Interspecies differences in coagulation profile

Jolanta M. Siller-Matula¹, Roberto Plasenzotti², Alexander Spiel¹, Peter Quehenberger³, Bernd Jilma¹

¹Department of Clinical Pharmacology, Medical University of Vienna, Austria; ²Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Austria; ³Department of Biomedical Research, Medical University of Vienna, Austria

Summary

Many animals are used in research on blood coagulation and fibrinolysis, but the relevance of animal models to human health is often questioned because of differences between species. The objective was to find an appropriate animal species, which mimics the coagulation profile in humans most adequately. Species differences in the coagulation profile with and without thrombin stimulation in vitro were assessed in whole blood by Rotation Thromboelastometry (ROTEM). Endogenous thrombin generation was measured in platelet-poor plasma. Measurements were performed in blood from five different species: humans, rats, pigs, sheep and rabbits. In humans and sheep, the clotting time (ROTEM) was in the same range with or without thrombin stimulation and a 100-fold lower dose of thrombin (0.002 IU) was required to cause a shortening in the clotting time as compared to rats, pigs and rabbits (0.2 IU) (p<0.05). Similarly, the endogenous thrombin potential (ETP) was in the same range in humans and sheep. The maximum clot firmness with or without thrombin stimulation was similar in rabbits and humans. The maximum lysis with or without thrombin stimulation was similar in humans and pigs. Significant species differences exist in the coagulation profile with or without thrombin stimulation. Most importantly, sheep had a clotting time most similar to humans and could thus be a suitable species for translational coagulation studies. Moreover, our findings confirm the potential usefulness of pigs as an experimental species to study fibrinolytic pathway and support the usefulness of rabbits as a species for examining platelets.

Keywords
Clotting time, endogenous thrombin potential, species differences, thrombin

Introduction

Most basic research in the field of haemostasis is conducted in animal models. However, the relevance of animal models to human health is often questioned because of differences between species (1). Accordingly, the lack of concordance between animal experiments and clinical trials has been reported and may be due to the failure of animal models to adequately represent human disease (1). In respect to translational research it is relevant which species are comparable to human. For instance, it is not yet known which animal species mimics the coagulation profile in humans most adequately. To address this question, we compared the thrombelastographic properties of clot formation and lysis of blood from five different species in vitro: humans, rats, sheep, pigs and rabbits. The second purpose of the study was a cross-species comparison of the endogenous thrombin generation, fibrinogen concentration, prothrombin time (PT), activated partial thromboplastin time (APTT) and platelet counts (PLT).

Materials and methods

Materials

Tubes with 3.8% citrate (BD Vacutainer; Becton Dickinson) were used for blood collection. Human thrombin (Tissucol Duo Quick®) was obtained from Baxter AG (Vienna, Austria). Start-TEM was purchased from Nobis (Endingen, Germany). Technothrombin and Normotest assays were obtained from Technoclone (Vienna, Austria). Fibrinogen and activated partial thromboplastin time assays were purchased from Diagnostica Stago (Roche, Vienna, Austria).

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Blood sampling
Blood samples from six animals of each species were obtained using a 21-gauge needle. Human venous blood was collected from an antecubital vein. In sheep (female Austrian mountain sheep, Department of Biomedical Research, Himberg, Austria) the jugular vein was used for blood collection, an ear vein was punctured in pigs (Austrian Landrace pig, Veterinary University of Vienna, Austria) and rabbits (New Zealand White, Charles River Laboratories, Wilmington, MA, USA). Blood from rats (male Sprague Dawley rats, Department of Biomedical Research, Himberg, Austria) was obtained by terminal heart puncture. The study was performed in accordance with the institutional guidance of the Ethics Committee of the Medical University of Vienna and with the Ethics Committee and Government on animal experimentation of Austria (Vienna). Volunteers provided written informed consent.

ROTEM, modified Rotation Thromboelastometry Analyzer
The ROTEM, Rotation Thromboelastometry Analyzer (Pentapharm, Munich, Germany) gives a graphic representation of clot formation and subsequent lysis. Blood is incubated at 37°C in a heated cup. As fibrin forms between the cup and the pin, the impedance of the rotation of the pin is detected and the trace is generated (2). The whole blood samples were collected into 3.8% sodium citrate tubes. Measurements were performed within one hour after blood sampling. Before running the assay, citrated blood samples were recalcified with 20 µl of 0.2 M CaCl2 (Start-TEM) and the NATEM test (a test without any activator = non-activated TEM) was started (3). The following parameters were analyzed: the clotting time (CT), the clot formation time (CFT), the maximum clot firmness (MCF), the maximum lysis (ML) and the alpha angle (αl). The clotting time (CT) characterises the period from analysis start until the start of clot formation. The clot formation time (CFT) describes the subsequent period until the amplitude of 20 mm is reached. The CT and CFT represent the activation and dynamics of clot formation (4). The maximum lysis (ML) represents the maximum fibrinolysis detected during the analysis (4). The maximum clot firmness (MCF) gives information about the clot strength and stability. MCF is largely dependent on fibrinogen and platelets and describes platelet function (5).

The endogenous thrombin potential (ETP)
The endogenous thrombin potential (ETP) was measured with the Technothrombin fluorogenic assay (Technoclone Vienna, Austria). Technothrombin is a thrombin generation assay based on the monitoring of the formation of thrombin in platelet-poor plasma by means of a fluorogenic substrate upon activation of the coagulation cascade by tissue factor (low concentration of phospholipid micelles containing 17.9 pM of recombinant human tissue factor in Tris-Hepes-NaCl buffer). The reaction was monitored by use of the FLX 800™ TC Fluorometer (BioTek Instruments GmbH, Bad Friedrichshall, Germany). Readings from the fluorometer were automatically recorded and calculated by Thrombinscope software (Maastricht, The Netherlands). The ETP was calculated from the area under the thrombin generation curve (7).

Other laboratory assays
Fibrinogen concentration, prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using the KC10 Coagulometer (Amelung GmbH, Lemgo, Germany). Fibrinogen concentration was measured using the Clauss method, in which human thrombin (70 NIH U/ml) is added to diluted citrated plasma (8). In the presence of excess of thrombin, the coagulation time is inversely proportional to the fibrinogen concentration. A standard of known concentration is used to generate a standard curve, from which concentrations of fibrinogen are deduced. Prothrombin time was measured with the Normotest according to the manufacturer’s instructions. The reagent, which contains rabbit brain thromboplastin and adsorbed bovine plasma (source of factor V and fibrinogen) is added to citrated plasma and the coagulation time is measured until clot formation. The coagulation activity is expressed as percentage of normal value of an adult population. The activated partial thromboplastin time (APTT) was determined according to the Lang- dell method (9). Determination of APTT involves recalcification of plasma in the presence of a standardized amount of cephalin (platelet substitute from rabbit cerebral tissues) and source factor (Kieselgur). The time is measured from the addition of calcium ions to the formation of clot. Platelets counts were quantified with an Abbott-Cell-Dyn counter 3500 (Abbott Diagnostic Division, Vienna, Austria).

Data analysis
The primary aim of this study was to compare the coagulation profile of different species to humans. Species differences are mostly presented by descriptive statistics. Data are expressed as mean of six determinations and the standard error of mean (SEM) or median and the range. Non-parametric statistics were applied. Statistical comparisons were performed with the Friedman ANOVA, repeated measures ANOVA, the Kruskal-Wallis test, the Wilcoxon signed rank test for post-hoc comparisons and paired Wilcoxon test. A two-tailed p-value of <0.0125 was considered significant to correct for multiple comparisons between humans and other species for the primary outcome parameter (CT+CFT in ROTEM). All other p-values provided are exploratory only. All statistical calculations were performed using commercially available statistical software (SPSS Version 14.0; Chicago, IL, USA).

Results
Interspecies differences measured by ROTEM
The clotting time without thrombin stimulation in humans (595 seconds [s]) was three-fold longer than in rats (207 s) and 2.5 fold longer than in rabbits (240 s) and pigs (244 s) (p<0.05) (Table 1, Fig. 1A). The clotting time without thrombin stimulation and the sum of the clotting time and the clot formation time (CT+CFT) were comparable in humans and sheep (Table 1, Fig. 1B).

Thrombin shortened the clotting time (CT) with the half maximal effective concentration (EC50) of 0.01 IU in humans, 0.02 IU in sheep, 0.03 IU in rabbits, 0.05 IU in pigs and 0.1 IU in rats (Fig. 2). Even the lowest thrombin dose of 0.0002 IU shortened the CT by 25% in humans and sheep (Fig. 1A). A thrombin
dose of 0.02 IU decreased the CT by 90% as compared to the control in humans and sheep (p<0.05). In rats, pigs and rabbits, 0.2 IU of thrombin was required to shorten the CT (p<0.05), which was a 100-fold higher dose of thrombin as compared to humans and sheep. An increase in thrombin dose greater than 0.2 IU did not have a significant additional effect on CT in all species.

Table 1: Comparison of the rotation thromboelastometry parameters between species at baseline. Data are presented as median and range.

<table>
<thead>
<tr>
<th>Species</th>
<th>CT (s)</th>
<th>CFT (s)</th>
<th>CT+CFT (s)</th>
<th>MCF (mm)</th>
<th>ML (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>595 (476–901)</td>
<td>200 (104–436)</td>
<td>733 (642–1197)</td>
<td>58 (49–65)</td>
<td>21 (2–24)</td>
</tr>
<tr>
<td>Rat</td>
<td>207 (63–352)***</td>
<td>55 (35–97)***</td>
<td>266 (102–449)***</td>
<td>75 (70–81)***</td>
<td>8 (3–13)***</td>
</tr>
<tr>
<td>Sheep</td>
<td>494 (344–1431)</td>
<td>182 (143–532)</td>
<td>685 (503–1963)</td>
<td>72 (61–77)***</td>
<td>2 (0–26)***</td>
</tr>
<tr>
<td>Pig</td>
<td>244 (146–296)***</td>
<td>52 (30–84)***</td>
<td>298 (176–349)***</td>
<td>74 (68–79)***</td>
<td>17 (12–31)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>240 (130–613)*</td>
<td>153 (72–751)</td>
<td>396 (247–1364)</td>
<td>64 (48–76)</td>
<td>2 (0–12)***</td>
</tr>
</tbody>
</table>

Figure 1: The effect of thrombin stimulation on clotting time (CT) (A), sum of clotting time and clot formation time (CT+CFT) (B), maximal clot firmness (MCF) (C) and maximal lysis (ML) (D) in different species. Data are presented as mean±SEM. Thrombin concentrations are international units (IU)/300 µl blood.

Figure 2: Concentration-response curves of thrombin on clotting time in different species. ED₅₀=effective thrombin dose that is required for a 50% decrease in clotting time (CT). The X-axis is given as a logarithmic scale.
Maximum clot firmness (MCF) without thrombin stimulation was similar in rabbits (64 mm) and humans (58 mm) (Fig. 1C). The MCF without thrombin stimulation was in the same range in rats, sheep and pigs (72–75 mm) (Fig. 1C).

A thrombin dose of 0.06 IU caused a 20% decrease in the MCF in humans and rabbits (p<0.05). One IU of thrombin caused a reduction in MCF by 45% in humans (p=0.004) and 35% in rabbits (p=0.02). Thrombin stimulation did not alter the MCF in rats, sheep and pigs (Fig. 1C).

The median maximum lysis (ML) without thrombin stimulation was similar in humans (21%) and pigs (17%) (Fig. 1D), which was on average nine-fold higher than in rabbits and sheep (both 2%) (p<0.001) and two- to three-fold higher than in rats (7%) (p=0.017).

Thrombin stimulation did not alter the ML in any species (Fig. 1D).

Endogenous thrombin potential (ETP)
The median area under the curve (AUC) of thrombin generation in humans (4235 nM·min) and sheep (4092 nM·min) was 25% higher than in rats (3075 nM·min; p=0.042) and 50% higher than in pigs (2043 nM·min; p=0.019). The AUC in rabbits was higher than in humans and other species (6295 nM·min; p=0.02) (Fig. 3).

The lag phase of thrombin generation (from addition of tissue factor until first burst of thrombin) was more than 90% higher in humans (12 min) than in other species (p<0.05) (Fig. 3).

Coagulation parameters at baseline
The fibrinogen levels in all species were in the normal range of human (180–390 mg/dl) (Fig. 4). The median activated partial thromboplastin time (APTT) was on average >50% longer in rabbits (76 s) than in other species (p=0.005). The median platelet counts (PLT) in rats (1,180 G/l), sheep (742 G/l) and pigs (430 G/l) were six-, four- and two-fold higher than in humans (187 G/l) (p<0.001), respectively. In humans and rabbits, the platelet counts (PLT) were in the same range (160–330 G/l). The median values of prothrombin time (PT) in humans (21 s) and rabbits (20 s) were two-fold lower than in sheep (40 s) (p<0.001) (Fig. 4). The prothrombin time (PT) in rats (24 s) and pigs (23 s) was slightly higher than in humans (p<0.05).

Discussion
A nice interspecies comparison for the clotting factors and other laboratory parameters of coagulation can be found in the thesis by Hoehle (10). Our study was focused on the interspecies comparison of two functional assays: whole blood clotting as measured by ROTEM and the endogenous thrombin potential (ETP).

Interspecies differences in the clotting time (CT), maximal clot firmness (MCF) and maximal lysis (ML) without thrombin stimulation
Our data show that the clotting time (CT) was most similar in humans and sheep (Table 1, Fig. 1A). In contrast to the CT, the maximum clot firmness (MCF) was in the same range in humans and rabbits (Table 1, Fig. 1C) and the maximum lysis (ML) was similar in humans and pigs (Table 1, Fig. 1D). These data are novel because to our knowledge there are no published data on interspecies differences in the clot formation and subsequent lysis by use of the NATEM test (a test without any species-specific activator).
Interspecies differences in the thrombin effects on coagulation profile in vitro

In humans and sheep, a 100-fold lower dose of thrombin (0.002 IU) was required to shorten the clotting time (CT) as compared to rats, pigs and rabbits (0.2 IU) (Fig. 1A). Moreover, thrombin doses higher than 0.2 IU did not have a significant additional effect on the clotting time in any species. The highest dose of thrombin was chosen on the basis of a previous report, which showed shortening in the clotting time after incubation of human and pig blood with 1 IU of thrombin (11). These data are novel and provide more complete understanding of interspecies differences in the thrombin effects on coagulation in vitro, which add to previously reported species differences of thrombin effects on platelet function (12–14).

Similar to the shortening of CT by thrombin, a thrombin dose >0.06 IU caused a decrease in the maximum clot firmness (MCF) in humans and rabbits (Fig. 1C). A likely explanation for this could be that high doses of thrombin may lead to platelet aggregation and thereby decrease platelet counts, which results in a reduction of MCF. In contrast to humans and rabbits, thrombin did not cause changes in the MCF in pigs, rats and sheep, which may be due to higher doses of thrombin required. This explanation is supported by other investigators (12–14).

Moreover, this study provides evidence that thrombin did not cause changes in the maximal lysis (ML) in any species. Therefore, thrombin had no anti/fibrinolytic properties in this in-vitro assay. A possible explanation of why thrombin did not alter fibrinolysis in our experiment might be an impaired activity of the thrombin-activable fibrinolysis inhibitor (TAFI) in vitro. The physiologic activator of TAFI is the thrombin-thrombomodulin complex (15). Thrombomodulin is a transmembrane protein expressed in endothelial cells. The extracellular region of thrombomodulin is digested by proteases into diverse-sized fragments collectively called soluble thrombomodulin (16). It has been reported that TAFI can be activated in vitro in the presence of either membrane-bound thrombomodulin or recombinant soluble thrombomodulin (17). In the NATEM test, the endothelial transmembrane receptor thrombomodulin was not present. Therefore, the concentration of plasma soluble thrombomodulin in the blood is conceivably too low for activation of TAFI.

Similarities between human and sheep

The data obtained in this study indicate that sheep could be a suitable species for coagulation studies. First, humans and sheep have very similar dynamics of clot formation. The clotting times (CT) and the clot formation times (CFT), which represent the dynamics of clot formation were in the same range with or without thrombin stimulation in both species (Fig. 1). In addition, thrombin shortened the clotting time (CT) with a similar half-maximal effective concentration (EC₅₀) of 0.01 IU for humans and 0.02 IU for sheep (Fig. 2). Second, humans and sheep had very similar AUC of the endogenous thrombin potential (ETP) (Fig. 3). ETP is triggered by the addition of exogenous tissue factor and is a parameter for plasma-based hypercoagulability (18). Third, activated partial thromboplastin time (APTT) is used to measure the activity of factors of the so called intrinsic coagulation system. Comparable APTT values in humans and sheep support the con-
cept that there are no major differences in the intrinsic system between those two species (Fig. 4).

These results thus lend further credence to studies on anticoagulant drugs in sheep including studies on heparin (10, 19–21), warfarin (22) and gabexate mesilate (23). Studies examining the efficacy of heparin in sheep have shown that heparin doses were directly applicable to humans (20). However, it has been shown that the anticoagulant activities of heparins with different molecular weight differ between humans and sheep (24). In particular, the efficacy of low-molecular-weight heparins was higher as compared to human (24).

A comparison of the intrinsic and extrinsic coagulation system as well as the fibrinolytic system of five different species (human, sheep, pig, dog and rabbit) has shown that the coagulation systems of humans exhibit similarities to that of sheep (10). In contrast to the other species, which all have enhanced coagulation, the coagulation system of sheep was characterised by a slightly decreased activation of the coagulation system as compared to humans (10). Thus, the results of our study confirm this observation and support the concept that sheep could be the most suitable animal species for coagulation studies (10).

However, there were also differences between humans and sheep in our study. In the endogenous thrombin generation assay, the lag phase of thrombin generation, which represents the clotting time, was shorter in sheep than in humans (Fig. 3). One possible explanation for this might be the different sensitivity of each species to the recombinant human tissue factor, which was the activator in the endogenous thrombin generation assay (7). Apparently sheep blood seems to have higher sensitivity to the human tissue factor than human blood.

In contrast to APTT, sheep had longer PT values than humans. This is in agreement with another study (25). Other publications reported shorter PT values than in our study (15–20 s); however, without direct comparison to humans (26, 27). This difference in study results may in part be explained by the assay used. We used a PT modified by Owen (Normotest), which is high sensitive to factor VII. As sheep are known to have decreased plasma levels of factor VII (10, 27), this might be a likely explanation for differences in study results.

Similarly, differences between human and sheep in platelet reactivity to different agonists as well as differences in the activity of the von Willebrand factor have been previously described (28–30). Nevertheless, sheep are a widely used species for testing of artery grafts (23), synthetic devices (31) and extracorporeal circulation (21) due to the similarities between human and sheep in platelet adhesion to foreign surfaces (32).

Differences between human and rat

A 100-fold higher dose of thrombin was required to cause a decrease in clotting time in rats as compared to humans (Fig. 1A). This observation is supported by previous studies using different methods. Platelets of rats were at least four-fold less responsive to thrombin when compared to human platelets when aggregometry was used (12, 13). Rats were three-fold less responsive to thrombin than human platelets as measured by flow cytometry (14). Also, anticoagulant and antiplatelet drug effects are different in rats and humans. A 40-fold higher dose of the potent inhibitor of factor Xa DX-9065a was required to inhibit thrombus formation in a rat thrombosis model as compared to humans (33). In another study, prasugrel’s active metabolite has been shown to inhibit platelet aggregation with a three-fold higher IC50 for rats as compared to humans (34). Thus, our results indicate that pronounced species differences between humans and rats are a limitation for the usefulness of rats in the research on blood coagulation.

Similarities between human and pig

Humans and pigs had similar values of the maximum lysis of the clot (ML) (Fig. 1D). Due to the similarities in the functional structure of coagulation proteins between humans and pigs (35), the usefulness of porcine plasmin for thrombolytic treatment in humans has been investigated (36). In addition, a cross-species comparison of the proteolytic activity of plasmin activated by the staphylokinase has shown that there are major similarities between humans and pigs (37). However, an in-vitro study has shown decreased sensitivity of porcine plasminogen to human tissue plasminogen activator (11). Thus, our findings confirm the potential usefulness of pig as an experimental animal species for examining the fibrinolytic pathway (38–43). Previous examples include studies examining the effects of plasminogen activators for prevention of the adult respiratory distress syndrome (39), urokinase in a disseminated intravascular coagulation model (40) and studies on tissue-type plasminogen activator (41, 42).

Similarities between human and rabbit

Rabbits and humans had similar values of the maximum clot firmness (MCF) with and without thrombin stimulation (Fig. 1C). The maximum clot firmness (MCF), which represents the clot strength and stability and describes platelet function, largely depends on platelet counts and fibrinogen (5, 6, 44). Our study shows that humans and rabbits had similar platelet counts (PLT) and fibrinogen levels, which explain the similar MCF values. Thus, our results support the usefulness of rabbits as an animal species for examining platelets, which has been shown in previous studies (34, 45–47). A cross species comparison of the antiplatelet effect of prasugrel shows similar IC50 values between humans, rabbits and dogs, higher IC50 value for monocyte and the highest for rats (34). Moreover, it has been shown that the use of rabbits for studies examining platelet activation and aggregation in vivo in a model of arterial thrombosis is more advantageous as compared to other animals (46). In addition, P2Y12 antagonists prevented arterial thrombosis in rabbits and suggested a reduction of thrombotic events in humans, which has been confirmed in clinical trials (48–50). However, it has been shown that platelet platelets do not contain PAI-1, which could be a limitation for the usefulness of rabbits for studies on platelets (51).

Study limitations

Possible imprecision in study results could have arisen from limitations inherent to the laboratory assays used, which are standardised for human blood. Different sensitivity of each species to the human thrombin (thrombin stimulated ROTEM, fibrinogen measurement), the human tissue factor (ETP assay), the rabbit brain thromboplastin and bovine plasma (PT) and the rabbit platelets (APTT) are inherent limitations of the assays used. Despite this limitation, the NATEM test without any activator showed
relevant differences between species. Secondly, a possible limitation for the comparison of the coagulation profile between humans and rats could be the blood sampling by heart puncture in rats, which could lead to positive test results for markers of coagulation activation due to tissue damage (52).

Conclusion

There are relevant differences in the coagulation profile with or without thrombin stimulation between the five different species tested: humans, rats, sheep, pigs and rabbits. The cross-species comparison indicates that sheep could be a suitable species for translational coagulation studies. In addition, our findings confirm the usefulness of pigs as an experimental species for examining the fibrinolytic pathway and support the usefulness of rabbits as a species for studies on platelets. Although rats are widely used animal species in coagulation studies, our study showed that they are least comparable to humans. In sum, our findings indicate that precautions must be taken in the interpretation of the results and in extrapolation of animal studies to humans in the field of haemostasis because of marked species differences.

References