Shear stress-induced total blood trauma in multiple species

Running Headline: Shear stress-induced blood trauma in multiple species

Abstract

The common complications in heart failure patients with implanted ventricular assist devices (VADs) include haemolysis, thrombosis and bleeding, which are linked to shear stress-induced trauma to erythrocytes, platelets and von Willebrand factor (vWF). Novel device designs are being developed to reduce the blood trauma, which will need to undergo *in vitro* and *in vivo* pre-clinical testing in large animal models such as cattle, sheep and pig. To fully understand the impact of device design and enable translation of pre-clinical results, it is important to identify any potential species-specific differences in the VAD associated common complications. Therefore, the purpose of this study was to evaluate the effects of shear stress on cells and proteins in bovine, ovine, and porcine blood compared to human. Blood from different species was subjected to various shear rates (0 – 8000 s^{-1}) using a rheometer. It was then analysed for complete blood counts, haemolysis by the Harboe assay, platelet activation by flow cytometry, vWF structure by immunoblotting, and function by collagen binding activity ELISA (vWF:CBA). Overall, increasing shear rate caused increased total blood trauma in all tested species. This analysis revealed species-specific differences in shear-induced haemolysis, platelet activation and vWF structure and function. Compared to human blood, porcine blood was the most resilient and showed less haemolysis, similar blood counts, but less platelet activation and less vWF damage in response to shear. Compared to human blood, sheared bovine blood showed less haemolysis, similar blood cell counts, greater platelet activation, and similar degradation of vWF structure, but less impact on its activity in response to shear. The shear-induced effect on ovine blood depended on whether the blood was collected via gravity at the abattoir or by venepuncture from live sheep. Overall, ovine abattoir blood was the least resilient in response to shear and

bovine blood was the most similar to human blood. These results lay the foundations for developing blood trauma evaluation standards to enable the extrapolation of *in vitro* and *in vivo* animal data to predict safety and biocompatibility of blood-handling medical devices in humans. We discourage the use of ovine abattoir blood and favour the use of bovine blood for *in vitro* device evaluation but multiple species could be used to create a full understanding of the complication risk profile of new devices. Further, this study highlights that choice of antibody clone for evaluating platelet activation in bovine blood can influence the interpretation of results from different studies.

Key Words: Shear stress, bovine, human, ovine, porcine, vWF, platelet activation, haemolysis, haematology, rheometry

Introduction

Ventricular assist devices (VADs) are an effective way to treat advanced heart failure in selected patients, both as temporary (bridge-to-transplant) and permanent (destination) therapy [1]. Although current VADs have benefited many patients, blood trauma associated with the non-physiological high shear flow within them remains a major clinical concern. The reported blood trauma includes haemolysis [2-4], platelet activation [5, 6], alteration of the coagulation cascade and thrombosis [7], leukocyte damage and release of microparticles [8-11], and degradation of von Willebrand factor (vWF) [12, 13] .

An increased understanding of shear-dependent blood trauma is therefore important in order to develop new, safer VADs with reduced complication rates. Before new VADs can be introduced for clinical application, device safety must be evaluated in large animal models. The animal models commonly used for this purpose include cow [5, 14-17], sheep [18-21] and pig [22]. There is no agreed industry standard, although sheep or pigs have been proposed to have the most similar haemostatic properties compared to humans [23]. Porcine blood has shown similar levels of haemolysis in response to shear stress as human [24], whereas the mechanical fragility of ovine and bovine erythrocytes is higher [25]. Potentially this is related to the cellular flexibility as porcine erythrocytes show similar flexibility compared to human, whereas ovine and bovine erythrocytes are relatively inflexible [26]. Bovine blood is the most thrombogenic when compared to porcine during extracorporeal membrane oxygenation (ECMO) [27], and to human using a clot signature analyser [28]. ECMO also caused a greater reduction in bovine than porcine platelet counts [27]. A study using an *in vitro* Couette type model system found that although platelet counts decreased with

increasing shear in both human and porcine blood, there was no difference between them [24]. The thrombogenicity of bovine blood is not possible to monitor with standard human coagulation tests, whereas the reagents work for porcine blood [27]. Bovine platelets are less activated compared to human in regards to shear based on flow cytometry of surface marker CD62P [29], they are ineffective in plugging a 150 µm hole in a polyethylene tube under shear stress compared to both ovine and human blood, and they are less responsive to collagen during whole blood aggregation [28]. To summarise, if bovine blood is the most sensitive to clotting, then the tests in current use are failing to monitor this and limit the usefulness of bovine blood. Because of the contradicting data described with bovine blood, and because most studies evaluate few species and/or few parameters it is difficult to compare the results. To date, there is a lack of a side-by-side comparison of total blood damage due to shear using blood from the three relevant large animal models against human.

To address this issue, we subjected human, bovine, ovine and porcine blood, to shear using rheometers, and analysed the effects on haemolysis, blood cell counts, platelet activation by surface marker expression, vWF multimer degradation and collagen binding activity (CBA). The knowledge gained from this study could be used to identify ideal animal model(s) for *in vitro* and *in vivo* VAD research and could aid the development of an evidence-based standard for use across the industry.

Materials and Methods

Blood preparation

Bovine, ovine, and porcine blood was collected from the carotid artery by gravity-filling at local abattoirs [30]. The animals were healthy as they would need to be to enter the food chain. However, the breed, sex and age were not available to us. In addition, ovine venepuncture blood (sourced from Ig-Innovations Ltd, Llandysul, UK, project licence (PPL) number 40/3538) was collected by venepuncture using an 11 G x 44 mm stainless steel needle from live sheep. The blood was collected into 14% Citrate Phosphate Dextrose Adenine anticoagulant solution and antibiotics / antimycotics (see Supplement 1: Buffers and solutions). Human whole blood (54 mL) was collected from different healthy volunteers into Vacuette® tubes containing sodium citrate 3.8% (455322, Grenier, Bio-one, Wemmel, Belgium). Use of human blood was approved by the Wales Research Ethics Committee 6. All volunteers gave informed written consent and were informed about the aims of the study in accordance with the Declaration of Helsinki. All haematocrit levels were adjusted to 30 ± 2% by dilution with phosphate buffered saline (Life Technologies Ltd., Paisley, UK) and transferred into the rheometer within 3 h of sample collection.

Shear stress exposure with double concentric rheometer

An AR-G2 rheometer (TA Instruments, New Castle, DE, USA) equipped with an anodised aluminium-coated aluminium double concentric geometry and a temperature-controlled (+37°C) aluminium cup was used to expose human, bovine, ovine and porcine whole blood to different shear rates (0, 2000, 4000, 6000 and 8000 s^{-1}) for 15 min. The double concentric geometry is a continuously rotating apparatus that is used to apply shear, without turbulent flow. However, Taylor vortices are possible at the high shear rate especially shear rate at 8000 s^{-1}. The surface roughness was unknown. The

sample volume loaded into the rheometer was 6.6 mL. Blood samples maintained in a water bath at +37°C for 15 min were used as static (0 s^{-1}) controls. The sheared blood samples were analysed subjected to a complete blood count analysis, haemolysis measurement and platelet activation analysis. The remaining plasma samples were stored at -80°C until used for vWF analysis.

Haemolysis (Harboe assay)

1 mL aliquots of sheared blood samples were centrifuged at 4700 g for 7 min to prepare platelet-poor plasma (PPP). 100 µL PPP was transferred from each aliquot into a deep well 96-well plate (StarLabs, Milton Keynes, UK) and diluted with 1 mL 0.1% Na_2CO_3 solution (Sigma-Aldrich). 170 µL diluted PPP was transferred into a 96-well flat bottom plate (ELISA plate, Greiner Bio-One, Stonehouse, UK) and the absorbance was measured at three wavelengths: 380, 415 and 450 nm (POLARstar Omega, BMG LABTECH Ltd, Aylesbury, UK). The plasma free haemoglobin (pfHb) was calculated (equation (1)) as described [31].

$$pfHb\left(\frac{g}{L}\right) = (167.2\ x\ A415 - 83.6\ x\ A380 - 83.6\ x\ A450)\ x\frac{1}{1000}\ x\ (1/\frac{Vol\ plasma}{Vol\ Na2CO3}) \quad (1)$$

Haematology analysis

Complete blood counts (leukocyte, platelet and erythrocytes) were determined using the clinical automated haematology analyser CELL-DYN Ruby (Abbott Diagnostics, Berkshire, UK) for human blood and the veterinary haematology analyser Abacus Junior vet 5 (Diatron, Budapest, Hungary) for bovine, ovine and porcine blood.

Platelet activation

Several different antibodies frequently reported in the literature for the individual species were employed in this assay to enable easy translation of results for other research groups. 20 µL aliquots of the sheared blood samples, along with a positive

control of blood treated with 5 μM phorbol 12-myristate 13-acetate (PMA) for 60 min at room temperature, were single-stained with the following antibodies according to Table 2: anti-CD62P, clone Psel.KO.2.5 [32]; anti-CD62P, clone Psel.KO.2.7 [29, 32]; anti-fibrinogen [33]; anti-CD42b-FITC [34]; GC5 (reported to bind activated bovine platelets but antigen still unknown [5]); CAPP2A (an anti-ruminant CD41/61 antibody [14, 18, 19, 32, 35, 36]). Antibodies were diluted in flow cytometry (FC) buffer (Supplement 1). The blood samples were incubated with the antibody on ice in the dark for 30 min. For CAPP2A and GC5, cells incubated with primary antibody were washed once with FC buffer and then further stained with PE-conjugated F(ab')2 fragment of goat anti-mouse IgG (H+L) antibody (0.25 μg/test, Life Technologies) in the dark on ice for 20 min. Red blood cells were then lysed with 2 mL BD FACS Lysing Solution (BD Bioscience) according to the manufacturer's instructions. Cells were washed once with FC buffer and fixed with 200 μL BD Stabilizing Fixative (BD Bioscience) according to the manufacturer's instructions and stored at +4°C overnight prior to acquisition using a 10-colour Navios flow cytometer (Beckman Coulter, High Wycombe, UK).

All events were displayed first as a forward scatter (FSC) versus side scatter (SSC) density plot on logarithmic scale (Supplemental Figure 1A-B). The platelet population was gated to include both platelets in a resting and activated state using the unstimulated control and the PMA-stimulated sample as guidance; 10,000 events within this gate were recorded and analysed for each sample. Surface CD62P and fibrinogen binding increase with platelet activation, so gates were drawn to determine the percentage of positive events (Supplemental Figure 1C-F and M-N). CAPP2a, CD42b and GC5 are expressed on resting inactivated platelets and decrease with platelet

activation. Markers were drawn on CAPP2a, CD42b and GC5 to measure the percentage of negative events, and thereby determine the percentage of activated platelets (Supplemental Figure 1G-L). Data analysis was performed with Kaluza 1.3 (Beckman Coulter).

Immunoblotting for high and low molecular weight vWF

PPP prepared from sheared and static samples was thawed and centrifuged at 15,000 g for 5 min to remove debris. 10 μL of PPP mixed in a ratio of 1:4 with non-reducing sample buffer (Supplement 1) was loaded into each well, and subjected to electrophoresis using high gelling temperature agarose (50041, Scientific laboratory Supplies, UK). The gel comprised of a 0.8% stacking gel and a 1.5% running gel. Electrophoresis was performed at room temperature for 20 hours at 60 volts and for 4 hours at 80 volts. The fractionated plasma proteins were then transferred from the gel to a polyvinylidene difluoride membrane (0.45 μm, IPVH304F0, Immobilon-P, Millipore Corporation, MA, USA) using capillary blotting. Free binding sites on the blot were blocked by incubating the blot with blocking buffer (Supplement 1) with agitation for 1 hour at room temperature. The membrane was incubated with horseradish peroxidase conjugated polyclonal rabbit anti-human vWF (P0226, DAKO, Denmark) diluted 1:1000 in blocking buffer for 1 hour at room temperature with mild agitation. The membrane was washed six times with Tris-buffered saline/Tween-20 (5 min each rinse) and developed using chemiluminescence substrate (170-5060, Bio-Rad, CA, USA) and visualized with the ChemiDoc XRS scanner (Model Number: universal hood II, Bio-Rad, CA, USA). The high molecular weight (HMW) vWF bands were counted and the loss of bands was compared to the 0 s^{-1} static control. The increase in the density of low molecular weight (LMW) vWF bands in each lane was quantified by densitometry

using Quantity One software, v4.6.8 (Bio-Rad, CA, USA) and normalised by dividing by total intensity of all vWF bands in each lane.

Collagen binding activity of vWF

PPP prepared from sheared and static samples was thawedand centrifuged at 15,000 g for 5 min to remove debris. The vWF collagen binding activity (vWF:CBA) in the samples was assessed using a Zymutest vWF:CBA enzyme-linked immunosorbent assay (ELISA) (Hyphen BioMed, Neuville-sur Oise, France) according to manufacturer's instructions. Absorbance was measured at 450 nm (POLARstar Omega, BMG LABTECH Ltd, Aylesbury, UK). Results were normalised to the total vWF and compared to the static control.

Data analysis

For each assay the data were standardised to the static control to evaluate the relative increase or decrease of the parameter caused by increasing shear rates whilst minimising donor-to-donor variability within species. For haemolysis and platelet activation, the background levels observed in the static control were subtracted from all other measurements. For haematology, vWF immunoblotting, and vWF:CBA, the data were expressed as a percentage of the static control.

The datasets consisted of repeated measurements of blood samples across 5 shear rates, and 4 species, and in the case of platelet activation measurements 2 to 4 antibodies. Linear mixed models were used to analyse the dataset. Shear and species or shear and antibodies (in the case of platelet activation) were regarded as fixed effects and the different subjects were treated as a random effect. Tukey's Range test was performed post hoc to further investigate the differences between species and shear rates where such a difference was found to be significant in the analysis. This method enables

comparison of the different species to each other at the same shear rate and comparison of the effect of shear within species. All the analysis was performed using the R statistical environment, v 3.0.2 [R Core Team, Vienna, Austria] [37], Version 0.99.896 [RStudio, Boston, MA, USA]) [38].

Results

Haemolysis

The haemolysis results differed significantly between the species ($p < 2 \times 10^{-5}$). The species could be divided into three groups: low haemolysis (ovine venepuncture, bovine and porcine), medium haemolysis (human), and high haemolysis (ovine abattoir) (Figure 1). Within the low haemolysis group there were no significant species differences. Ovine abattoir blood haemolysis was significantly greater than that found in human blood across all shear rates, and they both showed significantly greater haemolysis compared to the low haemolysis group ($p < 0.002$). The difference between the ovine abattoir and the low haemolysis species group became significant at a shear rate of ≥ 4000 s^{-1} ($p < 0.01$). At a shear rate of 8000 s^{-1} human blood haemolysis was significantly greater than ovine venepuncture and porcine ($p < 0.01$).

Within the species groups, shear had an effect on haemolysis, with each shear rate producing significant pfHb levels compared to the static control ($p < 0.038$). This effect was seen in increments from 0 – 2000 s^{-1}; 2000 – 6000 s^{-1}; and 6000 – 8000 s^{-1}, i.e. an increase in shear rate from 2000 – 4000 s^{-1} or from 4000 – 6000 s^{-1} did not produce a significant increase in haemolysis.

Haematology

Although there was an increase in pfHb in all species, there was no significant change in erythrocyte counts at any shear rate over the testing period indicating that they have remained intact throughout the shearing test (Figure 2). Nor was there a significant decrease in leukocyte numbers at any shear rate. Platelet numbers decreased slowly and at shear rates ≥ 6000 s^{-1} the decrease was significant across the species when compared to the static control ($p = 0.040$). Ovine abattoir platelets decreased in numbers (a

possible sign of activation) at all shear rates. This decrease was significantly more rapid than all other species ($p < 0.01$).

Platelet activation

Platelet activation was monitored using flow cytometry with a number of different antibodies specific for platelet cell surface antigens of the various species (Figure 3A-E). The results obtained were compared to those reported for the antibodies in the literature.

In human blood, shear rate significantly affected the platelet activation ($p < 2 \times 10^{-16}$), except when increasing from 2000 s^{-1} to 4000 s^{-1}. The activation measurements differed significantly between the CD62P antibodies (clones KO.2.5 and KO.2.7) and CD42b ($p < 10^{-8}$). This became apparent at a shear rate of 8000 s^{-1} with CD62P clone KO.2.7 showing the same high level of activation as CD42b, both significantly greater than the level detected with CD62P clone KO.2.5 ($p <0.03$) (Figure 3A).

In ovine abattoir blood, shear rate significantly affected the platelet activation up to a shear rate of 4000 s^{-1} ($p < 0.0002$), after which the activation plateaued. Activation measurements were significantly different depending on the antibody used: CD62P (clones KO.2.5 and KO.2.7) and CAPP2A ($p < 10^{-5}$). The activation level detected by the two CD62P antibodies was the same, but at shear rates > 2000 s^{-1} this was significantly greater than the level detected by the CAPP2A antibody ($p < 0.0003$) (Figure 3B).

In ovine venepuncture blood, there was a significant effect of shear ($p < 3 \times 10^{-8}$). Starting at 4000 s^{-1} there was significant platelet activation compared to the static control ($p < 4.2 \times 10^{-4}$). A shear rate of 8000 s^{-1} also had a significant effect compared

to 2000 s^{-1} ($p = 0.02$), but there were no other significant differences between the individual shear rates. The antibodies used for ovine venepuncture blood were the same as described for ovine abattoir blood. However, compared to the ovine abattoir blood, the antibody trend was reversed: CAPP2A reported the highest activation levels and the two CD62P antibodies reported the lowest levels. Interestingly, the maximum level of activation reported by CAPP2A appeared constant, with roughly 10% activation at 8000 s^{-1} in both abattoir and venepuncture blood (Figure 3C).

In bovine blood, a shear rate of $\geq$ 4000 s^{-1} had a significant effect on the antigen expression levels compared to the static control ($p < 1.4 \times 10^{-6}$), and every subsequent 2000 s^{-1} increment in shear rate caused further significant activation ($p < 1.4 \times 10^{-3}$). At a shear rate of $\geq$ 4000 s^{-1} a pattern emerged where both CD62P antibodies (clone KO.2.5 and KO.2.7) reported significantly greater platelet activation compared to both GC5 and CAPP2A ($p < 1 \times 10^{-7}$). There was no difference between GC5 and CAPP2A at any shear rate. CD62P KO.2.5 reported significantly lower levels of platelet activation compared to CD62P KO.2.7 ($p < 0.0002$) (Figure 3D).

In porcine blood, a shear rate of $\geq$ 2000 s^{-1} produced significant platelet activation ($< 2 \times 10^{-16}$). Activation also significantly increased with each 2000 s^{-1} increment in shear rate ($p < 3.1 \times 10^{-6}$). The two antibodies used to measure porcine platelet activation reported significantly different levels of activation from a shear rate of $\leq$ 4000 s^{-1}, where the CD62P clone KO.2.5 antibody reported greater platelet activation than the fibrinogen antibody (2.6×10^{-14}) (Figure 3E).

Since the CD62P (KO.2.5) antibody can distinguish between activated and resting platelets for all four species, we used this antibody to compare shear-induced

platelet activation in all species (Figure 4). There was a significant species-specific difference in the CD62P expression levels ($p < 1.92 \times 10^{-5}$). Based on the CD62P expression levels, the tested species were divided into three groups: low activation (ovine venepuncture), medium activation (human, ovine abattoir and porcine), and high activation (bovine). There was no significant difference between human platelet activation levels compared to porcine, or between porcine and ovine abattoir. However, human platelets were significantly more activated at 8000 s^{-1} than ovine abattoir platelets ($p = 0.027$). Ovine venepuncture platelets were significantly less activated ($p < 0.007$), and bovine showed significantly greater activation levels compared to all other species ($p < 0.008$). For all species, a shear rate of 2000 s^{-1} had no effect on activation, thereafter every increase of 2000 s^{-1} in the shear rate increased platelet activation significantly ($p < 1 \times 10^{-7}$). Increase from 6000 s^{-1} to 8000 s^{-1} provided no further effect. Specifically for ovine venepuncture platelets, there was no significant difference in shear-dependent platelet activation compared to the static control using CD62P (KO.2.5).

HMW vWF multimer degradation and LMW vWF multimer accumulation

A representative immunoblotting result of vWF multimers from multiple species is shown in Figure 5A, and the loss of HMW vWF bands in blood from different species exposed to various rates of shear stress compared to the static control in Figure 5B. This analysis divided the species into three groups: high loss (ovine abattoir), medium loss (human, ovine venepuncture, bovine), and no loss/increase (porcine) ($p < 1.03 \times 10^{-4}$). Ovine abattoir blood showed a significantly greater loss ($p < 4.5 \times 10^{-6}$), and porcine blood showed a significantly lower loss of HMW vWF bands ($p < 6.25 \times 10^{-4}$) compared to all other species.

Within the species, shear rate increments of $\geq 4000\ s^{-1}$ had a significant effect on the loss of HMW vWF bands ($p < 0.05$). Differences were not noticeable when comparing the loss by increasing shear rates in increments of $2000\ s^{-1}$. The significant findings were as follows: ovine abattoir HMW vWF showed a band loss at shear rates of $\geq 4000\ s^{-1}$ compared to its own static control ($p < 0.008$) and compared to porcine blood at the same shear rates ($p < 1.7 \times 10^{-5}$). At a shear rate of $\geq 6000\ s^{-1}$, the ovine abattoir blood showed a loss compared to all other species ($p < 0.036$). At $8000\ s^{-1}$, human and ovine venepuncture HMW vWF bands were decreased compared to the porcine blood ($p < 0.05$), but not to the static control.

Another way of analysing damage to the structure of vWF is to consider the second lowest LMW vWF band intensity in each lane compared to the static control (Figure 5C). The decrease in the HMW vWF band multimers and the increase in LMW band intensity may suggest that the HMW vWF bands were cleaved into smaller fragments, in a shear-rate dependent manner. The LMW vWF density divided the species into two groups: high density (ovine abattoir) and low density (remaining species) ($p < 0.001$). Bovine, ovine venepuncture and porcine blood showed similar behaviour to human blood. Within the species, shear rate differences of $4000\ s^{-1}$ resulted in significant increases in LMW vWF density ($p < 0.03$) compared to the static control.

vWF activity

The function of vWF was assessed by measuring its collagen binding activity (vWF: CBA) (Figure 6). The vWF:CBA activity declined with increasing shear rate in all species ($p < 2 \times 10^{-16}$). The most drastic decline was seen in ovine venepuncture blood, and was significantly lower than ovine abattoir ($p < 0.02$), porcine ($p < 0.0006$), and bovine ($p < 0.0008$), but not lower than human.

Discussion

It is important to understand total blood trauma in different test species blood in response to shear for *in vitro* testing of VADs and other blood handling devices. To address this issue, we conducted experiments of shear-induced total blood trauma comparing blood from humans, sheep (different sources: abattoir and venepuncture), cows and pigs (Table 3).

In our study, we found that ovine abattoir erythrocytes were the most sensitive to shear resulting in the highest levels of haemolysis, followed by human > bovine > porcine > ovine venepuncture. Ding *et al.* also collected animal blood from the abattoir and showed the same results (ovine abattoir > human > bovine > porcine) [39]. Jikuya *et al.* collected blood via venepuncture with the following haemolysis results: ovine venepuncture > human > bovine [25]. One potential explanation for the high haemolysis observed in ovine abattoir blood in our and Ding's study, could be related to the stress the animals encounter during the slaughter process. The ovine spleen contracts in response to an increase in adrenaline levels [40], and releases stored reserves of erythrocytes to enable increased oxygen consumption required for a quick escape. The splenic erythrocytes make up about 25% of the total erythrocyte pool [40] and are different from those in the circulation. They are younger and larger with a higher water content and are recognized by the spleen and removed from the circulation for storage [41]. Overhydrated erythrocytes have an increased volume whereas their surface area remains unchanged, thereby reducing cell deformability [42]. This, in turn, would increase blood viscosity and thereby shear stress [43]. Hence, we hypothesize that the increased haemolysis in the abattoir blood is due to the release of young erythrocytes with high water content, lower deformability, and therefore more haemolytic.

Supporting this theory, we found the baseline erythrocyte levels to be greater in the ovine abattoir blood versus the venepuncture blood ($11.67 \pm 0.81 \times 10^{12}$ /L vs $9.03 \pm 0.76 \times 10^{12}$ /L, $p < 0.0001$). The ovine venepuncture blood in Jikuya's study was collected from healthy barn bred sheep. Blood collection procedures are known to cause stress to sheep resulting in splenic contraction, unless the animals have been well socialised and allowed to get used to the procedure [44]. The ovine venepuncture blood used in our study was sourced from Ig-Innovations Ltd, a local company developing sheep derived polyclonal antibodies for human therapy. Their sheep are used to regular blood collections so they endure minimal stress during the procedure.

Although erythrocyte and leukocyte counts did not change for any species, platelet counts decreased for all species with increased shear rates. Again, the ovine abattoir blood showed the most damage, i.e. the lowest platelet counts. The reduction in platelet counts is likely due to activation, and this is supported by the flow cytometry results. Lu *et al*. showed that the platelet counts for bovine blood did not change significantly from the static control level even at high shear condition [29]. The discrepancy could be due to the relatively longer exposure time used in our study (15 min versus 2 min).

The use of the anti-CD62P clone KO.2.5 enabled direct comparison of the species revealing significant changes in platelet activation after exposure to shear rates ≥ 4000 s^{-1} compared to baseline levels, where bovine platelets showed the highest activation (Table 3). These results contradict the results published by Lu *et al.* who demonstrated a relative inactivity of bovine platelets compared to human platelets. We hypothesised that this discrepancy could be due to the antibody clone used. Lu *et al.* used the CD62P KO.2.7 clone to compare the platelet activation [29]. We used both the

KO.2.5 and the KO.2.7 clone to compare. However, both CD62P antibodies showed that the bovine platelet activation was greater than the human platelet activation, and the KO.2.7 consistently reported greater levels than the KO.2.5 clone in these two species. Instead, the discrepancy could have to do with the level of activation of the platelets caused by the longer exposure time used in our study (15 min versus 2 min). There may be an exposure time threshold below which bovine platelets are less activated compared to human, and above which the pattern is reverse.

The results we observed for ovine platelets using different sources of blood and the CAPP2A versus the CD62P antibodies were interesting. The CAPP2A antibody picked up similar levels of chemically induced platelet activation using the positive control PMA, as well as similar levels of mechanically induced platelet activation which increased with shear. However, the platelet counts differed (lower counts in abattoir blood) and the CD62P results differed (greater activation in abattoir blood). Thus, it appeared the CD41/CD61 antigen that is detected by the CAPP2A antibody is affected by mechanical shear in a robust manner, regardless of the overall condition of the blood. To our knowledge, the performance of CAPP2A against CD62P antibodies has not been evaluated in ovine or bovine blood before. Other studies using CAPP2A have employed it as a platelet marker to distinguish it from red blood cells, and then analysed CD62P expression on CAPP2A-expressing platelets [14, 18, 19, 32, 36]. In our protocol, we have used red blood cell lysis and a subsequent wash to remove red cell debris. Our results showed no difference between the two CD62P antibody clones when used with ovine blood, which is consistent with Johnson *et al.* who used them both to evaluate platelet activation *in vivo* during a paediatric VAD implant [32]. In bovine blood, we evaluated GC5 and CD62P antibodies, and we found that the percent positive platelet

levels were greater when CD62P was used compared to GC5. This finding is contradictory to Snyder *et al.* who found the number of activated platelets detected to be greater with GC5 when used to analyse *in vivo* samples from calves implanted with VADs, but in this study the antibody used to evaluate CD62P was a different clone (#NPL44-10) [14].

In this study, the results of vWF multimer analysis of four different species showed that ovine abattoir blood vWF is the most shear-sensitive; followed by ovine venepuncture, human, bovine, and finally porcine. Our results showed a significant loss of HMW vWF bands and a significant increase in LMW vWF bands in all species (except porcine) at shear rates $\geq$ 4000 s^{-1} compared to the static control. This observation is also consistent with work by Bartoli *et al.*, who found that LMW vWF bands increased significantly after exposing human blood to non-physiological shear stress [45]. In our previous work, we exposed human PPP to 4000 s^{-1}. The damage to HMW vWF was not statistically significant until after 4 hours of shearing which is due to the poorer resolution of bands of our previous method compared to that presented herein [46]. vWF function as assessed by vWF:CBA of human, ovine and bovine blood decreases with increasing shear rate, indicating shear stress-induced degradation of HMW vWF. However, what we have found in our study is that porcine vWF is the most resilient to shear, and possibly even activated by a shear rate of 4000 s^{-1}. At this shear rate, the HMW vWF multimers and vWF:CBA levels started to increase compared to the standard control. One explanation is that there may be an activation in platelets at this shear rate causing release of vWF from platelet α-granules [47, 48]. However, porcine blood did not show a peak in platelet activation at 4000 s^{-1} (Figure 4) to support this. In our study, ovine abattoir blood showed the highest platelet activation, but also

the highest vWF damage, thus the increased porcine vWF activity remains unexplained. Egger *et al.* [49], compared human and porcine vWF structure and function in sheared plasma, and found that although the functional assay indicated that porcine vWF was more resilient to shear stress, there was a similar loss in multimer bands. Since immunoblotting is not an easily reproducible assay, it may be difficult to compare results between different laboratories, and it could be that the indication of greater resilience and preserved function of porcine vWF compared to human are true, but more research would be needed to verify this.

The effect of shear-induced total blood trauma on four species (human, ovine [abattoir and venepuncture], bovine and porcine) was measured under the same conditions with the same shear rheometer. The reason for using a rheometer was to cause enough damage in a controlled manner to allow us to evaluate species-specific responses. We have previously tested several extracorporeal centrifugal pumps with bovine blood *in vitro* according to the ASTM guidelines [50]. The haemolysis level achieved after 15 min at 2000 s^{-1} matches that of the blood pumps after 6 hours of pumping (pfHb of around 10 mg/dL in the CentriMag [51], VentrAssist IRBP [8], and Bio-Medicus BPX-80 [3]). Previously, we did not detect platelet activation measured by CAPP2A caused by CentriMag pump during testing [51]. In this study, a shear rate of 6000 s^{-1} was required to detect significant activation. The damage to HMW vWF multimers became evident after 3 hours of pumping in the CentriMag whereas the rheometer caused a significant loss after 15 min at 4000 s^{-1} [51].

To summarise, in this study, bovine blood was the most similar to human blood and thus we would recommend it for *in vitro* testing of VADs. The haemolysis is lower, and the platelet activation is greater when CD62P KO.2.5 is used. Porcine blood and

ovine venepuncture blood showed less damage and may therefore underestimate the impact of the VAD on the blood components. Taking all results in consideration, we cannot recommend the use of ovine abattoir blood for in vitro testing due to the overall high damage (greater haemolysis, reduced platelet count, high vWF structural damage) compared to ovine venepuncture blood. However, this may only reflect the quality of blood from the abattoir available to us.

Cows, sheep, and pigs are all used as *in vivo* models for VAD testing. However, the choice of animal model for preclinical *in vivo* testing is not only dependent on the blood damage but also on practicalities (such as subject size, growth rate and temperament). The bovine model includes calves to ensure the heart is as close to human size as possible, but the calf's growth rate makes long-term studies practically difficult. The hearts of pigs and large (75-90 kg) sheep resemble human hearts in size, and use of adult animals eliminates the issue of growth rate. Unless the power source is wearable, the animal needs to be tethered for the duration of the study to prevent tangling and damage to wires. Calf and sheep tolerate long-term tethering, but pigs do not. Tethering of sows is a banned farm practice throughout the EU for animal welfare reasons [52] and the stress it causes has been found to create adaptations of the endogenous opioid systems [53]. Since the practical circumstances may limit the choice of the animal model for *in vivo* testing, it may be worth using a combination of species during *in vitro* testing for a translational approach to total blood damage.

Limitations

In this study, relatively low shear rates were used to establish the model. The maximum shear rate is 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 >20 000 s^{-1} [49]. Another limitation was the residence time (15 min) of the blood which is continuously sheared in the rheometer, whereas the blood passes through the VAD in a period of milliseconds. All animals were healthy as they would need to be for entering the food chain, but we did not have access to information such as breed, sex and age, which may influence the results. Differences in erythrocyte aggregation and deformability have been found between male and female rats and dogs [54], so it is likely that differences may also exist in the large animals used in this study. We did not measure the viscosity and density of the blood although the haematocrit was standardised to 30 ± 2% for all species. A few studies have used viscometers to analyse species-specific differences in blood with standardised haematocrit [43, 55]. The haemorheological behaviour shows that porcine blood is quite similar to human, whereas bovine and ovine show a different viscoelasticity pattern compared to porcine. This is likely due to the differences in cell size, bovine and ovine erythrocytes are smaller compared to human and porcine. Both studies show that the interspecies differences in viscosity decrease with increasing shear rate, and if all species' blood behaved in a non-Newtonian manner then the viscosity differences might fully diminish at the high shear rates that we are using. However, at 30% HCT, bovine and ovine blood behaves almost Newtonian, whereas porcine blood is more shear-thinning [43]. This might mean that the viscosity of ovine and bovine blood remains constant around 3.6-4.6 mPa s and that human and porcine blood viscosity continues to decrease. If this was the case, then bovine and ovine blood cells would be subject to the highest shear stress and we would expect them to show the highest haemolysis. Interestingly, this was not the case and more research is required to analyse the viscosity at higher shear rates using rheometers. However, this work provides a simple and accurate way to expose the

blood samples to controlled shear rates ranging from physiological to pathological levels which allows evaluation of the impact of shear rate on the overall blood damage. This is of value to provide further insight into complications in VAD patients. Our future work will focus on further exploration of the shear stress, time, flow regimes of overall blood trauma, especially at higher ranges of shear stress, shorter exposure times, different flow regimes: continuous flow (CF), and pulsatile flow (PF) on overall blood trauma responses.

Conclusion

Blood compatibility is essential to the successful development of VADs, however, there are few studies assessing the differences between animal and human blood in response to mechanical stress. In this study, we determined the shear-dependent differences in haemolysis, complete blood count, platelet activation and vWF structure and function between human blood and that from three large animals commonly used for *in vitro* and *in vivo* VAD testing. We found that bovine blood was the most similar to human overall. If ovine venepuncture or porcine blood is used for *in vitro* testing there is a risk that the blood damage could be underestimated as these blood types appeared more resilient to shear stress compared to human blood. We discourage the use of ovine abattoir blood due to the high level of overall damage. In case ovine venepuncture blood is used we recommend that it be collected from animals who are well socialised and used to the procedure. No single species tested had a shear-dependent total blood damage profile that mimicked human blood but we favour bovine blood for *in vitro* testing, at least as a starting point, as it behaved the most similar to human blood. Further, this study highlights that choice of antibody clone for evaluating platelet activation in bovine blood can influence the interpretation of results from

different studies. The multiplicative factors in Table 3 could serve as the basis for development of universal standards when extrapolating in vitro VAD total blood trauma data to predict its safety and biocompatibility for in vivo testing and further development.

Acknowledgments

[Acknowledgements cannot be mentioned without identifying the authors but are described in the Cover Letter]

Disclosure

[Conflict of interest disclosures cannot be mentioned without identifying the authors but are described in the Cover Letter]

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Tables

Table 1. Acronyms

ECMO	Extracorporeal membrane oxygenation
ELISA	Enzyme-linked immunosorbent assay
FC	Flow cytometry
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HMW	High molecular weight
LMW	Low molecular weight
PE	R phycoerythrin
pfHb	Plasma-free Haemoglobin
PMA	Phorbol 12-myristate 13-acetate
PPP	Platelet-poor plasma
SSC	Side scatter
VAD	Ventricular assist device
vWF	Von Willebrand factor
vWF:CBA	Von Willebrand factor collagen binding activity

Table 2. Antibodies for flow cytometry and immunoblotting

Antibodies	Human	Bovine	Ovine	Porcine
Anti-CD62P, clone Psel.KO.2.5 1 µg/test, PE-conjugate, MCA2418, AbD Serotec, Kidlington, UK	✓	✓	✓	✓
Anti-CD62P, clone Psel.KO.2.7 0.5 µg/test, AlexaFluor647-conjugate, MCA2419, AbD Serotec, Kidlington, UK	✓	✓	✓	
Anti-fibrinogen, clone 51G22 0.2 µg/test, FITC-conjugate, IMS09-038-335, Agrisera, Sweden				✓
anti-CD42b, clone HIP1 0.2 µg/test, FITC-conjugate, 11-0429-41, eBioscience, Hatfield, UK	✓			
GC5 0.1 µg/test Monoclonal Antibody Centre, Washington State University, Pullman, WA, US		✓		
CAPP2A 0.1 µg/test Monoclonal Antibody Centre, Washington State University, Pullman, WA, US		✓	✓	
PE-conjugated F(ab')2 fragment of goat anti-mouse IgG (H+L) antibody* 0.25 µg/test Life Technologies Ltd., Paisley, UK		✓	✓	

* Secondary antibody used with GC5 and CAPP2A

Table 3. The ratio of haemolysis, platelet count, platelet activation, HMW/ LMW vWF and vWF collagen binding activity of blood trauma in bovine, ovine and porcine blood compared to human blood after being subjected to a shear rate of 8000 s^{-1} for 15 min at +37°C in a rheometer. The ovine blood evaluated came from two sources: obtained via gravity-collection from sheep at the abattoir, or via venepuncture from live sheep.

Species	Haemolysis	Platelet count	Platelet activation (CD62P)	HMW vWF band loss	LMW vWF band density increase	vWF:CBA
Human	1.00	1.00	1.00	1.00	1.00	1.00
Ovine – abattoir	1.27	0.71	0.63	0.73	2.46	1.83
Ovine – venepuncture	0.32	1.05	0.11	0.96	1.23	0.85
Bovine	0.48	0.95	1.34	1.03	1.32	1.12
Porcine	0.41	0.99	0.75	1.25	0.95	1.18

Figure Legends

Figure 1. Shear-dependent haemolysis in multiple species. Human, ovine (abattoir and venepuncture), bovine and porcine whole blood exposed to increasing shear rates (0-8000 s^{-1}) for 15 min at +37°C in a rheometer. Haemolysis measured by the Harboe assay and plasma free haemoglobin release measured as mg/dL. Results expressed as mean ± SD, relative to the 0 s^{-1} static control.

Figure 2. Shear-dependent changes in complete blood counts in multiple species. Human, ovine (abattoir and venepuncture), bovine and porcine blood subjected to increasing shear rates for 15 min at +37°C in a rheometer and thereafter measured by automatic haematology analysis. Results expressed as mean ± SD, % relative to the 0 s^{-1} static control.

Figure 3. Shear-dependent platelet activation in multiple species. Whole blood subjected to increasing shear rates (0-8000 s^{-1}) in a rheometer for 15 min at +37°C and analysed by flow cytometry for changes in platelet activation markers. Human: two antibodies for CD62P (Psel.KO.2.5 and Psel.KO.2.7) compared to CD42b. Ovine from abattoir and venepuncture: the CD62P-antibodies compared to CAPP2A. Bovine: the CD62P-antibodies compared to CAPP2A and GC5. Porcine: CD62P (Psel.KO.2.5) compared to an anti-fibrinogen antibody. Results expressed as mean ± SD, % relative to the 0 s^{-1} static control.

Figure 4. Shear-dependent platelet activation in multiple species. Whole blood subjected to increasing shear rates in a rheometer (0-8000 s^{-1}) for 15 min at +37°C was analysed by flow cytometry for changes using CD62P (Psel.KO.2.5). Results expressed as mean ± SD, % relative to the 0 s^{-1} static control.

Figure 5. Measurement of vWF multimers from multiple species by immunoblotting. Whole human, ovine (abattoir and venepuncture), bovine and porcine blood subjected to increasing shear rates (baseline blood, BL, and 0-8000 s^{-1}) in a rheometer for 15 min at +37°C. Platelet-poor plasma was prepared from sheared blood and von Willebrand Factor (vWF) multimer expression analysed by immunoblotting. A) Representative immunoblot gel image. B) Loss of high molecular weight (HMW) vWF bands (analysed by counting). Results expressed as relative to the 0 s^{-1} static control. C) The increase in density in the 2nd lowest molecular weight (LMW) vWF band (analysed by densitometry) and normalised to total vWF. Results expressed as mean ± SD, % relative to the 0 s^{-1} static control.

Figure 6. Shear-dependent vWF:CBA in multiple species. Whole blood subjected to increasing shear rates in a rheometer (0-8000 s^{-1}) for 15 min at +37°C was analysed by vWF:CBA ELISA. Results normalised to total vWF (measured by densitometry) and expressed as mean ± SD, % relative to the 0 s^{-1} static control.

Supplemental figure 1. Gating strategy for platelet activation antibodies.

Left panel) untreated blood from various species. Right panel) blood treated with 5 µM PMA for 60 min at room temperature from various species. All ungated events were displayed on forward scatter (FSC) versus side scatter (SSC) density plots on logarithmic scale (A-B) to identify and gate the platelet population, here exemplified with human blood. Gated platelets were displayed on density plots with fluorescence on logarithmic axes versus SSC on logarithmic axes (C-N). Human platelets single-stained with either of two anti-CD62P antibodies (CD62P-PE, clone Psel.KO.2.5, or CD62P-AlexaFluor647, clone Psel.KO.2.7) or CD42b-FITC (C-H). Bovine platelets single-stained with CAPP2A or GC5, then further stained with PE-conjugated F(ab')2

fragment goat anti-mouse IgG (H+L) antibody (I-L). Porcine platelets single-stained with anti-fibrinogen-FITC (M-N). Note that CD62P and fibrinogen expression is up-regulated on activated platelets whereas CD42b, CAPP2A and GC5 expression is down-regulated on activated platelets.

Supplement 1.

Buffers and solutions

Antibiotic and antimycotic solution:

50 mg/dL Gentamycin and 10 mL/L Antibiotic antimycotic solution (A9909), both from Sigma-Aldrich, Poole, UK.

Flow cytometry (FC) buffer:

Dulbecco's phosphate-buffered saline, Life Technologies; 0.2% bovine serum albumin, and 0.05% sodium azide, both Sigma-Aldrich.

Non-reducing sample buffer:

0.01 M Trizma Base, 1 mM EDTA disodium salt dehydrate, 2% SDS (w/v), 0.05% Bromophenol blue (w/v) and 10% Glycerol (v/v), pH 8.0, all chemicals from Sigma-Aldrich.

Blocking buffer:

5% non-fat dry milk made in Tris-buffered saline/Tween-20 (T5030, Sigma-Aldrich)**.**