Brief Communication

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Measurement of Thrombin in Different Matrices: Whole Blood, Platelet-Rich and Platelet-Poor Plasma Using Fluorogenic Substrate ZGGR-AMC

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Methods for monitoring thrombin cleavage of fluorogenic substrate ZGGR-AMC over time in platelet-poor plasma (PPP) and platelet-rich plasma (PRP) samples allow real-time thrombin generation (TG) to be measured, allowing the option to deduce one's thrombin potential. Thrombin potential is an individual's ability to form a given amount of thrombin, depending on one's plasma proteome. The TG assay can detect hypercoagulability, clotting factor deficiencies and the effects of anticoagulants, as such, allowing the monitoring of drug therapy [1].

The need for centrifugation to separate plasma has limited the use of this method. Wolberg and Aleman [2] recently showed that the centrifugation step to isolate plasma may result in cell count variation, blood cell activation, platelet- and monocyte-derived microparticle production, and also heterologous blood cell aggregate removal, since variation in these factors have been shown to influence TG. Removing the need for centrifugation would discard these influencing factors and bring the protocol much closer to physiological conditions, thus allowing a greater number of cellular components to be assessed and, as such, improve TG analysis [3].

Whether or not fluorogenic substrate ZGGR-AMC offers the option to determine thrombin potential from samples containing erythrocytes has recently come under debate [4–7]. Ramjee [8] published an article suggesting the fluorogenic substrate ZGGR-AMC enabled coagulation to be measured from whole blood (WB), PRP and PPP. Tappenden et al. [4] described a modified version of the assay, which allowed TG to be measured in WB, with an acceptable error range [1]. Al Dieri and Hemker [6], however, challenged these findings as they were unable to reproduce them.

The aim of our work is to contribute to the exploration of the problems associated with WB TG methods when using fluorogenic substrate to deduce one's thrombin potential compared to PPP and PRP. Our direct and new approach will determine varied amounts of exogenous thrombin in WB using the commercially available fluorogenic substrate and compare the findings to those obtained from PPP and PRP of the same blood as well as buffer.

Venous blood of healthy individuals was collected into BD Vacutainer® Blood Collection Tubes (Becton Dickinson, Oxford, UK) containing 0.109 M trisodium citrate at a ratio of 9:1 v/v. An aliquot of WB was kept at 37°C and the remainder centrifuged at 100 *g* for 5 min at room temperature to obtain a PRP sample, and an aliquot of PRP was centrifuged further for 15 min at 1,500 *g* to obtain PPP.

Thrombin (Sigma-Aldrich Co., St. Louis, Mo., USA) dilutions were produced freshly from 1,000 NIH U/ml stock solution, using Hepes-NaCl buffer containing 1% bovine serum albumin to give final sample concentra-

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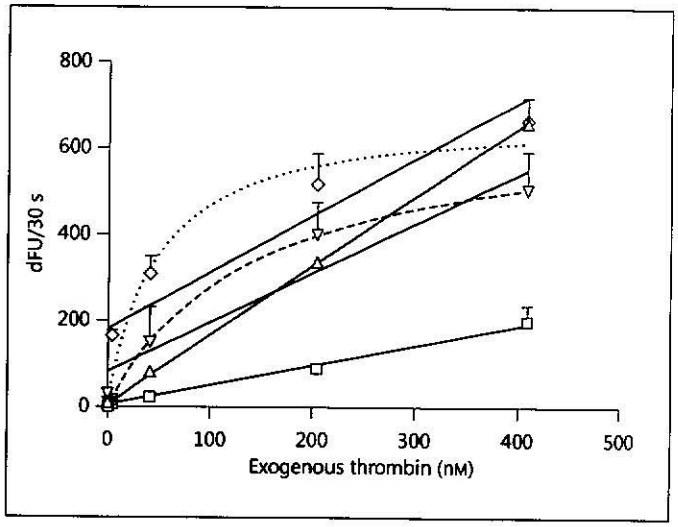

Fig. 1. Changes to dFU/30 s in WB, PRP, PPP and buffer in response to varied thrombin concentrations. Solid lines represent linear regression; the dotted line (PRP) and broken line (PPP) represent saturation curves. The measurements are expressed as mean ± SD. Square = WB; diamond = PRP; inverted triangle = PPP; triangle = buffer. WB, PPP n = 8; PRP n = 5; buffer n = 9.

Table 1. Parameters for the effect of dFU/30 s on endogenous thrombin concentration for linear and saturation curves

Sample	Linear regression line				Saturation curve		
	slope	y-intercept	R^2	slope relative to buffer, %	K	FU_{max}	R^2
WB	0.45±0.026	6.37±5.510	0.9223	28.001			
PRP	1.32±0.16	179.8±33.15	0.8260	82.20	45.79	681.9	0.8993
PPP	1.15±0.12	79.06±25.77	0.8125	71.71	145.1	681.5	0.8915
Buffer	1.60±0.054	6.98±10.86	0.9684	100			

tions of 408.66, 204.33, 40.866, 4.0866 nM and stored on ice until use. Sample volume was made from 20 µl matrix and 20 µl diluted thrombin. The reaction sequence was as follows: 20 µl of preincubated WB, PRP, PPP or buffer were added to wells of black, flat-bottomed, 96-well, polystyrene microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) in quadruplicates and plate incubated at 37°C for 5 min. Fifty microliters of substrate (Technothrombin TGA SUB, Technoclone GmbH, Vienna, Austria) was then added to the wells and the reaction initiated with 20 µl of the appropriate dilution of thrombin. Tissue factor was not added to the system to avoid endogenous TG. The fluorescent units at a wavelength of 460 nm were measured after excitation at 360 nm using a BioTek FLx 800 reader (BIO-TEK FLx800 TBI Fluorescence Reader, Technoclone GmbH) at 37°C every 30 s for 10 min, producing a total of 21 readings.

The delta fluorescent units (dFU)/30 s were calculated using KC4 software specified by the fluorometer and further statistical analysis was performed using Prism 5.0 (Graphpad Software, San Diego, Calif., USA). Paired t test was used to calculate significance.

A varied amount of exogenous thrombin was added to WB, PRP, PPP or buffer. The dFU/30 s derived from the detected light (FU) were plotted against exogenous thrombin concentration, linear regression lines were fitted for each matrix as well as saturation curves for PRP and PPP (fig. 1), and parameters were calculated (table 1). Of the linear regression lines the slope for buffer was greatest; with PRP, PPP and WB being 82, 71 and 28% of this. These differences were significant from the buffer: $p = 0.0424$, $p = 0.0004571$ and $p < 0.0001$, respectively. The difference between the slopes for PRP and PPP was not significant ($p = 0.4009$). PRP had the greatest intercept value followed by PPP, and these two were significantly different from each other ($p = 0.0002148$), while WB and buffer values were close to zero (table 1). The saturation curve parameters, FU_{max} and K (thrombin

concentration [nM] at half FU_{max}), were also analyzed. FU_{max} was almost the same for both matrices; however, the K for PPP was more than 3 times greater than in PRP.

Development of a TG assay with WB is ongoing [4–7]. Tappenden et al. [4] used a modified version of Hemker's method in order to monitor TG using WB [1, 4]. Al Dieri and Hemker [6] attempted to reproduce these findings but were unsuccessful. Our attempt to apply Tappenden's method was unsuccessful, and resulted in erratic curves where dFU/30 s could not be determined (results not shown). Al Dieri and Hemker [6] suggested these curves could be a result of moving clotted plasma; however, the formation of clots did not occur in our experiments. Blood contains several factors, including platelets and red blood cells, which may affect the activation of thrombin and the detection of fluorescence. Spiking various matrices with thrombin and measuring the recovery allows the effect of these factors to be seen and assessed to further understand TG.

Buffer was the preferred choice for comparison in our experiment because its matrix, in contrast to that of blood, is without interfering factors. Each data set was fitted with linear regression lines, as the development of fluorescence in the assayed time interval was best characterized by a linear response for buffer and allowed comparison of each matrix. PRP and PPP could also be fitted with saturation curves, giving a better fitting curve, but restricting comparability.

Linear regression slopes for PRP and PPP were not significantly different, indicating that the rate of light detection was similar in both matrices. The intercept value for PRP was greater than PPP indicating that the initial stages of coagulation differed between matrices. Platelet autofluorescence was tested; however, either in the presence or absence of thrombin the light detected from PRP or PPP did not change and meanwhile remained very low in WB (result not shown). The higher intercept could have been a result of phospholipids present on the activated platelet surface, which has been shown to be a major determinant leading to prothrombinase complex formation [9, 10]. PRP and PPP could be better fitted with a saturation curve and the parameters of these curves indicate the thrombin required to reach half FU_{max} in PRP was 3-fold lower than in PPP, which also shows a greater initial rate of thrombin conversion.

The very low rate of light detection observed for thrombin recovery from WB highlights why it has been disputed. To our knowledge no study has deduced the exact cause of the reduced fluorescent signal in WB; however, absorption characteristics of Hb [11] must be the underlying cause of the reduced rate of light detection because both the excitation and emission wavelength of this substrate fall within this range. In addition it was shown previously that red blood cells quench fluorescence [4].

In conclusion, although articles claim to have optimized TG in WB, PRP and PPP using fluorogenic substrate ZGGR-AMC, we suggest this substrate is not suitable for the measurement of TG in WB.

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