The Effect of Shear Stress on the Size, Structure, and Function of Human von Willebrand Factor


*Institute of Life Science, College of Medicine, Swansea University; and †Institute of Life Science, Calon Cardio-Technology Ltd, Swansea, Wales, UK

Abstract: Clinical outcomes from ventricular assist devices (VADs) have improved significantly during recent decades, but bleeding episodes remain a common complication of long-term VAD usage. Greater understanding of the effect of the shear stress in the VAD on platelet aggregation, which is influenced by the functional activity of high molecular weight (HMW) von Willebrand factor (vWF), could provide insight into these bleeding complications. However, because VAD shear rates are difficult to assess, there is a need for a model that enables controlled shear rates to first establish the relationship between shear rates and vWF damage. Secondly, if such a dependency exists, then it is relevant to establish a rapid and quantitative assay that can be used routinely for the safety assessment of new VADs in development. Therefore, the purpose of this study was to exert vWF to controlled levels of shear using a rheometer, and flow cytometry was used to investigate the shear-dependent effect on the functional activity of vWF. Human platelet-poor plasma (PPP) was subjected to different shear rate levels ranging from 0 to 8000/s for a period of 6 h using a rheometer. A simple and rapid flow cytometric assay was used to determine platelet aggregation in the presence of ristocetin cofactor as a readout for vWF activity. Platelet aggregates were visualized by confocal microscopy. Multimers of vWF were detected using gel electrophoresis and immunoblotting. The longer PPP was exposed to high shear, the greater the loss of HMW vWF multimers, and the lower the functional activity of vWF for platelet aggregation. Confocal microscopy revealed for the first time that platelet aggregates were smaller and more dispersed in postsheared PPP compared with nonsheared PPP. The loss of HMW vWF in postsheared PPP was demonstrated by immunoblotting. Smaller vWF platelet aggregates formed in response to shear stress might be a cause of bleeding in patients implanted with VADs. The methodological approaches used herein could be useful in the design of safer VADs and other blood handling devices. In particular, we have demonstrated a correlation between the loss of HMW vWF, analyzed by immunoblotting, with platelet aggregation, assessed by flow cytometry. This suggests that flow cytometry could replace conventional immunoblotting as a simple and rapid routine test for HMW vWF loss during in vitro testing of devices.

Key Words: von Willebrand factor—Shear stress—Platelet aggregation—Multimer analysis.

During the past few decades, ventricular assist devices (VADs) have emerged as an increasingly popular therapy in patients with advanced heart failure who do not respond to medical or resynchronization therapy. VADs are used either as a bridge-to-transplant or as destination therapy in patients deemed ineligible for cardiac transplantation, or those who cannot receive a transplant due to the limited supply of donors (1). While VADs have already benefitted many patients, VAD-related blood damage remains a major issue. This includes hemolysis (2,3), platelet activation (4), alteration of the coagulation cascade and thrombosis (5), reduced functionality of leukocytes (6), release of...
microparticles (7), and degradation of von Willebrand factor (vWF) (8,9).

Gastrointestinal (GI) bleeding is one complication associated with the placement of a VAD, with rates as high as 65% within the first year after VAD placement (10,11). The mechanism underlying this problem in patients on long-term continuous flow mechanical support is not well understood (1,9,11–22). This cannot be explained by the anticoagulation regimen alone, but may be symptomatic of acquired von Willebrand syndrome (AvWs) which could be the result of shear stress caused by the VAD. In a literature review of clinical studies describing patient populations suffering from GI bleeding, two populations in particular were found to stand out: those suffering from aortic valve stenosis and those implanted with axial flow VADs (Table 1). In aortic stenosis, there is a high level of shear stress and loss of HMW vWF multimers (23,24). In contrast to axial flow pumps, centrifugal flow VADs and total artificial hearts (TAHs) have fewer reported events of GI bleeding. The centrifugal pump with the most reports of GI bleeding is the novel HeartWare device (20,25–28). In patients implanted with VentrAssist and EVAHEART, there have been few reports of bleeding, although they have been diagnosed with AvWs, showing a loss of high molecular weight (HMW) vWF multimers and decreased ratios of collagen binding capacity and ristocetin cofactor activity to vWF antigen (17). Comparing the shear stress in these devices provides a partial explanation as the characteristic hydraulic performance of axial flow usually involves high rotational pump speed compared with centrifugal flow resulting in higher shear stress (29). Furthermore, TAHs typically have lower shear stress than centrifugal pumps due to their pulsatile operation (11). Thus, the hypothesis is that the higher the shear stress, the more damage caused to vWF, resulting in decreased platelet aggregation and thus bleeding.

In light of this, it is important to understand the point at which shear stress becomes damaging, and why centrifugal VAD patients do not suffer GI bleeding to the same extent, although obviously suffering impairment of vWF. It is difficult to calculate shear stress in VADs both in vitro and in vivo. Therefore, it is important to establish an in vitro laboratory model with greater sensitivity and the ability to better quantify HMW vWF multimer loss while furthering our understanding of the relationship between HMW vWF abundance and activity.

Research focusing on the impact of wall shear rate caused by perfusing normal plasma through long capillary tubing, and that of pumping plasma in a mock circulatory loop driven by a VAD has shown a relationship between the shear rate and the loss of HMW vWF multimers (8,30,31). However, neither of these models provide a way to subject the plasma to accurate and controlled levels of shear rate environment. A rheometer can be used to apply controlled levels of shear rate during timed intervals. Therefore, the purpose of this study was to: (i) quantify HMW vWF breakdown at specified levels of shear rate using a rheometer; (ii) correlate the loss of HMW multimers to vWF functionality; and (iii) investigate the effect of mechanical shear on platelet aggregates. This information would be of value to VAD developers who could modify device design to avoid damaging shear stress levels.

### TABLE 1. Literature review describing patient populations suffering from GI bleeding and vWF diagnostic methods

<table>
<thead>
<tr>
<th>Device or medical condition</th>
<th>GI bleeding</th>
<th>vWF diagnostic methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HMW vWF loss (multimer analysis)</td>
</tr>
<tr>
<td>CF-VAD</td>
<td>Axial flow</td>
<td>HeartMate II Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jarvik 2000 Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MicroMed DeBakey Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thoratec BVAD Yes</td>
</tr>
<tr>
<td>Centrifugal flow</td>
<td></td>
<td>VentrAssist No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CentriMag BiVAD N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HeartWare Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EVAHEART No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Durahart N/A</td>
</tr>
<tr>
<td>Pulsatile TAH</td>
<td></td>
<td>CardioWest No</td>
</tr>
<tr>
<td>No device</td>
<td>Aortic valve stenosis Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Artif Organs, Vol. 38, No. 9, 2014*
MATERIALS AND METHODS

Blood preparation
Peripheral blood (72 mL) was collected from six healthy volunteers in vacutainers containing 9NC coagulation sodium citrate 3.2% (455322, Greiner Bio-one, Wemmel, Belgium). Platelet-rich plasma (PRP) was prepared by centrifuging whole blood for 7 min at 500 \( \times g \) at room temperature. Platelet-poor plasma (PPP) was prepared by centrifuging PRP for 5 min at 13 000 \( \times g \) at room temperature. The PPP was extracted and analyzed using the automated hematology analyzer CELL-DYN Ruby (Abbott Diagnostics, Abbott Park, IL, USA) to ensure that the plasma was void of blood cells. PPP was used instead of whole blood to ensure that any potential effect of shear on vWF was not a result of cellular interactions. This study was approved by the South Wales Research Ethics Committee and all donors gave informed written consent.

Rheometry
PPP was subjected to shear stress rates of 4000 and 8000/s in an AR-G2 rheometer (TA Instruments, New Castle, DE, USA) at 37°C. The rheometer was equipped with double concentric geometry in which a rotating inner cylinder cup allows generation of uniformly established shear flow at a well-defined shear rate. The PPP was sampled at hourly intervals (6 h in total). Static PPP at 37°C was used as a control. The sheared PPP was analyzed by flow cytometry immediately after the shear tests and the remaining plasma samples were stored at −80°C for \( \leq 5 \) days prior to analysis for the vWF multimer by immunoblotting.

Plasma vWF multimer analysis by immunoblotting
Sheared and nonsheared PPP was subjected to electrophoresis on high gelling temperature agarose (0.6% agarose, w/v, Sea Kem, FMC Bioproducts, Rockland, ME, USA) in a horizontal gel apparatus (81–2325, Galileo Bioscience, Cambridge, MA, USA) at 4°C. Electrophoresis was performed at 30 mA for 30 min and then at 50 mA until the dye front had migrated 10–12 cm from the origin (total gel running time was 6 h). vWF multimers separated on agarose gel were transferred to polyvinylidene difluoride (PVDF) 0.45 \( \mu m \) membrane (IPVH304F0, Immobilon-P, Millipore Corporation, Billerica, MA, USA) for 15–17 h at 70 mA in the electroblotting tank (91–2020-TB, Galileo Bioscience). vWF detection using anti-human vWF primary (ab6994, Abcam) labeled with Pacific Blue (Z25041, Zenon Pacific Blue mouse IgG1 labeling kit, Life Technologies) in an 8-well borosilicate chamber slide (155411, Thermo Scientific, New York, NY, USA). Individual and aggregated fluorescent platelets adhered by vWF were fixed by Vectashield hardset mounting medium (H-1400, Vector Laboratories, Peterborough, UK) and examined with a confocal microscope (LSM 710, Zeiss, Jena, Germany). Images of green and red fluorescent platelets and blue fluorescent-stained vWF were captured and analyzed using ZEN imaging software 2012.

vWF ELISA
The concentration of vWF : antigen (vWF : Ag) in the sheared PPP was quantified by a vWF specific enzyme-linked immunosorbent assay (ELISA) kit (ab108918, Abcam) according to manufacturer’s
instructions and absorbance at 450 nm measured (POLARstar Omega, BMG LABTECH, Ortenberg, Germany).

Statistical analysis

Due to multiple measurements made per PPP sample at different time points and at different shear rates, a repeated measures analysis of variance on the data sets was conducted. The shear rates and the time points were treated as fixed effects and the sample as a random effect. Once significant effects were found, pairwise comparison tests were conducted, adjusted for the multiple comparisons using Bonferroni’s method. Analysis was performed using the RStudio v 0.97.551 (RStudio, Boston, MA, USA) and R statistical environment, v3.0.2 (R Core Team, Vienna, Austria).

RESULTS

Quantification of the rate of change of HMW and LMW vWF using densitometry

The rate of change of HMW and low molecular weight (LMW) multimers was quantified using densitometry (Fig. 1A–C, right panel) and the results are summarized in Fig. 2A (HMW vWF) and 2B (LMW vWF).

HMW vWF

Repeated measures analysis of variance shows a very significant dependence of the densitometry values on the shear rate \( (P < 10^{-6}) \) and on time \( (P < 10^{-6}) \). The interaction between time and shear rate is also significant \( (P < 0.002) \). Pairwise comparisons (with Bonferroni correction) confirm the
significant difference between any two levels of shear rate and show that significant differences are achieved between measurements that are at least 4 h apart. This is summarized in Fig. 2A which shows three distinct curves. The mean densitometry values in the control sample remain high while there is a decrease with time in the sheared samples, with a stronger decrease in the sample subjected to the higher shear.

LWM vWF

Repeated measures analysis of variance showed a significant dependence of the densitometry values on the shear rates ($P < 0.002$) and on time ($P<10^{-5}$). There was no significant interaction between shear rate and time in this case. Pairwise comparisons with Bonferroni correction show that the significant differences are between the static and high shear values, and that measurements need to be at least 5 h apart to show significant changes. This is summarized in Fig. 2B, which shows three distinct curves describing mean densitometry values of the three shear rates. The curves representing sheared samples exhibit an upward trend with time of the densitometry values. To summarize, the results reflect loss of HMW vWF at 4000 and 8000/s, compared with the static control at hourly intervals during 6 h at 37°C in Fig. 1 (left panel). There is a positive correlation between shear rate and degradation of vWF, with 8000/s causing the greatest loss of HMW bands compared with the static control. Similarly, there is a correlation between shear rate and an increase in LMW bands, suggesting that the HMW bands may be cleaved into smaller fragments. This conclusion is strengthened by the quantification of vWF antigen below.

Quantification of vWF antigen by ELISA

The total vWF antigen decreases during the 6-h test in the static control and the sheared samples (Fig. 3). However, as there is no difference between the groups, we can conclude that the HMW band loss demonstrated by immunoblotting (Fig. 1) in the sheared samples is due to a cleavage of HMW multimers into LMW multimers.
vWF functionality assessed using flow cytometry and visualized using confocal microscopy

The assessment of the vWF functionality was performed using a ristocetin-dependent flow cytometry assay of platelet aggregation. When equal concentrations of green and red platelets, resolved by the FITC and APC channels, respectively (Fig. 4A, left panel), were mixed in the absence of PPP, single green and red platelets dominated and double positive events (platelet aggregates) were negligible (<3%). These events were considered as a background effect and removed from the counting of aggregates when PPP was added. The number of aggregates at time 0 with PPP added was considered as the baseline, and all further counts were compared with this baseline. Repeated measures analysis of variance of the vWF:RCo activity revealed a significant effect of shear ($P < 10^{-6}$) and also a strong effect of time ($P < 10^{-6}$). Interaction between shear rate and time was also significant ($P < 0.0002$). Pairwise comparisons with Bonferroni correction further showed that there is a significant difference between any two shear rates in the following order of significance: none : 8000/s, 4000 s : 8000/s, and none : 4000/s. Pairwise comparisons with Bonferroni correction between time points show that significant difference in activity will be found between measurements that are at least 4 h apart. These results are evident in Fig. 5.

![Image: Quantification of vWF functionality by flow cytometry and confocal microscopy. Left panel: vWF functionality quantified by a ristocetin-dependent flow cytometry assay measuring the percentage of platelet aggregates. Right panel: Platelet aggregates of individually stained red or green platelets visualized by confocal microscopy. (A) No PPP negative control, (B) static control, (C) 4000/s, (D) 8000/s at 6 h, respectively.](image-url)
The vWF/platelet aggregates were then assessed by confocal microscopy that showed single green and red platelets in the negative control (no PPP) sample (Fig. 4A, right panel), and platelet aggregates in the samples to which static control or sheared PPP was added (Fig. 4B–D, right panel). The sheared samples resulted in several smaller platelet aggregates compared with the static control. Although there were also single platelets present in the static and sheared samples, they stayed at the surface whereas the aggregates sank to the bottom of the well, which meant that isolated, light platelets could not be visualized simultaneously (Fig. 4B–D, right panel).

There is a strong linear correlation between the loss of HMW vWF multimers as measured by immunoblotting and the reduction in functional activity of vWF as measured by flow cytometry (Fig. 6). In Table 2, the results show that the vWF functionality to antigen ratio (vWF : RCo / vWF : Ag) of the static control, 4000 and 8000/s at 6 h was 1.01, 0.67, and 0.42, respectively. These results prove that we were able to create an AvWs phenotype with human PPP in vitro. The vWF : RCo / vWF : Ag ratio for a normal healthy donor is close to 1.0, and for a type 2 von Willebrand disease patient it is less than 0.43 (17,33).

**Visual investigation of platelet aggregation by confocal microscopy**

vWF multimers interspersing platelets in aggregates can be visualized using confocal microscopy (Fig. 7). The structural platelet aggregations of nonsheared PPP (static control) were less dispersed...
and bigger in size compared with those of the postsheared PPP (at 8000/s, 6 h). The lower MW and lower activity vWF likely led to a smaller, more dispersed platelet aggregate (Fig. 8).

**DISCUSSION**

Current blood trauma research related to VADs often focuses on hemolysis with few reports of cleavage of vWF during in vitro testing (34). In this study, we have demonstrated shear dependent degradation of HMW vWF multimers and loss of vWF activity, although the total amount of vWF remains preserved. These results show that we were able to create an in vitro model of AvWs.

The molecular weight of vWF has also been shown here to be a major determinant of the adhesive functional activity causing platelet aggregation, and that there is a correlation between the loss of HMW vWF and vWF activity (Fig. 6). These results indicate that the assessment of vWF activity using flow cytometry, a faster and more sophisticated analysis than the gold standard vWF immunoblot, could be sufficient for the evaluation of VADs with regard to AvWs in comparative in vitro studies. In addition, the structural aggregation confocal microscopy analysis could provide more insight with regard to AvWs.

In this study, relatively low shear rates were used to establish the model. The maximum shear rate will be dependent on the rheometers used, and the researcher may want to select a model that spans a high range because the estimated shear condition in a centrifugal pump is \( >20000/s \) (8). Another limitation was the residence time (6 h) of the PPP which is continuously sheared in the rheometer, whereas the blood passes through the VAD in a period of milliseconds. However, this work provides a simple and accurate way to expose the blood plasma samples to controlled shear rates ranging from physiological to pathological levels which allow evaluation of the impact of shear rate on the vWF. This is of value to provide further insight into bleeding complications in patients with VAD. Our future work will focus on...
further exploration of the shear stress and time domains of vWF mechanoenzymatic stability, especially at higher ranges of shear stress and shorter exposure times. In addition, the use of whole blood instead of PPP is the next step to mimic physiological conditions, although shear stress might also damage or activate platelets causing release of ADP and vWF multimers into the plasma. Furthermore, the role of ADAMTS13, the protease known to break down vWF (31), in HMW vWF destruction during high shear stress will be considered.

CONCLUSIONS

The results shown here confirmed that the longer human platelet-poor plasma is exposed to high shear rate, the greater the loss of the high molecular weight von Willebrand factor multimers and decline in the functional activity of vWF will be. There is a strong linear correlation between HMW vWF multimer loss and the lower functional activity of vWF. Therefore, this study indicates that a simple, fast, accurate flow cytometric evaluation of the vWF activity is sensitive enough for a comparative acquired von Willebrand syndrome study for in vitro testing of different shear conditions. This work could improve in vitro ventricular assist device evaluation and bring it a step closer to assessment of total blood trauma. It is recommended that the effort of blood pump design should not only focus on mechanical stability and hemolysis, but also on the other blood components that could be damaged such as leukocytes, platelets, and soluble factors such as vWF and factor VIII. Future in vitro device evaluation could benefit from inclusion of assays such as this to provide a more complete picture of the overall effect on blood function.

REFERENCES


