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Assessment of Thrombelastography and Platelet Life Span in Ovin

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Abstract

Ovin are a common animal model for the study of cardiovascular devices, where consideration of blood biocompatibility is an essential design criterion. In the ovine model, tools to assess blood biocompatibility are limited and continued investigation to identify and apply additional assays is merited. Toward this end, the thrombelastograph, clinically utilized to assess hemostasis, was employed to characterize normal ovine parameters. In addition, platelet labeling with biotin was evaluated for its potential applicability to quantify ovine platelet life span. Mean ovine thrombelastograph values were reaction-time: 4.9 min, K-time: 2 min, angle: 64.1°, maximum amplitude: 68.6 mm, actual clot strength: 11.9 Kd/sc, and coagulation index: 1.5. Reaction time was significantly shorter and maximum amplitude, actual clot strength, and coagulation index were all significantly higher when compared to normal human thrombelastograph values suggesting some hypercoagulability of sheep blood. Biotinylation and reinfusion of ovine platelets allowed temporal tracking of the labeled platelet cohort with flow cytometry. These data indicated a mean ovine platelet life span of 188 h with a half-life of 84 h. The collection of these parameters for normal ovin demonstrates the applicability of these techniques for subsequent studies where cardiovascular devices may be evaluated, and provides an indication of normal ovine values for comparison purposes.

Keywords

ovine; platelet life span; thrombelastography; cardiovascular devices; biocompatibility assessment

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INTRODUCTION

The ovine is a frequently used preclinical animal model for the assessment of cardiovascular device function and biocompatibility [1–9]. Adult ovines have hearts that are similar in size to the human heart for evaluation of implantable devices such as ventricular assist devices (VAD). The cardiac output of a juvenile ovine more closely approximates the cardiac output for infants and small children in comparison to calves. Also, there are concerns about the juvenile bovine growth rate for longer term implant studies. The porcine model is generally not appropriate for long term cardiovascular device testing since devices with external power sources are not well tolerated due to the necessary tethering.

One of the primary endpoints of preclinical testing of blood contacting cardiovascular devices is to ensure adequate blood biocompatibility [6, 7, 9–14]. Previously flow cytometric assays to quantify activated ovine platelets have been reported, and shown to be sensitive to blood pump complications and when there was subsequent evidence of thromboembolism [3, 15, 16]. Wilhelmi and Foley have published normal ovine values for a number of coagulation parameters including prothrombin time, activated partial thromboplastin time, antithrombin III activity, and others [1, 17]. The characterization of additional assays, particularly those with clinical utilization, should be of value in providing an indication of the relative status of the hemostatic system in ovines, and how the ovine system may vary from humans in terms of specific assay parameters.

Thrombelastography (TEG) is a clinical tool that quantifies mechanical parameters in the clotting process such as time to achieve a defined clot strength, clot firmness, rate of clot strengthening, and others [18–20]. TEG has been shown to be effective in monitoring the coagulability of patients on anti-platelet and anticoagulants and in patients with clinical coagulopathies [19, 20]. Furthermore, TEG has been shown to decrease blood component transfusion in pediatric cardiac surgical patients and can aid in the prediction of mortality in trauma patients [21, 22]. Limited data exist describing the use of TEG in sheep. TEG was used as one parameter to assess the efficacy of anticoagulation in sheep as well as coagulation status following initiation of mechanical circulatory support [2, 5, 9]. In another report, TEG was used to assess platelet function before and after burn and smoke inhalation injury [23].

Increased platelet consumption is an established indicator of thrombogenicity in blood contacting cardiovascular devices [24–27]. Increased platelet turnover maybe the result of platelet deposition on blood contacting surfaces or the result of increased removal of platelets activated by the blood contacting surface [24]. Platelet life span was significantly reduced in calves implanted with VADs when compared to surgical shams indicating a level of platelet consumption that was directly attributable to the VAD [25]. Platelet half-life has previously been measured in sheep using radioactive isotopes; however these studies did not quantify platelet life span [26, 28]. Radiolabeling has several safety and regulatory concerns and may increase artifact due to the additional in-vitro processing necessary [24, 26]. Biotinylation of platelets to determine life span has been successfully applied in several animal models [29–32]. This method is an attractive alternative to radiolabeling as it avoids the use of radioactive materials and can label platelets in whole blood samples [24]. In this

report the objective was to establish normal values for the TEG in ovines and assess the applicability of a biotinylation method to determine ovine platelet life span.

METHODS

Blood collection and handling

All animal studies were performed in compliance with the University of Pittsburgh Institutional Animal Care and Use Committee (ref #: 0405954-1) and National Institutes of Health guidelines for the care and the use of laboratory animals. Ovine blood for the TEG studies was collected either by jugular venipuncture using an 18 gauge 1 ½" needle or through an indwelling jugular venous catheter. The first 3 mL of each sample was discarded and 2.7 mL blood was anticoagulated 1:10 with sodium citrate. A total of 53 sheep were used in this study. This cohort included ten juvenile and adult, ten Dorset-cross and Cheviot-cross, and ten male and female sheep. Healthy human volunteers (N = 130, 48% males) signed informed consent for venous phlebotomy. Donors were recruited from two states in the Midwestern United States and had an average age of 40.4 years ranging from 20–72 years of age. Human blood was collected into tubes containing 3.2% sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA).

Thrombelastography

TEG was performed with ovine and human blood using TEG 5000 Hemostasis Analyzers (Haemonetics, Braintree, MA, USA) according to the manufacturer's protocol. 1 mL of ovine or human blood was activated with kaolin (Haemonetics) and 340 µL of this blood was added to the TEG cup containing 20 µL of 0.2M calcium chloride (Haemonetics). Samples were tested within approximately 1 h of collection. The blood was analyzed by the TEG for the maximum duration permitted by the TEG software. The TEG parameters evaluated in this report are listed in Table 1.

Ex vivo biotinylation and flow cytometric determination of ovine platelet life span

Ovine blood (360 mL) was collected into a blood transfer pack containing 40 mL of Acid Citrate Dextrose anticoagulant. 150 mg sulfo-NHS-LC-LC-biotin (Pierce, Rockford, IL, USA) dissolved in 5 mL phosphate buffered saline (Gibco, Grand Island, NY, USA) was added to the bag and the blood gently mixed. The blood was incubated in the bag for at least 10 min. The biotinylated blood was then re-infused into the jugular vein of the sheep (N = 3). Blood samples were collected prior to the biotinylation (control), from the biotinylation bag, and one to two hours after the re-infusion. Samples were subsequently collected at least once daily by jugular venipuncture until the percentage of circulating, biotin-labeled platelets had reached 3%.

Biotinylated blood (diluted 1:10 in PBS, 60 µL) was incubated for 20 min with 20 µL of 100 µg/mL neutravidin-phycoerytherin (NA-PE; Invitrogen, Carlsbad, CA, USA), and 60 µL of Tyrode's buffer (Electron Microscopy Services, Hatfield, PA, USA) with bovine serum albumin (BSA). After incubation, the samples were washed with 1 mL of Tyrode's buffer containing 1% BSA and 0.106 M sodium citrate and centrifuged at $132 \times g$ for 10 min. The supernatant was then removed and the pellet was resuspended.

GB20A (3 μ L of 7.5 μ g/mL; Washington State University Monoclonal Antibody Center, Pullman, WA, USA), an antibody previously shown to bind ovine platelets, and 3 μ L of 30 μ g/mL goat anti-mouse IgG-Alexa 488 (Invitrogen) were then added and incubated for 20 min [3]. After resuspension of the pellet, the samples were fixed with 500 μ L of 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Blood samples were assessed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Johnson et al previously demonstrated that forward and side scatter alone was not enough to reliably detect ovine platelets therefore a platelet marker (GB20A) was employed in this study to identify platelets [3]. Single-platelet scattering events (5000 total) were identified using GB20A related Alexa 488 fluorescence and forward scatter (FSC) from each sample (Figure 1A) [3]. Each platelet event was then assessed for NA-PE fluorescence, which would indicate the presence of biotin on the platelet surface. A fluorescence intensity threshold was created and utilized to determine which platelets were positive for biotin (Figure 1B–1D). The percent of biotin-labeled platelets at the one or two hour time point was normalized to 100% and subsequent samples similarly analyzed to determine the relative percentage of biotin-labeled platelets remaining in circulation (Figure 1B). The FACScan flow cytometer was serviced every six to twelve months per manufacturer guideline by a senior Becton Dickinson field service engineer for instrument maintenance and to ensure machine quality control. WinList 6.0 (Verity Software House, Topsham, ME, USA) was used to analyze the flow cytometry data.

Statistical analysis

Statistical analyses were performed using SPSS 12.0.1 (Chicago, IL, USA). Comparison of means between sheep and human TEG values was done by t-test. Linear regression for the platelet life span plot was calculated using SPSS. Statistical significance was considered to exist at $p < 0.05$.

RESULTS

Ex vivo biotinylation and flow cytometric determination of ovine platelet life span

Platelets that were positive for biotin had a marked increase in fluorescence compared to unmarked platelets (Figure 1B). Figure 1C–1D show the decreasing number of platelets positive for biotin at 72 and 192 hours, respectively. The temporal courses of biotin-labeled platelets in the circulation for three sheep are shown in Figure 2A and the mean of the three studies is shown in Figure 2B along with a linear regression. Circulating biotin-labeled platelets decreased temporally with a half-life of 84 h and a total life span of 188 h. The linear regression yielded a R^2 of 0.95. The platelet life span of ovines was similar to human and longer than that of baboon, bovine, dog, mice, and rabbits as shown in Table 2.

TEG

TEG provides a number of parameters for evaluation, however in this study the following four independent parameters (R, K, α , and MA) and two calculated values (G, CI) were evaluated (Table 1). Average normal values obtained from healthy ovine donors (N = 53) compared to normal values obtained from healthy human donors are provided in Figure 3 and 4. The range of human TEG values of blood taken from healthy volunteers in three

additional studies: Scarpelini et al (N = 118), Gonzalez et al (N = 160), and Ji et al (N = 137) are also plotted on Figure 3 and 4 [18, 33, 34]. R-time was significantly shorter in ovines, whereas MA, G and CI were all significantly higher compared to humans. There was no difference for α or K between ovine and human blood.

DISCUSSION

TEG is used clinically to assess global hemostatic function in patients and has been employed to manage anti-thrombotic therapy, making this test attractive for use in ovines since it allows system-level comparisons to be made to humans [18]. The baseline ovine TEG values from this study were generally in agreement with values reported by other groups although these studies were not intended to establish normative ovine TEG values [2, 23]. When compared to human blood values, a significantly shorter R time and significantly higher MA, G and CI values were observed, suggesting that ovine blood might be relatively hypercoagulable with increased platelet function in response to strong pro-thrombotic stimuli such as kaolin [18]. The increased MA, G, and CI may be related to the higher platelet counts in sheep [1, 17]. Angle and K was not significantly different for ovine and human blood. An in vivo ovine response that is hypercoagulable to humans was also observed by Foley [17]. However, ovine platelet function was previously shown to be diminished in platelet adhesion to biomaterials in vitro [35, 36]. A portion of the human TEG data from healthy volunteers presented in this report was previously reported by Gonzalez et al as a range [33]. These data were in general agreement with other publications that reported on normative human TEG data [18, 33, 34].

Connell et al. demonstrated that TEG is a valuable tool in assessing the effects of anticoagulation and antiplatelet medications on sheep blood [2]. Weiss et al. effectively used the TEG to titrate heparin dose in sheep implanted with a pediatric VAD based on the R [5]. The normative ovine TEG values presented in this report will be useful as a baseline for comparison in future studies evaluating device biocompatibility in the ovine model. Additional information regarding antiplatelet medication efficacy in ovines could potentially be garnered by determining normative values for TEG platelet mapping assays, which determine platelet contribution to blood clotting in response to ADP or arachidonic acid [37].

Biotinylation is a readily-accepted protein and cell labeling method due to its relative ease of use and safe handling, avoiding the hazards and regulatory burden associated with the use of radioactive materials [24]. The biotinylation process may reduce artefactual activation as one can apply this method to whole blood rather than having to centrifuge blood to create platelet rich plasma as required when using radiolabeling. Three previous studies attempted to quantify ovine platelet half-life using radiolabeling (^{51}Cr and ^{111}In) [26–28]. The average sheep platelet half-life in two of these studies was 63 and 61 h. A third study compared platelet half-lives following contact with membrane oxygenators with varied surface coatings but did not investigate platelet half-life in the absence of contact with membrane oxygenators [27].

In this report, biotinylated platelets had a marked increase in fluorescence compared to unmarked platelets (Figure 1B) indicating excellent biotin labeling. The observed platelet half-life in this study was 84 h, which is approximately 20 h longer than previously reported [26, 28]. The discrepancy in the observed platelet half-lives may in part be due to the additional blood handling required for the radioactive studies. In fact, Lindon et al suggested that their method of blood collection may have actually induced some artifact leading to platelet activation and shortened platelet life span [26]. Neutravidin is a deglycosylated avidin derivative with reduced non-specific binding and high affinity for biotin; thus, the detection threshold for a labelled platelet may be improved over radiolabeling. Unfortunately, a double-labeling study employing both radiolabeling and biotinylation would be required to establish relative detection thresholds.

In this report there was generally good agreement in the observed life spans (174, 192, and 198 h) of the three ovines evaluated. Ovine platelet life span was similar to the reported human platelet life span of 7–10 days (Table 2) [38–40]. However, ovine platelet life span was longer than that of baboons, rabbits, mice, dogs, and bovines demonstrating some marked differences amongst the animal species. Baboon platelet life span was calculated using radiolabeling which could explain the difference between ovines. It was also somewhat surprising that the platelet life span of bovines, a fellow ruminant animal, was shorter than that of ovines. The temporal course of ovine platelet life span in this study was largely in agreement with the calculated linear regression, given the high coefficient of determination. It is worth noting that after approximately 150 h, the rate of biotinylated platelets disappearance may have slowed, creating a “tail phenomenon” indicating that platelet survival cannot be entirely predicted with linear interpolation [41]. Two studies in calves quantified platelet life span using this biotinylation method and demonstrated a marked decrease in platelet life span following the implant of ventricular assist devices [24, 25]. The demonstration of this technique in ovines provides another method by which the effects of device implant on the ovine hemostatic system might be quantified.

Biocompatibility assessment is critical in the successful development of blood contacting cardiovascular devices. Previously flow cytometric ovine platelet activation assays were evaluated and incorporated platelet agonists to assess platelet function after blood contact with pediatric VADs [3, 15, 16]. These assays were shown to be sensitive to pump complications as well as the presence and number of kidney infarcts [15, 16]. These assays are valuable in the armamentarium of ovine biocompatibility assessment but they do not include an assessment of the coagulation system or global hemostasis. The combination of these flow cytometric assays with TEG, platelet life span, and other tests such as aggregometry and clotting times, provide a more comprehensive assessment of the state of ovine platelets and have the potential to improve the quality and relevance of information gained in biocompatibility studies in the ovine model.

This study has several limitations. There have been previous studies that reported on the effects of stress on the composition of ovine blood [4, 42]. Johnson et al showed stress could result in increased platelet activation in ovines not accustomed to their surroundings [15]. Once a sheep becomes accustomed to their surroundings however, stress effects on blood are alleviated [4, 42, 43]. Placement of jugular venous lines also was shown to reduce

artefactual platelet activation as it mitigated the stress of blood collection for the animals [15]. The stress effect on ovine blood may have impacted the results of this report. In the three animals used for the platelet life span the ovine had resided in an onsite animal facility for at least 9 months and had become accustomed to both the surroundings and frequent blood draws and stress was not likely to have been a factor. In the TEG study however, it was not feasible to house the ovines for a long period of time nor to place indwelling jugular venous catheters, so stress may have altered the blood composition. However, for nearly every animal, samples from multiple blood draws were taken. The TEG values for the total number of blood draws for an animal were then averaged for that individual animal and the mean values for each animal were averaged for the entire animal cohort. Multiple blood draws for each animal may have mitigated the variability of blood composition associated with stress effects.

This was the first report of its kind to use biotinylation to assess ovine platelet life span. As a result there were no other studies available to corroborate the validity of the observed platelet life span. The sample size for this study was also low ($n = 3$), although the individual ovine values for life span were similar. Finally, this report presented assays that could enhance biocompatibility assessment in the ovine model however, these assays were not directly applied to the assessment of any blood contacting medical device. Future investigation could involve using platelet life span and TEG to evaluate the effects of blood contacting cardiovascular devices in vivo in order to assess the utility of the assays.

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Figure 1.

Ovine blood flow cytometric plot of GB20A fluorescence vs. forward scatter. B) Histogram of ovine platelet biotin fluorescence at 2 hrs. C) Histogram of ovine platelet biotin fluorescence at 72 hrs. D) Histogram of ovine platelet biotin fluorescence at 192 hrs.

Figure 2. Platelet life span determination by biotinylation of ovine whole blood. **A.** Plots of the percent of biotin-labeled platelets remaining in circulation from the three individual sheep studies are shown. **B.** The mean ovine platelet life span from the three studies; the gray line displays a linear regression to the data.

Figure 3.

Ovine TEG data for α and MA compared to human TEG values. The unit of measurement for each parameter is listed with the parameter name. The values presented for ovines (N = 53) and humans (N = 130) are the experimental mean $\pm$ standard deviation, while the values presented for the other human studies are presented as a normal range.

Figure 4.

Ovine TEG data for R, K, G, and CI compared to human TEG values. The unit of measurement for each parameter is listed with the parameter name. The values presented for ovines (N = 53) and humans (N = 130) are the experimental mean $\pm$ standard deviation, while the values presented for the other human studies are presented as a normal range.

Table 1

Thrombelastography (TEG) parameters

<u>TEG parameter</u>	<u>Abbreviation</u>	<u>Clinical significance</u>
Reaction time	R	Time it takes to reach initial clot formation (enzymatic portion of coagulation)
K-time	K	Time from initial clot formation to the time clot reaches a fixed clot strength of 20 mm (initial clot kinetics)
Angle	α	Rate of clot strengthening (fibrin cross-linking)
Maximum Amplitude	MA	Maximum clot strength (reflects platelet function)
Actual Clot Strength	G	Clot firmness
Coagulation Index	CI	Assessment of overall coagulation

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Table 2

Species Platelet Life Span (days)

Ovine	Human [38]	Human [39,40]	Baboon [31]	Bovine [24]	Bovine [25]	Dog [29]	Mice [30]	Rabbit [32]
7.83 ± 0.52	7.3–9.5	7–10	7.00	5.5 ± 0.85	5.98 ± 0.6	6 ± 1.1	4.5 ± 0.2	3.6 ± 0.3