Int J Clin Exp Med 2021;14(9):2333-2342
www.ijcem.com /ISSN:1940-5901/IJCEM0133019

Original Article

Exploration of appropriate anticoagulant reagents and reliability of porcine blood for *in vitro* evaluation of thrombogenicity

Marjolijn Sales[1], Johanna C Clauser[2], Thomas Schmitz-Rode[2], Torsten Linde[2], Ilona Mager[2], Judith Maas[2], Ulrich Steinseifer[2], Rüdiger Autschbach[1], Rachad Zayat[1], Jutta Arens[2,3*], Heike Schnöring[1*]

[1]Department of Thoracic and Cardiovascular Surgery, RWTH University Hospital Aachen, Aachen, Germany; [2]Department of Cardiovascular Engineering, Medical Faculty, Institute of Applied Medical Engineering, Helmholtz Institute, RWTH Aachen University, Aachen, Germany; [3]Chair of Engineering Organ Support Technologies, Department of Biomechanical Engineering, Faculty of Engineering Technology, University of Twente, Netherlands. [*]Equal contributors.

Received March 11, 2021; Accepted August 5, 2021; Epub September 15, 2021; Published September 30, 2021

Abstract: Background: A validated *in vitro* testing system is crucial to evaluate the thrombogenicity of new medical devices such as heart valve prostheses, since thromboses and thromboembolisms remain limiting factors in clinical application. The testing fluid, animal species, and anticoagulants may affect preclinical analyses. Our study aimed to investigate the use of unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) in reference to blood anticoagulation by citrate using porcine blood to determine their applicability in preclinical thrombogenicity testing. Methods: In a static experimental setting, material specimens of glass and polymethylmethacrylate were used to simulate different degrees of clotting activation. The specimens were exposed to porcine blood anticoagulated by either citrate, UFH or LMWH. Porcine blood obtained from the abattoir was compared with blood obtained from laboratory swine. Monitoring included complete blood count, activated partial thromboplastin time (aPTT), prothrombin time (PT), and fibrinogen time. Results: UFH-anticoagulated blood showed significant decreases in aPTT, PT, and thrombocyte count, most notably after glass-induced, strong activation of clotting. Blood anticoagulated with LMWH showed significant decreases in aPTT, PT and thrombocytes as well, but no significant differences between the effects of different clotting-activating materials were recorded. No relevant changes were observed with the use of citrate. Abattoir-derived blood had huge variability in baseline values; for example, thrombocyte count showed a 25.85% standard deviation from the mean value in citrated blood, 15.54% in UFH-anticoagulated blood, and 21.69% in LMWH-anticoagulated blood, whereas laboratory-derived blood varied only around 2.01%. Conclusions: We demonstrated the applicability of porcine blood in thrombogenicity testing. Special caution is required to standardize blood withdrawal methods and eliminate preanalytical interference. No distinct advantage of either type of heparin was detected.

Keywords: Thrombogenicity evaluation, *in vitro*, heparin, citrate, porcine blood

Introduction

In vitro evaluation of thrombogenicity is an indispensable element in the progress of design and development of medical devices that are meant to be applied inside the cardiovascular system. Continuous exposure of the blood system to a foreign object facilitates thrombosis and thromboembolism. For example, the clinical relevance of evaluation of thrombogenicity in prosthetic heart valve replacement, which often remains the only therapeutic option in advanced heart valve disease, can easily be recognized. Despite six decades of experience, thromboembolism and anticoagulation-associated hemorrhage remain the Achilles' heel of this treatment [1]. Because the ideal prosthetic valve [2] has not yet been developed, improvement of valve design remains ongoing.

Analytical methods to study the functionality of heart valve prostheses (HVP) range widely from numerical flow simulations, *in vitro* studies,

with animal research and clinical trials. The specific requirements for medical device testing are outlined by public authorities [3], with precise details for animal and clinical trials, as well as for many preclinical *in vitro* tests, such as durability testing. *In vitro* evaluation of thrombogenicity remains rather heterogeneous. Recently, a selected preclinical method for assessment of the thrombogenic potential of HVP underwent successful validation [4], focusing on reproducible and physiologic hemodynamic conditions. To evaluate the thrombogenicity of HVP, many factors in addition to flow dynamics, such as the testing fluid, animal species, and anticoagulation method play a key role. These parameters vary widely among different research groups, and their impact on experimental results may not always have been regarded closely. In addition to human blood, regularly used testing fluids are porcine and bovine blood as well as nonsanguineous liquids such as enzyme-activated milk [5]. The use of blood in an artificial circuit usually requires anticoagulation. Different anticoagulants such as citrate or heparin have been commonly used in experimental settings.

The present *in vitro* study aimed to investigate the use of unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) in reference to blood anticoagulation by citrate, using porcine blood to determine their applicability in preclinical thrombogenicity testing. Additionally, porcine blood from the abattoir was compared with blood obtained from laboratory swine to determine the effect of a possible preactivation of the abattoir-derived blood.

Materials and methods

A simplified experimental setting was employed to simulate different degrees of clotting activation using discriminative material specimens. The experiment was designed to evaluate which of the reviewed anticoagulants is more suitable during *in vitro* thrombogenicity testing and to assess whether the conditions of blood withdrawal affect blood quality and coagulation behavior.

Anticoagulants

The selection of anticoagulants was based on current clinical guidelines [6, 7]. UFH (heparine-sodium-25000-ratiopharm® [ratiopharm GmbH, Ulm, Germany]) and LMWH (enoxaparin sodium Clexane® multidose [Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany]) were compared with a reference of citrate (3.13% trisodium citrate solution Eifelfango® [Eifelfango, Bad Neuenahr-Ahrweiler, Germany]).

Blood

Taking account of comparability with animal trials and clinical data, ethical aspects, availability, and costs, human blood was abandoned as a testing fluid and replaced by porcine blood, which has similar hemostatic properties to human blood [8].

Blood samples were obtained from Duroc and Piétrain pigs at the abattoir by exsanguination. The blood was collected in 1000 mL polyethylene bottles prepared with 100 mL of anticoagulant solution, resulting in 1300 IU/L of UFH, 2000 IU/L of LMWH and 0.313% citrate, which had comparable anticoagulant effects. Each bottle contained blood from a single animal and was capped when full to the brim without entrapped of air. The temperature was maintained at about 37°C during transport with the use of an insulated container.

Additional blood samples were obtained from three laboratory animals (crossbred German Landrace × Piètrain), that were under general anesthesia for other experimental research at the time of blood sampling via a central venous line. The blood was collected in three 50mL syringes, which were prepared with 5 mL of an anticoagulant solution, resulting in equivalent dosages to the abattoir-derived blood.

Material specimens

To simulate different degrees of clotting activation, small tubes (height 30 mm, diameter 10 mm, wall thickness 2 mm) of either glass or acrylic glass (polymethylmethacrylate, PMMA) were used. Whereas glass is known to be a strong initiator of coagulation processes [9, 10], PMMA does not show substantial thrombocyte or coagulation activation [11].

Experimental protocol

Six stagnant testing chambers (polypropylene tubes of 75 mm height and 13 mm diameter)

Table 1. Baseline mean values ± SD of coagulation parameters from abattoir porcine blood and their respective *p*-values

Parameter	Citrate		UFH		LMWH		Citrate vs. UFH	Citrate vs. LMWH	UFH vs. LMWH
	Mean	SD	Mean	SD	Mean	SD	*p*-value	*p*-value	*p*-value
aPTT (s)	13.43	1.25	66.76	22.92	34.49	5.90	**< 0.0001**	**< 0.001**	**< 0.0001**
Fibrinogen time (s)	11.17	2.89	13.96	3.77	14.57	1.70	0.063	**0.018**	0.872
PT (s)	8.41	0.88	8.09	0.44	8.29	0.54	0.304	0.846	0.621
Thrombocytes (10^3/µL)	257.8	66.6	216.5	33.7	228.0	49.4	0.217	0.496	0.845

Boldface indicates a significant difference (P < 0.05). aPTT, activated partial thromboplastin time; LMWH, low-molecular-weight heparin; PT, prothrombin time; SD, standard deviation; UFH, unfractionated heparin.

were each filled with 3 mL of porcine blood anticoagulated by either UFH, LMWH, or citrate. Two chambers of each anticoagulant were closed without an additional material specimen for comparison. Another two chambers of each anticoagulant were supplemented with either glass or PMMA specimen and subsequently closed. All chambers were placed inside a warming cabinet to maintain temperatures between 36.8°C and 37.3°C. Every 30 minutes, all chambers were carefully swiveled to ensure homogeneous distribution and counteract sedimentation in this static setting. After a total duration of 150 minutes, the material specimens were extracted. The remaining blood was withdrawn, and in case of prior heparinization, citrate was added to prevent ongoing coagulation until laboratory tests were performed. Primarily citrated blood was unaltered and used for further testing.

Laboratory tests

Differential blood count was determined by the automated hematology analyzer Celltac α (Nihon Kohden, Tokyo, Japan) with its original reagent solutions and settings for porcine blood.

Further clotting tests were performed with thrombocyte-depleted citrated plasma, which was obtained by double centrifugation at 1500 relative centrifugal force (rcf) at room temperature for 15 minutes. All analyses were performed on the semiautomatic ball coagulometer MC10 (MERLIN medical® ABW Medizin und Technik GmbH, Lemgo, Germany) with test cuvettes and balls from Tcoag Ireland Limited (Dublin, Ireland) and the following reagents from Siemens Healthcare Diagnostics Products GmbH (Marburg, Germany).

Activated partial thromboplastin time (aPTT) was determined with the use of Dade Actin FS and calcium chloride solution.

Prothrombin time (PT) was measured with the use of Dade Innovin. Because prior calibration with a range of porcine standard plasma was not applicable, time measurements were not converted into prothrombin ratio or international normalized ratio.

Fibrinogen was identified with the use of Dade Thrombin and Owren's Veronal Buffer. Because translation into fibrinogen concentration was not applicable due to required porcine standard calibration, we refer to the coagulometric measured fibrinogen times.

Data collection and statistics

Data were collected manually using the spreadsheet application Numbers (Apple, Cupertino, CA, USA). Sample size was determined by two-way analysis of variance (ANOVA) with repeated measures based on three pilot tests with a significance level $\alpha = 0.05$ and a test power $1-\beta = 0.8$. Calculations were performed with statistical software SAS Version 9.2 (Statistical Analysis System, SAS Institute, Cary, NC, USA). Including the minimum sample size for three out of four variables as well as dropout of up to two measurements, a sample size of $n = 11$ was determined. Statistical analyses were performed with Prism 7 (GraphPad Software, La Jolla, CA, USA). Analysis of continuous variables was based on two-way ANOVA with repeated measures to a level of significance $P < 0.05$.

Results

Baseline measurements

For porcine blood from the abattoir, the three anticoagulants showed generally homogeneous baseline values of performed clotting analyses, with the exception of aPTT for all three anticoagulants and fibrinogen time for the comparison of citrate and LMWH (**Table 1**).

Table 2. Baseline mean values ± SD of blood count and coagulation parameters from abattoir and laboratory porcine blood and their respective *p*-values

Parameter	Citrate		UFH		LMWH		LAB		LAB vs. citrate	LAB vs. UFH	LAB vs. LMWH
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*p*-value	*p*-value	*p*-value
Leukocytes (10^3/µL)	22.49	6.90	18.71	4.06	16.70	4.35	13.99	0.42	**0.007**	0.436	0.616
Erythrocytes (10^6/µL)	6.79	1.21	6.64	0.47	6.61	0.57	4.36	0.19	**< 0.0001**	**< 0.0001**	**< 0.0001**
Hemoglobin (g/dL)	12.14	2.12	11.63	0.77	11.95	0.85	7.50	0.38	**< 0.0001**	**< 0.0001**	**< 0.0001**
Hematocrit (%)	36.30	5.76	35.09	2.02	36.14	2.40	22.94	0.98	**< 0.0001**	**< 0.0001**	**< 0.0001**
MCV (fL)	53.78	3.49	52.95	2.63	54.90	4.40	52.67	0.07	0.932	0.997	0.175
Thrombocytes (10^3/µL)	257.8	66.6	216.5	33.7	228.0	49.4	181.8	3.7	**< 0.001**	0.186	0.184
Fibrinogen time (s)	11.17	2.89	13.96	3.77	14.57	1.70	18.37	3.08	**< 0.0001**	**0.003**	**0.012**

Because of the lower sample size of n = 3 of laboratory animals these measurements were not subdivided into the different anticoagulant groups. Therefore, only non-anticoagulant dependent parameters are shown. The columns referring to particular anticoagulants include only abattoir-obtained blood. Boldface indicates a significant difference (P < 0.05). LAB, laboratory-derived porcine blood; LMWH, low-molecular-weight heparin; MCV, mean corpuscular volume; SD, standard deviation; UFH, unfractionated heparin.

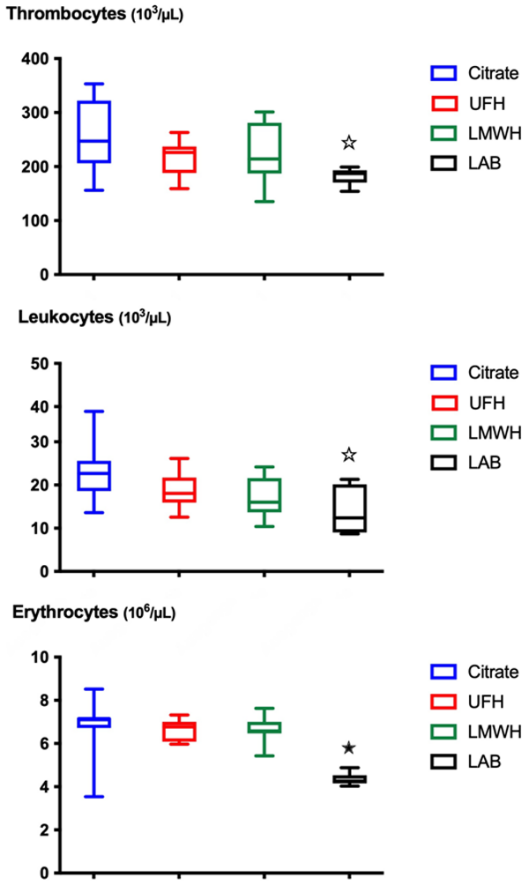

Figure 1. Distribution of cell count measurements from abattoir-obtained (separated per anticoagulant) and laboratory-obtained porcine blood (composition of all anticoagulant groups); boxes outline median, first quartile, and third quartile and the whiskers indicate minimum and maximum; black stars indicate a significant difference from all groups of abattoir-obtained blood, and white stars indicate a significant difference only from citrated blood. LAB, laboratory-derived porcine blood; LMWH, low-molecular-weight heparin; UFH, unfractionated heparin.

The initial hematologic and coagulation parameters revealed distinct differences between blood from laboratory swine and pigs from the abattoir (**Table 2**). Compared with abattoir-obtained blood, erythrocyte count (4.36 ± 0.19 × 10^6/µL; P < 0.0001), hemoglobin (7.50 ± 0.38 g/dL; P < 0.0001), and hematocrit (22.94 ± 0.98%; P < 0.0001) were significantly reduced in blood from laboratory swine, whereas fibrinogen time (18.37 ± 3.08 s) was significantly higher (P ranging from < 0.0001 to 0.012). Leukocyte (13.99 ± 0.42 × 10^3/µL) and thrombocyte (181.8 ± 3.7 × 10^3/µL) mean values were also reduced in blood from laboratory swine, but were significantly reduced only compared with citrated abattoir-derived blood (leukocytes P = 0.007, thrombocytes P < 0.001).

Overall, the data from laboratory-derived blood were less variable (**Figure 1**, box plots for cell counts).

Measurements after exposure to different clotting-activating materials

Figure 2 illustrates all coagulation parameters from abattoir- and laboratory-obtained porcine blood. Citrated blood showed nearly constant measured values, with the sole exception of PT in abattoir-obtained blood, which declined significantly in all experimental approaches (baseline 8.41 ± 0.88 s; glass 7.92 ± 0.43 s [p = 0.004]; PMMA 7.78 ± 0.45 s [P < 0.001]; no specimen 7.79 ± 0.41 s [P < 0.001]).

UFH-anticoagulated blood showed significant decreases in aPTT, PT, and thrombocyte count, which were greatest in the approaches using a

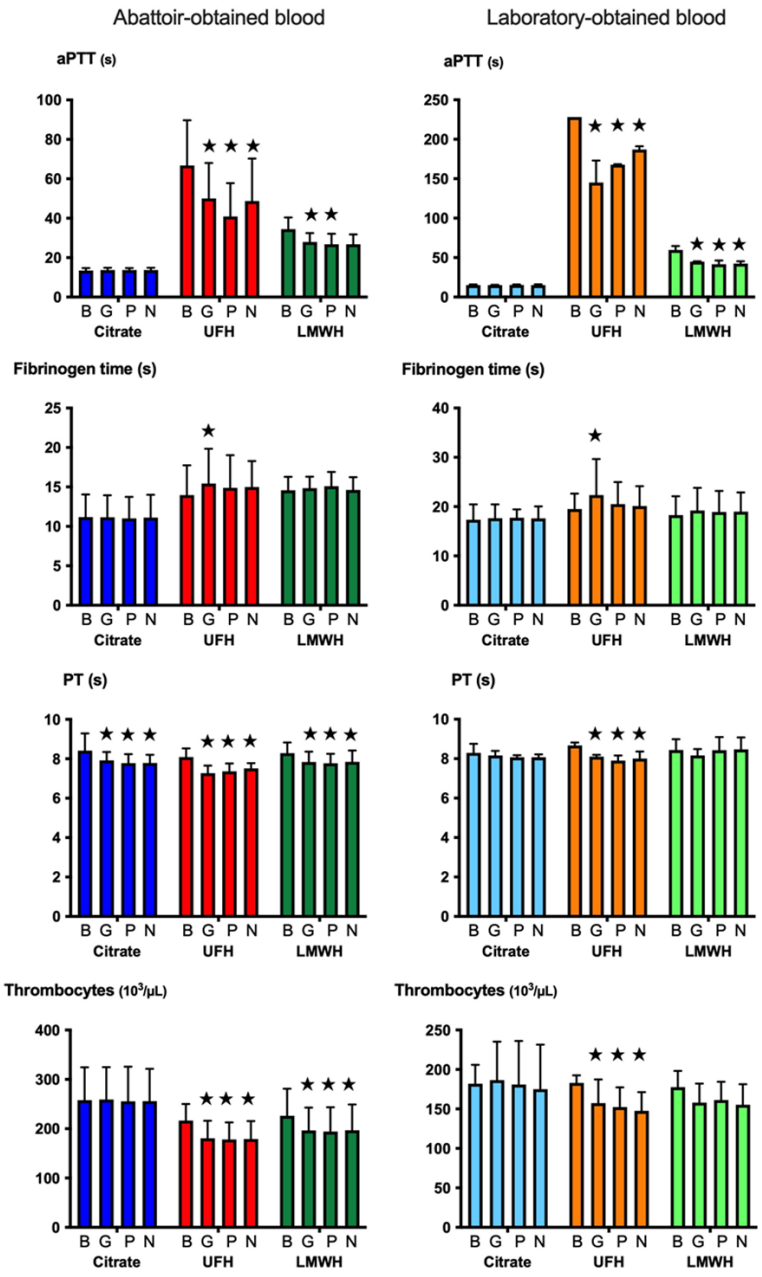

Figure 2. Coagulation parameters from abattoir-obtained and laboratory-obtained porcine blood (attention must be paid to the different y-scalings). The four columns per anticoagulant represent the different experimental approaches: baseline (B), glass (G), PMMA (P), and no material specimen added (N). Each column shows the mean value, whiskers show the standard deviation, and black stars indicate significant changes compared with baseline measurements. aPTT, activated partial thromboplastin time; LMWH, low-molecular-weight heparin; PMMA, polymethylmethacrylate; PT, prothrombin time; UFH, unfractionated heparin.

glass specimen (aPTT: baseline 66.76 ± 22.92 s, glass 47.03 ± 20.05 s [P < 0.0001]; PT: baseline 8.09 ± 0.44 s, glass 7.27 ± 0.38 s [P < 0.0001]; thrombocytes: baseline 216.5 ± 33.7 × 10^3/µL, glass 178.3 ± 34.5 × 10^3/µL [P < 0.0001]). Fibrinogen time was significantly prolonged with the application of glass as well (baseline 13.96 ± 3.77 s, glass 15.43 ± 4.42 s [P = 0.002]). Similar results were obtained with blood from laboratory swine.

Abattoir-derived, LMWH-anticoagulated porcine blood showed significant declines in aPTT, PT, and thrombocyte count with the application of glass and PMMA (aPTT: baseline 34.49 ± 5.90 s, glass 26.80 ± 5.29 s [P = 0.048], PMMA 26.80 ± 4.99 s [P = 0.048]; PT: baseline 8.29 ± 0.54 s, glass 7.84 ± 0.53 s [P = 0.008], PMMA 7.77 ± 0.48 s [P = 0.002]; thrombocyte count: baseline 228.0 ± 49.4 × 10^3/µL, glass 195.6 ± 46.7 × 10^3/µL [P < 0.0001], PMMA 197.2 ± 49.2 × 10^3/µL [P < 0.0001]). Fibrinogen time increased slightly. Similar results were obtained with blood from laboratory swine, in which only decrease in aPTT reached statistical significance (P < 0.0001).

Discussion

Swine show substantial similarities in anatomy, size and physiology to humans [12]. Therefore, the pig is well accepted as a model for cardiovascular, metabolic and neurological disease research. Despite numerous successful implementations of the porcine model in cardiovascular surgical research [13-15], profound limitations have been described as well. Long term adequate anticoagulation with coumarins proved to be quite challenging in pigs with mechanical valves and resulted in major hemorrhagic complications

Table 3. Selected blood parameters of porcine species from literature references

Author	Grabowski et al. 1977 [32]		Klaus et al. 2002 [8]	Kixmöller 2004 [19]				Höhle 2000 [33]		Müller et al. 2001 [21]		Roussi et al. 1996 [20]	
Pig breed	-		-	Piétrain		Duroc		GLxGE		-		Large White (Yorkshire)	
Parameter	Mean	SD	-	Mean	SD	Mean	SD	Mean	95%-RR	Mean	SD	Mean	SD
Leucocytes (10^3/μL)	21.7*	4.8*	7-20*	20.3	3.1	20.1	3.0						
Erythrocytes (10^6/μL)			6.4*	7.03	0.59	6.89	0.60						
Hemoglobin (g/dL)			13.7	12.56*	0.97*	12.08*	0.81*						
Hematocrit (%)	32.0	0.9	39	37	0.3	37	0.3						
Thrombocytes (10^3/μL)	497	180	220-620*	526	161	381	100			343*	88*		
Fibrinogen (g/L)								2.01*	1.33-2.68*	1.3	0.2	1.73	0.18
aPTT (s)								22	11.7-32.3	17	0.6	23.7	0.3
INR										1	0		

*Data converted into standardized dimensions. Pig breeds are according to original reference. aPTT, activated partial thromboplastin time; GLxGE, crossbred German Landrace × German Edelschwein; INR, international normalized ratio; RR, reference range; SD, standard deviation.

[16]. Furthermore, the physiologically rapid growth of the animals caused increased paravalvular leaks, thus limiting long-term study design [17]. Additionally, the use of porcine blood during *in vitro* testing revealed considerable and unpredictable preactivation of clotting processes in abattoir-obtained blood samples [18].

This inhomogeneity among abattoir-derived porcine blood is consistent with our findings. Baseline measurements of thrombocyte count in citrated blood showed a standard deviation of 25.85% of mean value (15.54% in UFH-anticoagulated and 21.69% in LMWH-anticoagulated blood), whereas corresponding analyses in laboratory swine demonstrated more stable measurements that varied by only about 2.01%.

Potential causes of the observed differences in variation of baseline measurements include the technique of blood withdrawal, since exsanguination at the abattoir facilitates tissue contact and thus induction of clotting activation. Moreover, physical and psychological stress during transportation and procedures at the abattoir has been proven to influence blood quality in swine [19]. Differences in the temperature and duration of animal transport might facilitate dehydration and thus alteration of blood scores.

However, the mean values of abattoir-derived blood in our study were all within published normal ranges (**Table 3**), whereas hematocrit, hemoglobin, and platelet count in blood derived from laboratory animals were clearly lower (**Table 2**) and exceeded the lower normal range in the case of hemoglobin and platelet count (**Table 3**).

Preoperative treatment or anesthesia of the laboratory animals could have affected their blood values, although an effect of general anesthesia on coagulation has not been proven in published studies [20]. Although general anesthesia itself may have no impact on blood scores, the often associated fluid management could very well influence hematologic parameters. Our blood samples from laboratory swine were acquired through a central venous line. This technique of withdrawal can be prone to errors, such as dilution of blood samples from previous or simultaneous fluid administration through a more distal portion of the same intravenous line.

The exclusion of strongly deviant and nonphysiological blood samples would improve the homogeneity of our baseline measurements. Any hypercoagulable state (for example, due to prior clotting activation) or any lack of coagulation components (such as in thrombocytopenia or hypofibrinogenemia) will greatly influence the experimental course and results. Our analysis strongly underlines the need for a pre-experimental quality inspection of porcine blood in the evaluation of thrombogenicity, which has now been implemented in our laboratory routine.

Additionally, methods of laboratory analysis might impact measurement results. Because

routine test systems are implemented for use in humans, their transfer to different species remains arguable. Investigation of thrombogenicity during extracorporeal circulation in pigs and calves showed reliable analyses of coagulation with the use of standard diagnostics in pigs but not in calves [21]. However, the use of different methods and reacting thromboplastins led to unequal prothrombin times in swine [22]. By using the same analytical methods for all our blood samples, comparisons between samples should be feasible, whereas transferring our results to other studies requires caution regarding analytical limitations.

Furthermore, the characteristics of the swine used can affect blood measurements. Our investigations and published studies (**Table 3**) showed wide ranges of variation within coagulation and hematologic parameters. Differences in breed and age can influence blood count and clotting parameters [19, 23]. In preclinical evaluation of thrombogenicity, as well as in our study, different pig breeds have been used, and sometimes no explicit information regarding breed or age is given, thus precluding reliable comparisons.

Evaluation of interactions between cardiovascular devices and their recipients' blood usually requires anticoagulation. Citrate and UFH are commonly used as anticoagulating agents [24-26]. Recently, LMWH has become increasingly popular for *in vitro* tests and animal studies [18, 27] because of its simplified dosage and application as well as its low rate of undesirable interactions with thrombocytes [28]. Although LMWH has not yet been approved for patients with HVP by recent European guidelines [29], off-label use allows for limited application.

As expected, our experiments with citrated blood, using both abattoir- and laboratory-obtained blood samples, showed nearly unchanging measurements after material exposure compared with baseline values. Citrate reversibly binds to calcium ions in the blood, thereby efficiently inhibiting the plasmatic coagulation cascade. Therefore, citrate serves well as a negative control reference, since coagulation is blocked entirely and can be reversed by calcium supplementation, thus allowing for laboratory coagulation analysis. As anticipated, a discrimination of clotting initiation by the use of different material samples, that are known to cause strong (glass) or no (PMMA) coagulation of blood, was not feasible in our experimental setup.

UFH-anticoagulated blood allowed quite accurate discrimination of graduated coagulation. Our measurements indicated the consumption of thrombocytes and fibrinogen (by prolonged fibrinogen time) as well as clotting activation during exposure to clotting activating materials by shortening of aPTT and PT compared with baseline in laboratory analyses of abattoir- and laboratory-derived porcine blood. These findings were most pronounced with exposure to glass, followed by PMMA, although the differences between these materials were not themselves significant. Even with heparinization, the presence of a strong activator could induce outright thrombosis. Therefore, UFH can be considered suitable for evaluation of thrombogenicity as it enables graduation of coagulant activity.

LMWH has been rarely used for *in vitro* investigations of thrombogenicity [20], although it is increasingly favored in clinical practice and animal trials [30]. LMWH predominantly inhibits factor Xa, commits to fewer unspecific (antithrombin-3-independent) bindings and therefore has less impact on platelet function than UFH [31]. Our experiments with blood from the abattoir showed significant consumption of thrombocytes and shortening of PT and aPTT (only with the use of glass or PMMA), but no reliable distinction between the material specimens could be made. Blood derived from laboratory animals only showed a significant shortening of aPTT from baseline measurements.

Both types of heparin provided sufficient anticoagulation while still enabling measurable coagulation processes in our experimental design. Although several parameters changed significantly compared with baseline measurements, direct comparison of material samples did not show outstanding differences. The presumed benefit of fewer interactions of platelets with LMWH was not verified in our experiments. Therefore, no definite preference for one type of heparin could be substantiated.

Our experimental setup itself might also influence coagulation analyses. Measurement changes in our testing chambers without mate-

rial samples could indicate possible influences of stasis, time, and chamber surface.

Although hemostasis and thrombosis are highly dynamic processes *in vivo*, our simplified static experimental setting can only record material- and surface-induced thrombogenicity and excludes flow-induced thrombus formation. Moreover, the multitude of participating and interacting biochemical processes *in vivo* can never be fully represented in an *in vitro* model. The functions of thrombocytes, plasmatic coagulation, tissue factors, endothelium, and blood flow are manifoldly intertwined, thus limiting *in vitro* evaluation of thrombogenicity in highly simplified models.

The testing chambers were made of polypropylene, which despite its biocompatibility might slightly activate coagulation itself, thereby obscuring subtle differences between the procoagulant effects of glass and PMMA. Furthermore, the experimental duration of 150 minutes might limit coagulation processes.

Nevertheless, our experiments can contribute to an improvement of preclinical evaluation of thrombogenicity by emphasizing the impact of blood quality and anticoagulant agents on test results.

In conclusion, we demonstrated the general applicability of porcine blood in thrombogenicity testing, although special caution is required to standardize blood withdrawal methods and to eliminate preanalytical interference. Pre-experimental quality control of blood samples and exclusion of unphysiological deviates will greatly contribute to reliable test conditions. Although both UFH and LMWH seem to be suitable for *in vitro* evaluation of thrombogenicity, no distinct advantage of either type of heparin was detected in our simplified, static experimental setup. Further investigations, including different animal species and anticoagulant agents, are required to improve the validity of *in vitro* thrombogenicity testing methods.

Acknowledgements

Helmholtz Institute of Applied Medical Engineering, RWTH Aachen University, Pauwelsstraße 20, D-52074 Aachen. Department of Thoracic and Cardiovascular Surgery, University Hospital RWTH Aachen, Pauwelsstraße 30, D-52074 Aachen. Institute of Laboratory Animal Science, University Hospital RWTH Aachen, Pauwelsstraße 30, D-52074 Aachen.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Marjolijn Sales, Department of Thoracic and Cardiovascular Surgery, RWTH University Hospital, Pauwelsstr. 30, 52074 Aachen, Germany. Tel: +49 241 80-89221; Fax: +49 241 80-82454; E-mail: mguemmer@ukaachen.de

References

[1] Cannegieter SC, Rosendaal FR and Briet E. Thromboembolic and bleeding complications in patients with mechanical heart valve prostheses. Circulation 1994; 89: 635-641.

[2] Harken DE. Heart valves: ten commandments and still counting. Ann Thorac Surg 1989; 48: S18-19.

[3] DIN EN ISO 5840-2:2016-05. Herz- und Gefäßimplantate - Herzklappenprothesen - Teil 2: Chirurgisch implantierter Herzklappenersatz. Beuth Verlag GmbH, 2015.

[4] Linde T, Clauser J, Meuris B and Steinseifer U. Assessing the thrombogenic potential of heart valve prostheses: an approach for a standardized in-vitro method. Cardiovasc Eng Technol 2019; 10: 216-224.

[5] Scharfschwerdt M, Thomschke M and Sievers HH. In-vitro localization of initial flow-induced thrombus formation in bileaflet mechanical heart valves. ASAIO J 2009; 55: 19-23.

[6] Nishimura RA, Otto CM, Bonow RO, Carabello BA, Erwin JP 3rd, Guyton RA, O'Gara PT, Ruiz CE, Skubas NJ, Sorajja P, Sundt TM 3rd and Thomas JD; American College of Cardiology/American Heart Association Task Force on Practice Guidelines. 2014 AHA/ACC guideline for the management of patients with valvular heart disease: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 2014; 63: e57-185.

[7] Nishimura RA, Otto CM, Bonow RO, Carabello BA, Erwin JP 3rd, Fleisher LA, Jneid H, Mack MJ, McLeod CJ, O'Gara PT, Rigolin VH, Sundt TM 3rd and Thompson A. 2017 AHA/ACC Focused Update of the 2014 AHA/ACC guideline for the management of patients with valvular heart disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. J Am Coll Cardiol 2017; 70: 252-289.

[8] Klaus S, Korfer S, Mottaghy K, Reul H and Glasmacher B. In vitro blood damage by high shear flow: human versus porcine blood. Int J Artif Organs 2002; 25: 306-312.

[9] Kratz A, Stanganelli N and Van Cott EM. A comparison of glass and plastic blood collection tubes for routine and specialized coagulation assays: a comprehensive study. Arch Pathol Lab Med 2006; 130: 39-44.

[10] Rapaport SI, Vermylen J, Hoylaerts M, Saito H, Hirsh J, Bates S, Dahlback B and Poller L. The multiple faces of the partial thromboplastin time APTT. J Thromb Haemost 2004; 2: 2250-2259.

[11] Coli L, Cianciolo G, Feliciangeli G, De Sanctis LB, Zanchelli F, Dalmastri V, De Pascalis A, Sestigiani E, Donati G and Stefoni S. Anticoagulation and platelet activation in hemodialysis: clinical results with PMMA. Contrib Nephrol 1999; 125: 111-119.

[12] Gallegos RP, Nockel PJ, Rivard AL and Bianco RW. The current state of in-vivo pre-clinical animal models for heart valve evaluation. J Heart Valve Dis 2005; 14: 423-432.

[13] Kim CH, Steinseifer U and Schmitz-Rode T. Thrombogenic evaluation of two mechanical heart valve prostheses using a new in-vitro test system. J Heart Valve Dis 2009; 18: 207-213.

[14] Lester PA, Coleman DM, Diaz JA, Jackson TO, Hawley AE, Mathues AR, Grant BT, Knabb RM, Ramacciotti E, Frost CE, Song Y, Wakefield TW and Myers DD Jr. Apixaban versus warfarin for mechanical heart valve thromboprophylaxis in a swine aortic heterotopic valve model. Arterioscler Thromb Vasc Biol 2017; 37: 942-948.

[15] Tuzun E, Pennings K, van Tuijl S, de Hart J, Stijnen M, van de Vosse F, de Mol B and Rutten M. Assessment of aortic valve pressure overload and leaflet functions in an ex vivo beating heart loaded with a continuous flow cardiac assist device. Eur J Cardiothorac Surg 2014; 45: 377-383.

[16] Schomburg JL, Medina EM, Lahti MT and Bianco RW. Dabigatran versus warfarin after mechanical mitral valve replacement in the swine model. J Invest Surg 2012; 25: 150-155.

[17] Grehan JF, Hilbert SL, Ferrans VJ, Droel JS, Salerno CT and Bianco RW. Development and evaluation of a swine model to assess the preclinical safety of mechanical heart valves. J Heart Valve Dis 2000; 9: 710-719; discussion 719-720.

[18] Linde T. Experimentelle und numerische Evaluierung thrombosegefährdeter Bereiche in Herzklappenprothesen: Rheinisch-Westfälische Technische Hochschule Aachen. 2016.

[19] Kixmöller M. Labordiagnostische Referenzbereiche bei unterschiedlichen Schweinerassen sowie histopathologische und immunhistochemische Untersuchung von Gehirnen älterer Sauen und Eber auf transmissible spongiforme Enzephalopathie im Rahmen der TSE-Studie: Ludwig-Maximilians-Universität München. 2004.

[20] Roussi J, Andre P, Samama M, Pignaud G, Bonneau M, Laporte A and Drouet L. Platelet functions and haemostasis parameters in pigs: absence of side effects of a procedure of general anaesthesia. Thromb Res 1996; 81: 297-305.

[21] Mueller XM, Tevaearai HT, Jegger D, Tucker O and von Segesser LK. Are standard human coagulation tests suitable in pigs and calves during extracorporeal circulation? Artif Organs 2001; 25: 579-584.

[22] McGlasson DL, Brickey DA and Doe RH. Oral anticoagulant therapy and international normalized ratios in swine. Lab Anim Sci 1998; 48: 371-373.

[23] Pliszczak-Krol A, Rzasa A, Gemra M, Krol J, Luczak G, Zyzak A, Zalewski D, Iwaszko-Simonik A and Graczyk S. Age-related changes of platelet and plasma coagulation parameters in young pigs. J Vet Diagn Invest 2016; 28: 561-567.

[24] Mueller MR, Schima H, Engelhardt H, Salat A, Olsen DB, Losert U and Wolner E. In vitro hematological testing of rotary blood pumps: remarks on standardization and data interpretation. Artif Organs 1993; 17: 103-110.

[25] Linde T, Hamilton KF, Navalon EC, Schmitz-Rode T and Steinseifer U. Aortic root compliance influences hemolysis in mechanical heart valve prostheses: an in-vitro study. Int J Artif Organs 2012; 35: 495-502.

[26] Gregoric ID, Eya K, Tamez D, Cervera R, Byler D, Conger J, Tuzun E, Chee HK, Clubb FJ, Kadipasaoglu K and Frazier OH. Preclinical hemodynamic assessment of a new trileaflet mechanical valve in the aortic position in a bovine model. J Heart Valve Dis 2004; 13: 254-259.

[27] McKellar SH, Abel S, Camp CL, Suri RM, Ereth MH and Schaff HV. Effectiveness of dabigatran etexilate for thromboprophylaxis of mechanical heart valves. J Thorac Cardiovasc Surg 2011; 141: 1410-1416.

[28] Salzman EW, Rosenberg RD, Smith MH, Lindon JN and Favreau L. Effect of heparin and heparin fractions on platelet aggregation. J Clin Invest 1980; 65: 64-73.

[29] Baumgartner H, Falk V, Bax JJ, De Bonis M, Hamm C, Holm PJ, Iung B, Lancellotti P, Lansac E, Rodriguez Munoz D, Rosenhek R, Sjogren J, Tornos Mas P, Vahanian A, Walther T, Wendler O, Windecker S and Zamorano JL; ESC Scientific Document Group. 2017 ESC/EACTS Guidelines for the management of valvular heart disease. Eur Heart J 2017; 38: 2739-2791.

[30] McKellar SH, Thompson JL 3rd, Garcia-Rinaldi RF, Macdonald RJ, Sundt TM 3rd and Schaff HV. Short- and long-term efficacy of aspirin and clopidogrel for thromboprophylaxis for mechanical heart valves: an in vivo study in swine.

J Thorac Cardiovasc Surg 2008; 136: 908-914.

[31] Hirsh J, Warkentin TE, Shaughnessy SG, Anand SS, Halperin JL, Raschke R, Granger C, Ohman EM and Dalen JE. Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. Chest 2001; 119: 64S-94S.

[32] Grabowski EF, Didisheim P, Lewis JC, Franta JT and Stropp JQ. Platelet adhesion to foreign surfaces under controlled conditions of whole blood flow: human vs rabbit, dog, calf, sheep, pig, macaque, and baboon. Trans Am Soc Artif Intern Organs 1977; 23: 141-151.

[33] Hoehle P. Zur Übertragbarkeit tierexperimenteller endovaskulärer Studien: Unterschiede der Gerinnungs- und Fibrinolyse-Systeme bei häufig verwendeten Tierspezies im Vergleich zum Menschen: Rheinisch-Westfälische Technische Hochschule Aachen. 2000.