Modification of the ASTM Platelet and Leukocyte Assay – Use of an Alternative and Clinically Relevant Anticoagulant: An In-vitro Hemocompatibility Assessment of Blood Contacting Medical Devices

Tim Schatz, Abby Beltrame, Sarah Howard, Mark Smith
American Preclinical Services, Minneapolis, MN

1 Background
The purpose of this test method is to assist in the determination of the thrombogenic potential of medical materials exposed to human whole blood. By evaluating surface-induced activation, platelet adherence to a material, and platelet and leukocyte depletion from blood, a material’s potential for thrombus formation can be assessed. If a significant decrease in platelets and/or leukocytes is observed in whole blood when compared to a blank control, the tested material has the potential to induce an in-vivo thrombogenic response.

The present standard for the testing of platelet and leukocyte response to cardiovascular materials, ASTM F2888-13, Standard Test Method for Platelet Leukocyte Count - An In-Vitro Measure for Hemocompatibility Assessment of Cardiovascular Materials [1], mandates the use of several reference materials in the presence of blood anticoagulated with sodium citrate. This study was designed to address the relevance of the assay method when using a potent anticoagulant, 3.2% sodium citrate, to evaluate the thrombogenic potential of medical devices. Current studies on this question are under investigation at the FDA also with the intent of improving the standard methods for this assay by evaluating blood anticoagulated with 2-3 IU/mL of heparin [2]. For this study, the effects of several biomaterials were evaluated when exposed to blood anticoagulated with sodium citrate and, concurrently, an even lower dose of heparin at 1 IU/mL also used by our laboratory in a new circulating in-vitro assay for thrombogenicity [3]. We believe this test method allows for a sensitive assay that can more accurately predict potential thrombogenic outcomes of cardiovascular materials, while maintaining appropriate responses in both positive and negative control materials.

2 Methods
ASTM F2888-13 recommended positive controls were utilized: black rubber, natural rubber latex, and crushed borosilicate glass which were prepared in triplicate for each assay (4 assays, n=3 per sample per anticoagulant type). The blank consisted of an empty polypropylene tube and the negative control consisted of medical-grade high density polyethylene (HDPE).

Fresh human blood was drawn into blood collection tubes with either 3.2% sodium citrate or sodium heparin at a final concentration of ~1 IU/mL. A complete blood count (CBC) was obtained to qualify the blood samples and ensure that the platelet and leukocyte counts fell within a normal range (platelets 116-329 k/µL and leukocytes 3.4-8.4 k/µL) and then they were stored on ice until use. Each control was exposed to blood at ratios of 12 cm³/mL (HDPE, latex) or 1 gram/mL (black rubber, glass) and incubated at 37±2 °C for 1 hour±5 minutes with continuous agitation. A minimum of 1 mL of blood was used for each sample.

Following exposure, the tubes were removed from incubation and immediately placed on ice. Ethylenediaminetetraacetic acid (EDTA) was added to each tube to stop further clotting activity, and the blood was then decanted from each article and placed into a new, pre-chilled polypropylene tube. All biomaterials were removed from the tubes and articles with a visible clot (typically only the positive controls in heparin treated blood) were placed in weigh boats. The samples were subjected to a CBC (Hemavet HV950) to determine platelet and leukocyte counts. The cell counts from each of the biomaterials were compared per anticoagulant and the mean percentage of blank results were compared by use of one-way ANOVA with a post hoc Tukey analysis of means.

3 Results
Citrated Blood: The three readings from each run were averaged and compared to each assay’s respective blank control, which had a mean platelet count of 229 k/µL (229,000 cells/µL blood) and a mean leukocyte count of 4.81 k/µL. For HDPE, the mean platelet count was 211 k/µL and the mean leukocyte count was 4.7 k/µL, which were 92.1% and 97.7% of the blank control, respectively. For black rubber, the mean platelet count was 166 k/µL and the mean leukocyte count was 4.98 k/µL, which were 72.5% and 104% of the blank control, respectively. For glass, the mean platelet count was 232 k/µL and the mean leukocyte count was 4.89 k/µL, which were 101% and 102% of the blank control, respectively. For latex, the mean platelet count was 231 k/µL and the mean leukocyte count was 4.95 k/µL, which were 101% and 103% of the blank control, respectively (See Figure 2).

Heparinized Blood: Samples were tested as above and the blank control had a mean platelet count of 245 k/µL and a mean leukocyte count of 5.32 k/µL. For HDPE, the mean platelet count was 225 k/µL and the mean leukocyte count was 4.75 k/µL, which were 91.8% and 89.3% of the blank control, respectively. For black rubber, the mean platelet count was 73 k/µL and the mean leukocyte count was 3.37 k/µL, which were 29.8% and 63.3% of the blank control, respectively. Additionally, black rubber induced visible clots for each assay (See Figure 1). For latex, the mean platelet count was 155 k/µL and the mean leukocyte count was 3.71 k/µL, which were 63.3% and 69.7% of the blank control, respectively. For glass, the mean platelet count was 215 k/µL and the mean leukocyte count was 4.86 k/µL, which were 87.8% and 91.4% of the blank control, respectively (See Figure 2).

![Figure 1 – A representative photo of induced clots from the black rubber control seen at the completion of the 1 hour exposure. Only these controls demonstrated clot formation and only in the heparinized blood.](Image)
4 Interpretation

Each experiment met the assay validity criteria stipulated in ASTM guidance document F2888-13 [1]. The donor whole human blood was within normal ranges and was used within 4 hours of collection. Each donor’s blood was free from systemic anticoagulants for 10 days along with no alcohol or tobacco product ingestion within 12 hours prior to collection. The individual readings of the blank control, HDPE and positive controls were within ± 20% of the mean value of the triplicate readings for each run completed. The average platelet and leukocyte counts for the negative reference controls (HDPE) were within 80-120 % of the blank control, therefore all assays included in this study were considered valid.

For citrated blood, none of the expected positive biomaterials elicited a platelet reduction response ≥ 50% of that of the negative control material or produced a visible clot. Only black rubber showed a statistically significant change in mean platelet count. Per ASTM 2888-13, these materials would not meet criteria as positive controls and as such, all four of the individual assays would be considered invalid. In contrast, one biomaterial tested, black rubber, produced consistent results reducing platelet count in the heparinized blood. The black rubber control exhibited a visible clot for each individual assay and the reduction of mean platelet count was ≥ 50% when compared to the negative control. Latex produced more variable responses which resulted in differential final outcomes. Glass did not appear to generate a platelet count reduction for the heparinized system and would therefore not be recommended as a positive control material.

It is worth noting that, due to inherent differences in the final geometry of the black rubber and natural rubber latex controls in the sampling tube, inconsistent clot sizes are possible. Per ASTM 2888-13, the presence of a visible clot is sufficient evidence of a valid positive response, thus negating the necessity of meeting the criteria that all replicates of the positive control must fall within ± 20% from the mean platelet and/or leukocyte value(s). This therefore would allow the test to meet acceptance criteria and for unknown materials to pass the test even though the positive controls were not within the ± 20% limit.

5 Conclusion

The selection of the potent anticoagulant sodium citrate used in ISO 10993 biological assessment of medical devices and their effects on blood plays a critical role in the final outcome of this ASTM test. The change to a less potent anticoagulant, heparin (at 1 IU/ml) proposed within this study has the potential to significantly improve the predictive power of the ASTM Platelet and Leukocyte assay, as devices that have been screened in studies using sodium citrate as an anticoagulant may produce substantially fewer “thrombogenic” results than if they were retested in blood anticoagulated with low level heparin [2]. The findings presented within this study suggest a possible additional method to assess thrombogenic potential for devices in which other methods may not provide predictive relevant results (e.g. in fully citrated blood) or prove otherwise geometrically unfeasible to test via in vitro or in vivo thrombogenicity methods (e.g. high profile in design or complex geometry). While recent advances in thrombogenicity testing, including an in vitro circulating blood loop model can suggest a low risk of potential thrombogenicity which is consistent with observed clinical data a catheter-like devices [3], devices that are not linear or catheter-like in geometry such as stents or valve and pump components may benefit from this platelet and leukocyte depletion assay in allowing rank ordering materials or coatings with respect to thrombogenic potential.

This study confirms the potential improvement in sensitivity of the assay when the anticoagulant used for blood is changed to a less potent form. The capability of black rubber to produce consistent positive results in a heparinized blood system and not in a citrated system further supports the use of heparin at levels as low as 1 IU/mL as the anticoagulant of choice for the ASTM Platelet and Leukocyte Assay. We recommended that further investigation be performed on clinically relevant test devices with known clinical histories using heparinized blood and that this method should be more generally applied for routine biocompatibility testing in the future.

References