO_2$</td>
<td>$y = 3.1956x - 42.924$</td>
<td>0.6432</td>
<td>17.03</td>
</tr>
<tr>
<td>$pO_2$</td>
<td>$y = -0.4286x + 67.732$</td>
<td>0.4094</td>
<td>7.16</td>
</tr>
</tbody>
</table>

Predictions are based on the use of formulas generated from this study. Lactate production rate and pH on day 5, used in generating predicted recovery and survival values, respectively, came from the data published by AuBuchon et al. [27].

Table 3 Predicted and measured values of recovery and survival time for control and test platelets treated with Mirasol® PRT

<table>
<thead>
<tr>
<th></th>
<th>Mirasol PRT</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted recovery (%)</td>
<td>49.2 ± 5.6</td>
<td>61.5 ± 3.2</td>
</tr>
<tr>
<td>Observed recovery (%)</td>
<td>50.0 ± 18.9</td>
<td>66.5 ± 13.4</td>
</tr>
<tr>
<td>Predicted survival (h)</td>
<td>112 ± 15</td>
<td>149 ± 12</td>
</tr>
<tr>
<td>Observed survival (h)</td>
<td>104 ± 26</td>
<td>142 ± 26</td>
</tr>
</tbody>
</table>

Predictions are based on the use of formulas generated from this study. Lactate production rate and pH on day 5, used in generating predicted recovery and survival values, respectively, came from the data published by AuBuchon et al. [27].
during storage. In this work, the correlations obtained in this manuscript are being used as a potential guide to determine if changes are in the desired direction relative to predicted outcomes in vivo. Future work will determine how well these correlations hold and thus how much further utility such correlations may have in predicting in vivo performance.

References

5. Rinder HM, Smith BR: In vitro evaluation of stored platelets: is there hope for predicting posttransfusion platelet survival and function? Transfusion 2003; 43:2–6
21. Johnson RB, Napyanchak PA, Murphy S, Snyder EL: In vitro changes in platelet function and metabolism following increasing doses of ultraviolet-B irradiation. Transfusion 1993; 33:249–255