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Assessment of the Stability of von Willebrand Profile Clotting Factors and Platelet Dense Granule Testing Following Air Transport

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Abstract

The purpose of this study was to determine the reliability of test results dependent upon blood and plasma sample stability when shipped by airfreight courier for reference laboratory assessment. Of particular interest was evaluation of von Willebrand profile assays and platelet dense granule storage pool analysis. Peripheral venous blood was obtained from healthy volunteers. von Willebrand factor (VWF) activity, VWF antigen, and factor VIII coagulant activity assays were performed immediately following venipuncture with additional aliquots of plasma frozen and stored at −70°C for subsequent analysis 48 hours later. One frozen aliquot was shipped via airfreight for analysis 48 hours later, with another frozen aliquot that remained on-site. Blood was also collected to enumerate platelet dense granules to determine whether shipment would affect results. Statistical analysis of all test results demonstrated significant correlation between immediately assayed samples and samples that were stored for 48 hours at −70°C ($P < .0001$), or frozen and shipped on dry ice ($P < .0001$) for analysis upon return to our laboratory. No difference was found in the mean number of platelet dense granules between samples retained in our laboratory or samples analyzed upon return of shipment ($P = .751$).

Keywords

bleeding, blood coagulation factors, platelet dysfunction, laboratory tests, platelet storage pool

Introduction

von Willebrand disease (VWD) is regarded as the most common bleeding disorder with a reported incidence of 1% of the population; it is a quantitative or qualitative von Willebrand factor (VWF) protein defect and laboratory diagnosis of VWD is not always straightforward, not well standardized, and may be prone to error.[1-3] Patients suspected of having VWD usually present with nonspecific symptoms of mucocutaneous bleeding, including epistaxis, easy bruising, gum bleeding associated with teeth brushing, and heavy menstrual bleeding.[4,5] These identical symptoms are seen in patients with platelet δ granule storage pool deficiency (δ-SPD).[6,7] Patients exhibiting clinical symptoms of a potential platelet disorder should be queried for a complete medical history including evaluation of liver and renal function, medication history, and hemostatic challenges such as assessment of menstruation, surgical procedures, trauma, and especially for bleeding symptoms in family members. In conjunction with the medical history, a bleeding assessment tool may be utilized to screen for a potential inherited bleeding diatheses.[8-10] Subsequently, laboratory evaluation with general hemostasis screening tests, including a complete blood cell count that includes a platelet count with the mean platelet volume, a prothrombin time (PT), activated partial thromboplastin time (aPTT), and a platelet function screening test (PFA100), is essential. These tests may reveal the underlying etiology of the mucocutaneous bleeding symptoms. If a platelet disorder is suspected, a VWD profile should be employed to determine VWF activity, VWF antigen concentration, and factor VIII coagulant activity.[3] In addition, evaluation of the platelet storage pool should also be considered. We have previously reported a significant association of δ-SPD with heavy menstrual bleeding in a cohort study of young girls

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in which only 1 was diagnosed with VWD.[11] Not all hospital laboratories have the equipment and facilities required to perform VWD-specific testing and even fewer laboratories have the ability to assess platelet dense granules by electron microscopy (EM). As a result, many laboratories utilize reference laboratories for VWD-specific testing, often requiring air-freight transportation.

There are concerns regarding potential false-positive VWD profile results due to cold storage of citrated plasma, potentially due to cold-induced activation of platelets or degradation of VWF.[12-14] While reference laboratories are usually proficient in VWD testing, transport of samples at cold temperatures may lead to false identification of the disease.[14,15] There are issues and controversies related to VWD diagnosis including laboratory error, insufficient assays utilized, and most often, incorrect interpretation of test results.[16-18] A recent report described a significant effect of freezing of plasma for values of PT, aPTT, factors V, VIII, and antithrombin activity when compared to fresh sample analysis, but determined not to be of clinical significance.[19] It has been reported that the stability of frozen plasma for reliable VWD profile testing lasts for months,[20] but such studies are limited in the literature. The aim of our study was to evaluate the stability of VWF activity factor, VWF antigen, and factor VIII coagulant activity in thawed plasma that had been frozen at −70°C for 48 hours, with samples retained in our laboratory that had been assayed soon after venipuncture or frozen, thawed, and assayed 48 hours later. We also wanted to evaluate the effect of air transport upon the platelet dense granule storage pool. Similarly, we compared the mean number of platelet dense granule in whole blood samples; also, we shipped via airfreight courier at ambient temperature, with blood retained in our laboratory and processed within hours of venipuncture or processed 48 hours later when the shipped samples had been received. Our hypothesis was that freezing and/or transport by airfreight would not induce a significant difference in VWD profile results or platelet dense granule numbers compared with assay results of aliquots of fresh plasma or platelets analyzed soon after venipuncture in our laboratory.

Methods

Sample Collection

We obtained institutional review board approval according to the Helsinki agreement for human research protocols to solicit blood samples for study. We had no study exclusions nor did we collect any identifying information or medical history from volunteer participants. Blood was obtained from 35 healthy student volunteers, all in their 20s with a balance of both sexes. This was a single acquisition of blood with no possibility of volunteer follow-up if individuals with low VWD profile results actually had VWD. Samples were obtained using standard phlebotomy techniques using four 3.0 mL light-blue-top 3.2% sodium citrate vacutainer tubes (Becton Dickinson [BD], Franklin Lakes, New Jersey) and two 10 mL yellow-top solution A, acid dextrose citrate vacutainer tubes (BD).

Sample Processing

Platelet-free plasma (PFP) for VWD profile testing was obtained using the 4 sodium citrate tubes via centrifugation at 3500 rpm for 10 minutes within 1 hour of venipuncture. Plasma from each tube was combined and centrifuged again at 3500 rpm for an additional 10 minutes. Each double-centrifuged plasma sample was immediately divided into three 1.5 mL aliquots for analysis. Our VWD profile consisted of a VWF activity assay, a VWF antigen assay, and a factor VIII coagulant activity assay.

Experimental design. One aliquot was analyzed immediately following the final centrifugation. The other aliquots were immediately frozen at −70°C, with one sample stored on-site for analysis 48 hours postvenipuncture and another one shipped airfreight to Kansas City, Missouri, overnight with a sufficient amount of dry ice to last 72 hours in transit. Each shipment (each containing 5-7 samples and 1 shipment per week) was immediately reshipped to our laboratory for analysis. Thus, triplicate samples of PFP were analyzed with 1 fresh and ambient temperature aliquot assayed immediately the day of venipuncture and the 2 aliquots that were frozen 48 hours later. Frozen aliquots were warmed to 37°C and were adequately mixed before the VWD profile assays were performed at ambient temperature.[18] Our study was intended solely to evaluate the stability of frozen plasma shipped via air transport on dry ice for assay at reference laboratories for comparison with samples assayed under ideal and immediate processing postvenipuncture or plasma stored on-site in a freezer; we did not evaluate different storage temperatures on-site as variance in temperature could not be controlled for samples shipped on dry ice.

Factor VIII coagulant activity assay. A Stago STA R Analyzer was also utilized to assess FVIII coagulant activity. The method employed STA-deficient VIII plasma (Cat. No. 0725), as substrate, to determine percent activity from the standard curves prepared with appropriate control plasma (PTT Automate 5, Cat. No. 0480; STA-ImmunoDef VIII, Cat. No. 0728). Our normal reference range for FVIII coagulant activity is 60% to 140% activity.

von Willebrand factor antigen (VWF: Ag) assay. von Willebrand factor antigen was determined using an immunoturbidimetric assay. A Stago STA-R Analyzer (Diagnostica Stago, Inc, Parsippany, New Jersey) was employed using a STA Liatest VWF Kit (Cat. No. 0518), with appropriate controls. Our laboratory normal range is 50% to 140%.

von Willebrand factor activity assay. The assay measures the ability of a patient's plasma to agglutinate formalin-fixed platelets in the presence of ristocetin. The rate of ristocetin-induced

agglutination is related to the concentration of VWF and ristocetin cofactor activity. Helena lyophilized platelets (Helena Laboratories, Beaumont, Texas; Helena Ristocetin cofactor assay kit, Cat. No. 5370) were reconstituted in Tris-buffered saline for use in a Helena AggRAM Platelet Aggregometer to assess VWF activity at 2 different dilutions for comparison to standard curves of both ristocetin cofactor control plasma and abnormal control plasma. Our laboratory normal range is 55% to 185%.

Electron microscopy. Acid citrate dextrose (ACD) tubes of blood were obtained to evaluate platelet dense granule numbers by EM.[21] One tube was processed immediately for a whole mount preparation of air-dried platelets; the second vial was shipped with the frozen PFP aliquots but packaged to ensure these vials remained at ambient temperature and subsequently processed for EM 48 hours postvenipuncture. Preparations of platelet whole mounts required low-speed centrifugation to obtain platelet-rich plasma, which was subsequently incubated upon EM support films for 5 minutes followed by a brief distilled water wash and then air-dried.[22] Enumeration of δ-granules using a Philips CM 10 electron microscope was performed; the average number of δ-granules was determined by counting the total number of 100 platelets contiguously observed, excluding platelets that were obscured by debris or partially overlaying grid bars.[23]

Sample Transport

The experiment was designed to simulate the process that many hospitals utilize to ship samples to reference laboratories for analysis. Our first shipment of 6 samples was intended to be shipped via airfreight for overnight delivery back to our laboratory; however, the package was returned via ground delivery the following day due to zip code proximity sorting. We were unable to identify a carrier, including commercial airlines that would ship our samples to a national sorting facility for return to our laboratory the following day. Therefore, all subsequent shipments were transported by airfreight to a relative of one of the authors living in Kansas City who immediately returned each package to our laboratory for analysis within 48 hours of venipuncture.

Statistical Analysis

Statistical analysis of both VWD profile test results and platelet dense granule number determination was evaluated using SPSS (v4.5) descriptive statistics. Using the Shapiro-Wilk test, our data did not have a normal distribution. Therefore, we utilized a logarithmic transformation to determine correlations among fresh and ambient temperature aliquot assayed immediately the day of venipuncture and the 2 frozen aliquots that were assayed 48 hours later for each of the VWD profile assays. The platelet dense granule results did not have a normal distribution as well and were therefore evaluated using a Mann-Whitney rank sum test.

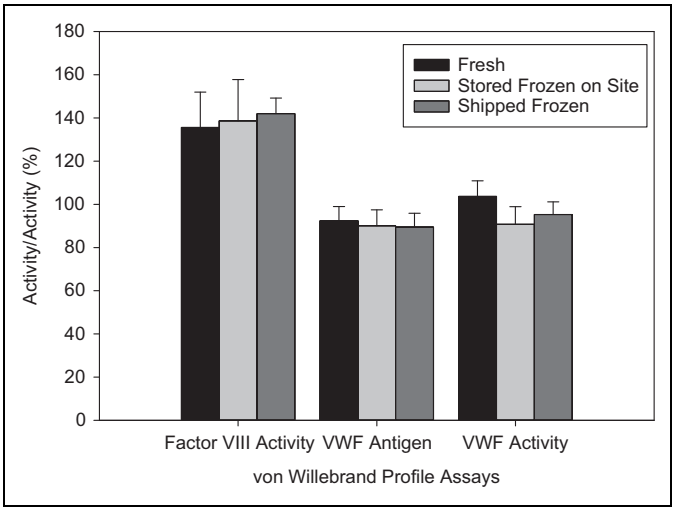

Figure 1. Comparison of von Willebrand profile test results obtained in fresh plasma samples assayed immediately postvenipuncture with results obtained in frozen plasma samples stored on-site or shipped and assayed 48 hours later.

Results

von Willebrand Disease Profile

To determine whether freezing and/or transport significantly affected vWD profile, we completed a correlation study for each of the 3 assays aliquoted for each volunteer's blood plasma. Thus, 3 samples for each participants were compared for each of the 3 VWD profile assays: a FVIII coagulant activity, a VWF antigen assay, and a VWF activity assay.

Factor VIII Coagulant Activity Assay

Factor VIII coagulant activity for frozen aliquots, shipped via airfreight or remaining on-site in a −70°C freezer, was essentially identical with plasma processed and assessed immediately (Figure 1) for each volunteer (n = 35). For samples assessed immediately postvenipuncture, the average FVIII coagulant activity was 135.6% ± 16.4% activity, whereas aliquots frozen and stored at −70°C in our laboratory and processed 48 hours postvenipuncture had a mean activity of 138.7% ± 19.1%, and the frozen sample (48S) sent through airfreight and assayed with the stored sample had a mean of 142.0% ± 7.2% activity (n = 31, 1 package thawed due to insufficient dry ice [−78.5 °C)]. The correlation coefficient between groups 2 hours and 48 hours was 0.93 with a confidence interval (CI) from 0.86 to 0.97, the coefficient between frozen plasma groups 48 hours and 48S hours was 0.474 (CI: 0.14-0.71), and between 2 hours and 48S hours was 0.574 (CI: 0.28-0.77). While the individual correlation between the frozen groups of samples (48 hours and 48S hours) and the immediately analyzed and frozen shipped samples (2 hours and 48S hour) was lower, the overall coefficient indicated correlation between the 3 groups. The intraclass correlation coefficient of average measures was 0.799, with a 95% CI of 0.63 to 0.88. There was no statistical difference observed between the 3 groups as the

correlation P value of <.0001 indicates significant agreement for the FVIII coagulant activity groups.

von Willebrand Factor Antigen (VWF:Ag) Assay

The mean of all aliquots assayed to determine VWF:Ag was essentially identical with samples assessed immediately following venipuncture at 92.4% ± 6.6% (n = 35) and the frozen samples assayed 48 hours later at 90.1% ± 7.4% (n = 35) and 89.5% ± 6.4% (n = 31), respectively (Figure 1). There was a correlation coefficient of 0.97 (CI: 0.95-0.99) between groups 2 hours and 48 hours, a coefficient of 0.97 (CI: 0.94-0.99) between groups 48 hours and 48Shours, and a coefficient of 0.94 (CI: 0.87-0.97) between groups 2 hours and 48S hours. The intraclass correlation coefficient of average measures was 0.979, with a 95% CI from 0.97 to 0.99. There was significant correlation for the 3 VWF:Ag groups (correlation $P < .0001$ indicates significant agreement).

von Willebrand Factor Activity Assay

The VWF activity was determined for each aliquot within hours or venipuncture and again 48 hours postvenipuncture using frozen PFP, with one sample having been shipped airfreight and the second frozen sample stored in a −70°C freezer in our laboratory. The mean VWF activity for samples processed and assayed immediately was 103.7% ± 6.2% (n = 35), whereas the frozen samples processed and analyzed 48 hours postvenipuncture were 90.8% ± 8.1% (n = 35, on-site at −70°C) and 95.3% ± 5.9% (n = 31, shipped on dry ice at −78.5°C; Figure 1). The VWF activity assay also demonstrated correlation. The correlation coefficient between groups 2 hours and 48 hours was 0.962 (CI: 0.92-0.98), between groups 48 hours and 48S hours was 0.484 (CI: 0.16-0.72), and between groups 2 hours and 48S hours was 0.378 (CI: 0.03-0.65). The intraclass correlation coefficient of average measures was 0.71, with a 95% CI of 0.5 to 0.84. Again, despite low individual correlation between the frozen groups (48 hours and 48S hour) and the fresh and frozen shipped group (2 hour and 48 S hours), the overall coefficient between the 3 VWF activity assay groups had significant correlation ($P < .0001$).

von Willebrand Factor Activity/Antigen Ratios

Ratios for VWF activity and VWF antigen were calculated for each of the 3 different assay sample groups. The VWF, activity/VWF, and Ag were found to be 1.12, 1.01, and 1.06, respectively.

Electron Microscopy

We also evaluated stability of the number of dense granules per platelet (DG/PL) determined by EM with processing and assessment on the day of venipuncture for comparison with samples that were shipped and then returned to the laboratory 48 hours later. The mean number of dense granules per platelet for samples processed within hours of venipuncture was 4.4 ± 0.2 DG/PL (n = 35), whereas the samples processed and assessed 48 hours postvenipuncture had 4.3 ± 0.2 DG/PL (n = 35; Figure 2). The minimum mean number of DG/PL was found to be 2.4 DG/PL (same day processing and analysis) or 1.8 DG/PL (processed and analyzed 48 hours postvenipuncture), and the maximum mean number of DG/PL was 6.7 DG/PL or 6.5 DG/PL. As the groups were not normally distributed by the Shapiro-Wilk normality test, a Mann-Whitney rank sum test was used. There was no significant difference between dense granule number in samples assessed at the day of venipuncture compared with blood that was shipped and analyzed 48 hours postvenipuncture ($P = .751$).

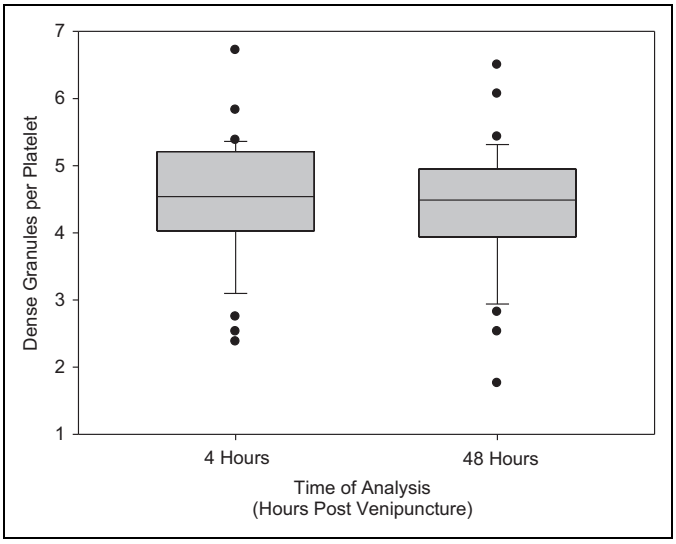

Figure 2. Comparison of the mean number of platelet dense granules in platelets evaluated by electron microscopy immediately following venipuncture with whole blood shipped via airfreight carrier and subsequently processed and platelet dense granules evaluated 48 hours postvenipuncture.

Discussion

The diagnosis of VWD is challenging due to the necessity of complex testing methods required, variability in techniques, lack of standardization of cutoff values, sample acquisition, shipping and transport of samples, and interpretation of test results.[2,3,5,7,12,18] There have been limited reports regarding the stability of coagulation testing of frozen plasma, a requirement of sample processing for shipment to reference laboratories.[19,24] The aim of our study was to evaluate the stability of frozen plasma for VWD profile assays compared with results obtained in our laboratory for aliquots of fresh plasma processed within 2 hours of venipuncture. Our volunteers were all healthy adults, which might be considered a limitation of the study since no one included was known to have a bleeding diathesis. Therefore, we did not assess any participant known to have VWD; the project was intended only to evaluate blood sample stability and not to compare normal values with individuals with VWD. We also sought to investigate the stability of the platelet dense granule storage pool in peripheral blood

shipped via airfreight compared with the results of whole-mount platelet EM determined from preparations made within hours of venipuncture. To our knowledge, there is no report in the literature describing the stability of the dense granule content of platelets sent to a reference laboratory via airfreight. Our hypothesis was that shipping of frozen plasma for VWD profile testing and the dense granule content of platelets would not be affected by airfreight shipment. Plasma samples transported for VWD profile analysis are usually frozen at −70 or −80°C and shipped when necessary on dry ice (−78.5°C) for no more than 3 days. Whole blood collected in ACD vacutainer tubes and shipped at ambient temperature is usually prepared for analysis within 24 to 48 hours of venipuncture.

As we hypothesized, we did not find a statistically significant difference between our groups of plasma tested for VWD parameters, indicating little effect from the freezing and transport of the samples. The greatest correlation was observed between the samples analyzed 2 hours after venipuncture and those frozen and left on-site for 48 hours. The lower degree of correlation between 48-hour frozen samples and 48-hour frozen shipped samples suggests that transport may play a role in decreased correlation of assay results. However, this decrease was not statistically significant and likely would also not be clinically significant. These results indicate that storage and transport of samples at −78.5°C and within 48 hours, respectively, are adequate for the stability of VWD profile results.

Similarly, we found no difference between the number of dense granules determined from samples prepared immediately postvenipuncture and those shipped via airfreight and processed 48 hours postvenipuncture. Our data confirm our hypothesis that platelets obtained from whole blood and shipped to our laboratory within 48 hours are stable for the assessment of the dense granule storage pool.

Conclusion

Transport of plasma samples for von Willebrand profile analysis to specialized reference laboratories is often necessary due to lack of on-site resources at many hospitals. The reliability of coagulation testing has often been questioned in the literature due to a variety of concerns including the effect of freezing plasma and shipment, although literature that demonstrated the stability of some coagulation proteins for weeks to months does exist. We report that samples collected at our institution, processed immediately and aliquoted for VWD profile analysis, were stable if frozen for 48 hours when compared to fresh samples assayed within hours of venipuncture. Our volunteers were all considered healthy participants; thus, our results reflect stability of normal test values only. We also report that platelets shipped via airfreight may be reliably assayed for the dense granule storage pool within 48 hours of venipuncture.

Authors' Note

Denise Bichuyen Mai performed the research, analyzed the data, and wrote the paper. Mary R. Smith performed the research and contributed essential reagents and tools. William T. Gunning III designed the research study, performed the research, analyzed the data, and wrote the paper. The authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Declaration of Conflicting Interests

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