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Elevated anti-human factor Xa activity in rabbit and rodent plasma: Implications for preclinical assessment of human factor X in animal models of hemostasis

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ABSTRACT

A wide variety of animal models on thrombosis and hemostasis are used in thrombosis and hemostasis research for the preclinical assessment of hemostatic agents. While the vertebrate coagulome is highly conserved, human and animal plasmas differ considerably when evaluated in coagulation assays such as prothrombin time (PT), activated partial thromboplastin time (APTT), and calibrated automated thrombography (CAT). Here, we have aimed to provide a reference framework for the evaluation of coagulation assays and inhibition of activated human FXa (hFXa) in various animal plasmas. To do so, a side-by-side evaluation of the extrinsic and intrinsic pathway of coagulation was performed by means of PT, APTT, and CAT measurements on (diluted) pooled plasmas from goats, pigs, rabbits, rats, mice, and humans. Plasma anti-FXa activity was assessed by determining the rate of recombinant hFXa inhibition through chromogenic activity analyses and immunoblotting. In general, rabbit, rat, and mouse plasmas exhibited robust clotting upon stimulation of both the extrinsic and intrinsic pathway, produced more thrombin during CAT upon plasma dilution, and displayed relatively high hFXa inhibitory activities. By comparison, goat, porcine, and human plasma displayed a similar profile in PT and APTT assays, produced less thrombin during CAT upon plasma dilution, and displayed comparable hFXa inhibitory activities. In conclusion, the observed differences in clotting parameters and anti-hFXa activity point to a higher anticoagulant threshold in plasma from rabbits, rats, and particularly in mice relative to human, goat, and porcine plasma. Finally, rat plasma was found to be more relevant to the preclinical assessment of human FX(a) in comparison to murine plasma.

1. Introduction

The vertebrate coagulome comprises a wide variety of (pro)enzymes, cofactors, and inhibitors that maintain an intricate balance between pro-, and anticoagulant pathways. While individual components of the coagulation cascade are highly conserved between mammals, large variations in response to various procoagulant stimuli have been reported [1–3]. For example, mouse and rabbit plasma have been observed to exhibit reduced rates of thrombin generation upon tissue factor-dependent activation of coagulation [4,5]. While interspecies differences in coagulation factor levels that regulate thrombin formation may be fundamental to these observations, detailed comparisons

Abbreviations: APTT, activated partial thromboplastin time; AT, antithrombin; BSA, bovine serum albumin; CAT, calibrated automated thrombography; CTI, corn trypsin inhibitor; Dil, dilution; ELISA, enzyme-linked immunosorbent assay; ETP, endogenous thrombin potential; FluCa, fluorescent substrate with calcium; FX, factor X; FXa, activated coagulation factor X; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hFXa, activated human factor X; hFXa-i, activated human factor X intermediate; HMW, high molecular weight; IACUC, individual institutional animal care and use committees; $k_{hFXa/AT}$, rate of activated human factor X-antithrombin complex formation; k_{obs}, pseudo-first-order rate constant; k_2, second order rate constant; M, molar; PT, prothrombin time; PCPS, small unilamellar phospholipid vesicles; RVV-X, Russel's viper venom activator of Factor X; s, seconds; TF, tissue factor; TFPI, tissue factor pathway inhibitor; ttPeak, time to thrombin peak; ZPI, Z-dependent protease inhibitor.

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between coagulation factor levels and coagulant activity across vertebrate species are sparse. Improved knowledge of global coagulation assays for rodent and non-rodent plasmas will facilitate the use and interpretation of animal models for studies in the field of thrombosis and hemostasis [6]. Moreover, the evaluation of specific coagulation parameters in various species will directly impact the selection of animal models for in vivo studies that are essential in the development of procoagulant therapeutics, such as for instance coagulation factor X (FX) and variants thereof [7–9].

Coagulation factor Xa (FXa) is an essential serine protease that plays a pivotal role in the initiation and propagation of the coagulation cascade [10,11]. We recently developed a novel variant of human coagulation factor X (FX-C) that can propagate blood coagulation in the presence of direct FXa-inhibitors such as apixaban, edoxaban, or rivaroxaban [8]. FX-C is currently in preclinical development as a reversal agent for patients that use direct FXa-inhibitors and require restoration of blood coagulation. In order to identify a suitable animal model for the preclinical assessment of FX variants, we have evaluated the in vitro global clotting parameters of mouse, rat, rabbit, porcine, and goat plasma in a side-by-side comparison with human plasma, using assays and reagents designed for analysis of human proteins. In addition, the anticoagulant potential of these plasmas was examined by determining the rate of human FXa inhibition.

2. Material and methods

2.1. Materials and reagents

Unless stated otherwise, a single batch of platelet-poor pooled plasma per species was used for all experiments to avoid intra-assay variability. Mouse (wild-type C57BL/6, WT-A), rat (Sprague-Dawley), rabbit (New Zealand White), domestic pig, and goat (sub-species not defined) plasmas (3.2% (w/v) sodium citrate) were purchased from Innovative Research (Novi, MI, USA). Mouse, rat, and rabbit pooled plasmas were collected via jugular vein extraction, which is preferred over cardiac puncture extraction [12]. Domestic pig and goat pooled plasmas were obtained via arterial blood extraction. Mouse TFPI-low and wild-type C57BL/6 (WT-B) pooled plasmas were generous gifts from dr. George Broze (Washington University School of Medicine, St Louis, MO, USA) and dr. Cornelis van 't Veer (Amsterdam University Medical Centers, location Academic Medical Center, Amsterdam, The Netherlands). Antithrombin (AT)-low C57BL/6 pooled mouse plasma was a kind gift from dr. Bart van Vlijmen (LUMC, Leiden, The Netherlands) [13]. All procedures were approved by the individual institutional animal care and use committees (IACUC). Normal human platelet-poor pooled plasma (3.2% (w/v) sodium citrate) was from Sanquin (Amsterdam, The Netherlands). The peptidyl substrate SpectrozymeXa was obtained from Sekisui Diagnostics (Stamford, CT, USA). Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg L-phosphatidylcholine and 25% (w/w) porcine brain L-phosphatidylserine (Avanti Polar Lipids, Alabaster, AL, USA) were prepared and characterized as described previously [14]. Calibrator and fluorescent substrate (FluCa) were from Thrombinoscope (Maastricht, The Netherlands). Dade Innovin prothrombin time (PT) reagent (purified recombinant human tissue factor (TF) with synthetic phospholipids, calcium, buffers and stabilizers) was obtained from Siemens (Newark, NY, USA). Neoplastine CI Plus 10 PT reagent (lyophilized fresh rabbit brain thromboplastin hydrated with a calcium-containing solvent and heparin inhibitor), Triniclot automated activated partial thromboplastin time (APTT) reagent (containing platelet factor III from rabbit brain phospholipids and micronized silica as particulate activator) and CK Prest 5 APTT reagent (rabbit cephalin reagent with kaolin as particulate activator) were obtained from Diagnostica Stago (Paris, France). All functional assays were performed in HEPES-buffered saline (HBS: 20 mM HEPES, 0.15 M NaCl, pH 7.5) supplemented with 0.1% PEG 8000 (dilution buffer) and filtered over an 0.2 μm membrane.

2.2. Proteins

Plasma-derived human AT and corn trypsin inhibitor (CTI) were from Haematologic Technologies (Essex Junction, VT, USA). Recombinant tissue factor (TF, Innovin) was obtained from Siemens (Newark, NY, USA). RVV-X activator and the matched-pair antibody set for the enzyme-linked immunosorbent assay (ELISA) of murine AT were obtained from Diagnostica Stago. Recombinant human FX (hFX) was prepared, purified, and characterized as described previously [8]. Purified hFX was activated with RVV-X (0.1 U/mg hFX), and hFXa was isolated by size-exclusion chromatography on a Sephacryl S200 HR column (V_t: 460 ml) and stored at −20 °C in HBS containing 50% (vol/vol) glycerol.

2.3. Coagulation assays

PT and APTT assays were performed on the Diagnostica Stago ST4 Coagulation Analyzer. PT samples were prepared by diluting plasma 2-, 6-, and 12-fold in dilution buffer supplemented with 0.1% bovine serum albumin (BSA). Samples (50 μl) were incubated for 60 s at 37 °C, and coagulation was initiated by addition of 50 μl PT reagent at 37 °C. APTT samples were prepared by diluting plasma 2-, 3- and 4-fold in Owren-Koller buffer (Diagnostica Stago). Samples (50 μl) were incubated for 180 s at 37 °C with APTT reagent. Coagulation was initiated with 50 μl of 25 mM $CaCl_2$ at 37 °C.

2.4. Calibrated automated thrombography analysis

Calibrated automated thrombography (CAT) was performed as described [15]. Briefly, thrombin generation curves were obtained by supplementing 80 μl (1.5-fold dilution) or 20 μl (3-fold dilution) of plasma with 20 μl of HBS containing TF (2, 6, or 20 pM final), CTI (70 μg/ml final), and PCPS (20 μM final) in 96-well round-bottom plates (Thermo Fisher Scientific, Waltham, MA, USA). Thrombin generation was initiated by adding 20 μl substrate buffer containing $CaCl_2$ supplemented with a thrombin fluorogenic substrate (FluCa). Thrombin formation was measured every 15 s for 60 min and corrected for the calibrator using Thrombinoscope software. For experiments with supraphysiological concentrations of AT, normal human pooled plasma was spiked with AT (0.625–2.5 μM; 125–200%) and assayed as described.

2.5. Inhibition of human FXa in pooled plasma

The rate of hFXa inhibition was essentially determined as previously described for AT inhibition in the absence of calcium [16]. Briefly, 25 μl of recombinant hFXa (10 nM) was added to 25 μl of plasma that was 2- to 100-fold diluted in dilution buffer, upon which the mixture was incubated for 5–60 min at ambient temperature. hFXa incubated in dilution buffer for 60 min at ambient temperature was used as reference. The residual enzyme activity as a function of time was determined after the addition of 50 μl SpecXa (500 μM) upon which the initial steady state increase in absorbance at 405 nm was monitored. The pseudo-first-order rate constants of hFXa inhibition (k_{obs}: nM s^{-1}) were determined by fitting the decrease in enzymatic activity to a single exponential function with a finite end-point as described [17]. Second order hFXa inhibition rate constants (k_2: nM s^{-1} dil^{-1}) were obtained by plotting k_{obs} against the plasma dilution and subsequent analysis by linear regression using GraphPad Prism 7 (San Diego, CA, USA). Analysis of hFXa inhibition in wild-type (WT-A, WT-B), TFPI-low, or AT-low C57BL/6 mouse plasma was determined by incubating hFXa (10 nM) for 0–45 min in 25 μl of 4- to 200-fold diluted pooled plasma.

2.6. Immunoblotting of FXa-AT complexes

The rate of hFXa-AT complex formation was assessed by incubating 5 nM of hFXa in 12-fold diluted plasma for 1–90 min at ambient temperature. Samples were quenched by the addition of sample buffer with

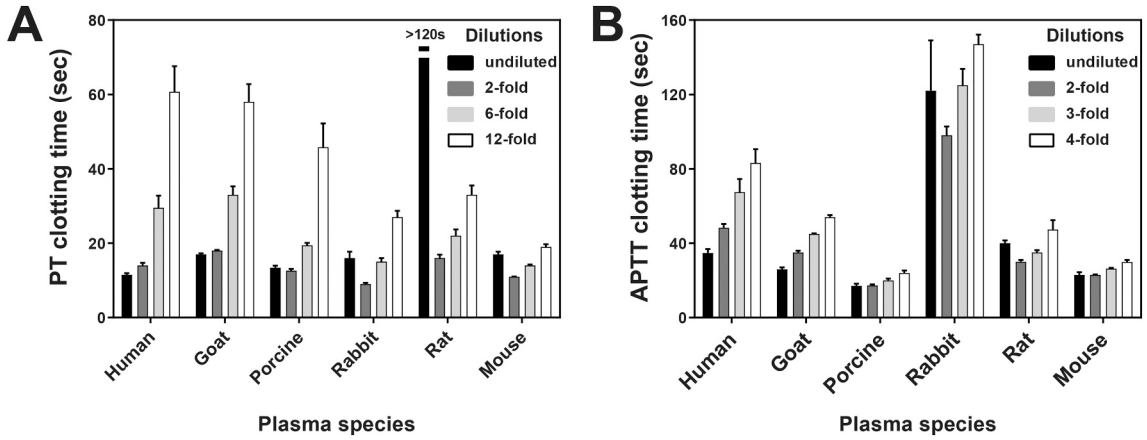

Fig. 1. Analysis of the extrinsic and intrinsic pathways of coagulation in human and animal plasmas. (A) The extrinsic pathway of coagulation was assessed on undiluted and diluted (2-, 6-, and 12-fold) human, goat, porcine, rabbit, rat, or mouse plasma employing a PT-based clotting assay with a rabbit brain thromboplastin reagent (Neoplastine CI Plus) as described in 'Materials and methods'. (B) The intrinsic pathway of coagulation was assessed on undiluted and diluted (2-, 3-, and 4-fold) human, goat, porcine, rabbit, rat, or mouse plasma using an APPT-based clotting assay with platelet factor III from rabbit brain phospholipids and micronized silica (TriniClot) as described in 'Materials and methods'. Data analysis was performed with GraphPad Prism 7 and the coefficient of variation (CV = μ / σ) was calculated for all means. A mean CV of 10% or higher was observed for the PT or APTT clotting times of the following groups: PT of human plasma at 6- and 12-fold dilution and APTT at 3-fold dilution, PT of porcine plasma at 12-fold dilution, PT and APTT of undiluted rabbit plasma, PT of undiluted rat plasma, and APTT of 4-fold diluted rat plasma. The data represent the average clotting time ± 1 S.D. from six individual measurements.

reducing agent (DTT; Invitrogen, Carlsbad, CA, USA), denatured for 10 min at 72 °C, subjected to electrophoresis on pre-cast 4–12% Bis-Tris gels using the MOPS buffer system (Invitrogen, Carlsbad, CA, USA), and transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The membrane was first probed with a polyclonal goat-anti-human FX antibody (Affinity Biologicals, Ancaster Canada), followed by an IRDye 680LT conjugated donkey-anti-goat polyclonal antibody (LI-COR Biotechnology, Lincoln, NE, USA) and detection at 700 nm using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences). Relative band intensities of hFXa-AT were counted automatically, corrected for background, and analyzed by non-linear regression using GraphPad Prism 7 (San Diego, CA, USA) by fitting the data to a one-phase decay curve, whereby Y0 was constrained to zero.

3. Results

3.1. Rabbit, rat, and mouse plasma exhibit robust clotting parameters

The extrinsic pathway of coagulation was evaluated in pooled plasma from humans, goats, pigs, rabbits, rats, and mice employing PT-based clotting analyses. Previously, it has been demonstrated that the procoagulant response is more robust in diluted mouse plasma relative to undiluted plasma [4]. Therefore, clotting assessment was performed in serially diluted plasma using the electro-mechanical clot detection method (viscosity-based readout). In line with previous findings, we observed a shortening of the PT-based clotting time upon either 2- or 6-fold dilution of mouse and rabbit plasma compared to the undiluted samples (Fig. 1A). Furthermore, undiluted rat plasma displayed an exceedingly prolonged clotting time (>120 s), which was markedly shortened upon plasma dilution and close to previously reported values [2]. As expected, serial plasma dilutions resulted in a prolongation of the clotting time for all species. However, the extent to which the extrinsic clotting time was prolonged varied, with rabbit, rat, and mouse plasma displaying a similar profile that substantially differed from that observed for human, goat, and porcine plasma (Fig. 1A). In case of the latter set of plasmas, the extrinsic clotting time of maximally diluted plasma (12-fold) was up to 5-fold prolonged relative to undiluted plasma (e.g. human plasma: 60.7 ± 6.9 s vs. 11.5 ± 0.4 s, respectively). In contrast, extrinsic clotting was at most 2-fold prolonged in rabbit and mouse plasma upon maximal dilution (e.g. rabbit plasma: 27.4 ± 1.7 s vs. 16.4 ± 1.7 s for undiluted plasma). Similar trends in PT clotting times were observed using an alternative thromboplastin reagent (Supplementary Fig. 1A). Collectively, these results suggest a consistently robust TF-induced fibrin clot formation in rabbit and mouse plasma.

To verify the observed relationship between plasma dilutions and clotting time, the intrinsic pathway of coagulation was evaluated employing APTT-based analyses. While human and goat plasma displayed prolonged APTT clotting times upon serial dilution, no substantial prolongation was observed following dilution of rat plasma (Fig. 1B). However, analogous to previous reports we observed exceptionally short intrinsic clotting times in undiluted porcine plasma (17.1 ± 1.1 s) and mouse plasma (23.6 ± 1.4 s) [18,19]. Furthermore, the APTT clotting times were more or less unperturbed upon serial dilutions of these plasmas. Similar trends in APTT clotting times of (diluted) rat and mouse plasma were observed using a contact activation reagent containing kaolin (Supplementary Fig. 1B). Rabbit plasma, on the other hand, demonstrated a weak response of the intrinsic pathway (undiluted plasma: 122.0 ± 27.0 s). In contrast, rabbit plasma exhibited a very short clotting time (undiluted plasma: 23.8 ± 0.5 s) using the kaolin-based reagent. Subsequent dilution of rabbit plasma nonetheless resulted in a more prolonged clotting time relative to human plasma (Supplementary Fig. 1B), suggestive of a weaker intrinsic clotting pathway [2]. Overall, assessment of the intrinsic pathway of coagulation confirmed that mouse plasma displays relatively robust fibrin clot formation relative to human and goat plasma.

3.2. Dilution of rabbit, rat, and mouse plasmas enhances thrombin generation

To examine the effects of plasma dilution on TF-induced coagulation in more detail we assessed thrombin generation employing CAT analysis. The extrinsic pathway of coagulation was triggered using human recombinant TF [20], which was previously shown to be compatible with murine plasma [4,21]. In addition, the use of human TF facilitated screening of clinically relevant animal plasma models for compatibility with reagents specific for human proteins. CAT analysis was performed using either a standard (80 μl plasma, 1.5-fold diluted) or reduced sample volume (20 μl plasma, 3-fold diluted), and thrombin generation was initiated with a low (2 pM) or high (6 pM) TF trigger [4,22]. Interestingly, thrombin generation in 3-fold diluted rat and rabbit plasma displayed an increased thrombin peak, a shorter lag time and

Table 1
Calibrated automated thrombography parameters. Thrombin generation was measured in human, goat, porcine, rabbit, rat, or mouse (WT-A) plasma using a plasma volume of 80 μl (1.5-fold diluted plasma) or 20 μl (3-fold diluted plasma) following a 6 pM or 2 pM TF trigger. Thrombin generation was initiated with $CaCl_2$ and a thrombin fluorogenic substrate as detailed in 'Materials and methods'. The lag time, thrombin peak, time to peak (ttPeak), and the velocity of thrombin generation were assessed. Values represent averages of three independent experiments ± 1 S.D.

	Lag Time	Peak	ttPeak	ETP	Velocity
Mouse	min ± SD	nM ± SD	min ± SD	nM·min ± SD	nM/min ± SD
2pM TF					
80 μl	2.7 ± 0.2	118.2 ± 13.4	6.0 ± 0.2	824.2 ± 72.6	36.4 ± 4.1
20 μl	6.7 ± 0.2	45.7 ± 7.4	10.6 ± 1.1	493.0 ± 47.5	12.1 ± 2.9
6pM TF					
80 μl	1.5 ± 0.1	238.7 ± 7.2	3.2 ± 0.1	974.1 ± 76.3	140.2 ± 7.6
20 μl	3.4 ± 0.1	88.3 ± 6.3	5.4 ± 0.2	676.6 ± 47.5	46.4 ± 7.1
Goat					
2pM TF					
80 μl	2.7 ± 0.2	26.9 ± 8.9	7.1 ± 0.8	231.3 ± 61.7	6.0 ± 1.0
20 μl	3.8 ± 0.3	31.8 ± 3.5	9.3 ± 0.3	338.6 ± 47.4	5.9 ± 0.7
6pM TF					
80 μl	1.6 ± 0.3	84.2 ± 6.8	4.2 ± 0.4	495.1 ± 54.8	32.8 ± 4.2
20 μl	1.9 ± 0.1	84.8 ± 2.8	4.4 ± 0.2	569.2 ± 39.6	35.0 ± 1.7
Porcine					
2pM TF					
80 μl	1.4 ± 0.1	98.1 ± 7.5	2.6 ± 0.1	247.5 ± 8.1	85.2 ± 15.0
20 μl	2.9 ± 0.3	52.4 ± 5.7	4.6 ± 0.4	257.0 ± 20.9	31.6 ± 4.9
6pM TF					
80 μl	0.9 ± 0.1	113.7 ± 4.4	2.0 ± 0.1	251.4 ± 7.7	106.4 ± 16.8
20 μl	1.6 ± 0.1	67.5 ± 4.6	3.1 ± 0.1	289.6 ± 15.3	46.6 ± 5.7
Rabbit					
2pM TF					
80 μl	5.2 ± 0.1	6.6 ± 1.8	8.4 ± 0.9	60.0 ± 13.0	2.2 ± 1.0
20 μl	4.5 ± 0.1	37.8 ± 4.6	8.0 ± 0.6	284.2 ± 19.1	11.2 ± 2.3
6pM TF					
80 μl	2.8 ± 0.1	27.7 ± 4.1	4.5 ± 0.4	141.0 ± 8.0	16.9 ± 2.7
20 μl	2.5 ± 0.1	77.4 ± 6.7	4.3 ± 0.3	359.0 ± 19.7	42.4 ± 5.2
Rat					
2pM TF					
80 μl	1.8 ± 0.3	36.2 ± 3.9	5.3 ± 0.4	380.0 ± 15.9	10.5 ± 1.6
20 μl	1.5 ± 0.1	68.1 ± 4.4	3.2 ± 0.1	380.2 ± 46.8	41.0 ± 1.9
6pM TF					
80 μl	1.3 ± 0.1	48.4 ± 11.5	4.0 ± 0.1	393.9 ± 38.6	18.2 ± 5.8
20 μl	1.0 ± 0.1	76.9 ± 5.8	2.5 ± 0.1	387.8 ± 53.4	53.6 ± 2.2
Mouse					
2pM TF					
80 μl	0.0	0.0	0.0	0.0	0.0
20 μl	1.8 ± 0.1	42.7 ± 19.1	3.5 ± 1.5	244.3 ± 43.0	24.0 ± 19.2
6pM TF					
80 μl	0.0	0.0	0.0	0.0	0.0
20 μl	0.8 ± 0.1	82.5 ± 7.1	1.9 ± 0.2	212.7 ± 19.8	71.1 ± 6.9
20pM TF					
80 μl	0.0	0.0	0.0	0.0	0.0
20 μl	0.4 ± 0.1	83.7 ± 0.7	1.3 ± 0.1	174.3 ± 14.0	104.6 ± 7.7

time to peak, and an enhanced velocity index relative to the parameters observed using 1.5-fold diluted plasma (Table 1). On the other hand, these parameters were either unaffected in 3-fold diluted goat plasma, or reduced (thrombin peak, velocity index) and prolonged (lag time, time to peak) in human and porcine plasma, irrespective of the TF concentration used. Remarkably, thrombin generation in mouse plasma was only observed following a 3-fold plasma dilution, as no detectable amounts of thrombin were observed for the 1.5-fold diluted plasma. Similar findings were obtained following initiation with 20 pM TF, consistent with previous reports [4]. Importantly, undiluted mouse plasma was shown to clot normally in the PT assay (Fig. 1A), which uses excess amounts of TF to stimulate the extrinsic pathway. In contrast, in CAT analysis picomolar TF concentrations are used, which do not lead to detectable thrombin generated in all of the conditions employed. This apparent discrepancy between the PT and CAT assays suggests an elevated anti-coagulant threshold in mouse plasma that can be overcome by a robust extrinsic trigger. In conclusion, current observations demonstrate that serial dilution of rabbit, rat, and mouse plasma leads to enhanced thrombin generation. As such, plasma dilution increases the clotting potential in a manner that is independent and upstream from fibrin clot formation.

3.3. *Plasma dilution mitigates antithrombin-dependent inhibition of thrombin generation*

The anticoagulant serpin antithrombin (AT) is known to strongly regulate TF-induced thrombin generation and is the main plasma inhibitor of FXa [23,24]. To investigate whether elevated plasma levels of AT may affect thrombin generation in diluted plasma, we analyzed normal pooled human plasma in the presence of increasing plasma concentrations of human AT. TF (6 pM) stimulation of 1.5-fold diluted plasma supplemented with 625 nM AT (125% final plasma concentration) typically displayed a 65% reduced thrombin peak (65 ± 8 nM) compared to that of 1.5-fold diluted plasma without additional AT (183 ± 12 nM) (Fig. 2) [25]. However, employing similar conditions, thrombin generation in 3-fold diluted plasma resulted in a 15% reduced thrombin peak (70 ± 8 nM with 125% AT vs. 82 ± 8 nM with 100% AT). Further assessment of the thrombin peak and velocity index at AT plasma levels of up to 200% revealed a stronger procoagulant response in thrombin generation parameters of 3-fold diluted plasmas relative to

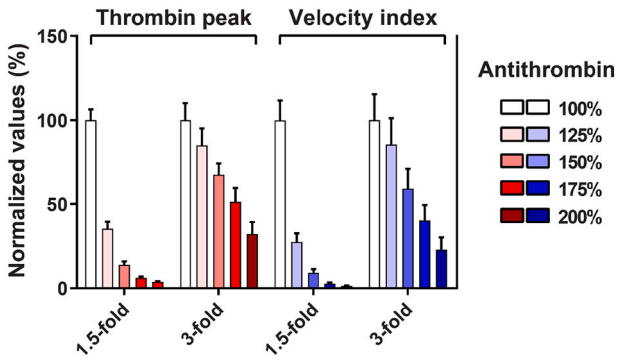

Fig. 2. Calibrated automated thrombography of human plasma spiked with antithrombin. Thrombin generation was measured in either 1.5-fold (standard plasma volume of 80 μl) or 3-fold (plasma volume of 20 μl) diluted normal human plasma with 100–200% AT (final plasma concentrations) and 6 pM TF and 20 μM PCPS (final reaction concentrations). Thrombin generation was initiated with $CaCl_2$ and a thrombin fluorogenic substrate as detailed in 'Materials and methods'. Percentages AT reflect final plasma concentrations of AT. The thrombin peak and the velocity index of thrombin formation were derived from individual experiments and normalized to conditions without 100% AT final plasma levels. Values represent the normalized averages ± 1 S.D. of three independent experiments.

the 1.5-fold dilution (Fig. 2). Taken together, these results demonstrate that plasma dilution mitigates AT-mediated anticoagulation following TF-initiated thrombin formation. As such, these findings corroborate our observations on the enhanced extrinsic clotting potential in diluted plasmas (Fig. 1A; Table 1). Furthermore, AT-mediated inhibition of FXa, and the reduction thereof upon plasma dilution, could potentially be at the basis of the attenuated thrombin formation observed for rabbit, rat, and mouse plasma.

3.4. Enhanced inhibition of human FXa in rabbit, rat, and mouse plasma

To assess anti-FXa activity in the various animal plasmas in more detail, we assayed the time-dependent inhibition of recombinant human FXa (hFXa, 5 nM) in a series of diluted plasmas (Fig. 3A, B). Assessment of the second order rate constants (k_2) revealed similar rates of hFXa inhibition in human (k_2 9.2 ± 0.4 M s^{-1} dil^{-1}), porcine (k_2 8.2 ± 0.5 M s^{-1} dil^{-1}), and goat (k_2 9.6 ± 1.6 M s^{-1} dil^{-1}) plasma. Rat (k_2 22.2 ± 1.6 M s^{-1} dil^{-1}) and rabbit (k_2 24.9 ± 3.4 M s^{-1} dil^{-1}) plasma, on the other hand, displayed a 2- to 2.5-fold higher rate of hFXa inhibition relative to human plasma (Fig. 3C). Notably, analysis of hFXa inhibition in mouse plasma required shortened hFXa incubations and higher plasma dilutions (Fig. 3B), resulting in a hFXa inhibition that was over 13-fold higher compared to human plasma (WT-A, k_2 118 ± 5 M s^{-1} dil^{-1}; WT-B, k_2 119 ± 18 M s^{-1} dil^{-1}) (Fig. 3D). These results show that inhibition of hFXa is enhanced in rabbit, rat, and particularly in mouse plasma.

As wild-type C57BL/6 mice are known to comprise 10- to 20-fold higher plasma levels of Tissue Factor Pathway Inhibitor (TFPI) relative to human plasma [26], a plasma pool of TFPI-low C57BL/6 mice was examined to assess the contribution of TFPI to hFXa inhibition [27]. Remarkably, no significant difference in hFXa inhibition rate was observed between TFPI-low plasma (k_2 125 ± 10 M s^{-1} dil^{-1}; 2-fold diluted PT 11.4 ± 0.4 s; 2-fold diluted APTT 35.8 ± 1.1 s) and a wild-type C57BL/6 plasma pool derived from the same breeding facility (WT-B, k_2 119 ± 18; PT 2-fold diluted 12.5 ± 0.8 s; 2-fold diluted APTT

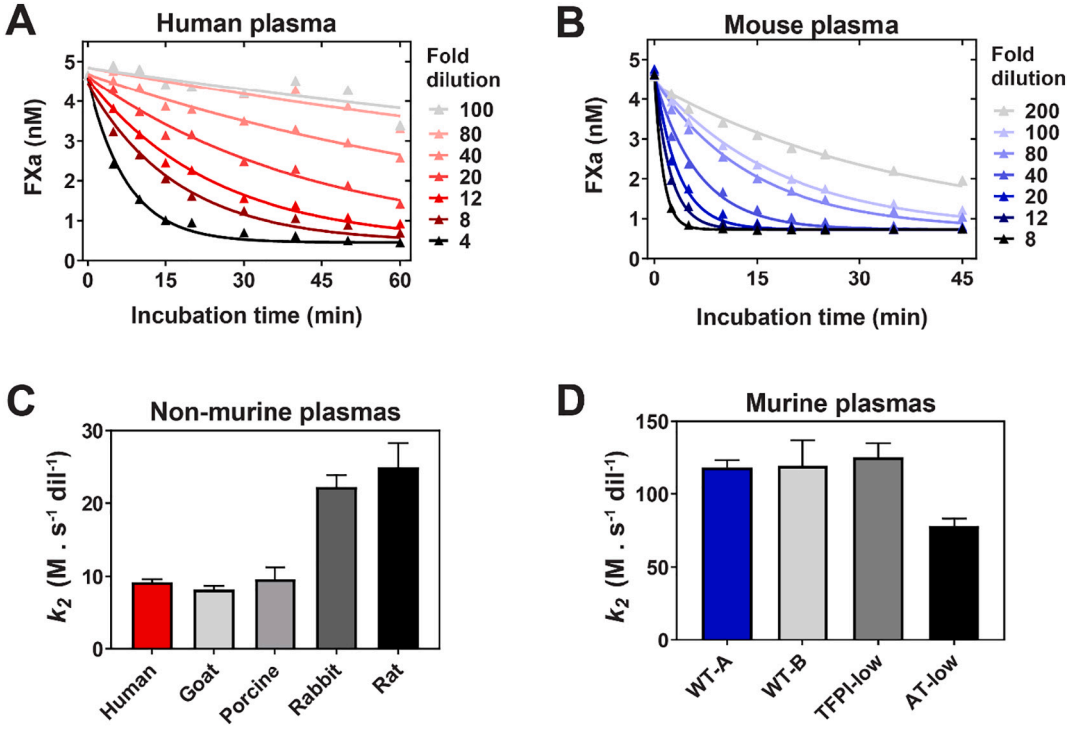

Fig. 3. Inhibition of human FXa in human and animal plasmas. The rate of hFXa inhibition was measured under pseudo-first order conditions. hFXa (5 nM) was incubated in 4- to 100-fold diluted human plasma (A) or 8- to 200-fold diluted mouse plasma (B, WT-A) for up to 60 min at ambient temperature. At various time points, residual enzyme activity was measured using the chromogenic substrate SpecXa. The lines are drawn following analysis to equations detailed in 'Materials and methods'. (C, D) hFXa (5 nM) was incubated in diluted human, goat, porcine, rabbit, or rat plasma (C, 4- to 100-fold diluted in dilution buffer) or in diluted murine WT-A, WT-B, TFPI-low, or AT-low plasma (D, 8- to 200-fold diluted in dilution buffer) for up to 60 min at ambient temperature. At various time points, residual enzyme activity was measured using the chromogenic substrate SpecXa, and the first order rates (k_{obs}, M s^{-1}) of hFXa inhibition were determined as detailed in 'Materials and methods'. Second order rate constants of hFXa inhibition (k_2, M s^{-1} dil^{-1}) were obtained by plotting k_{obs} against the plasma dilution and subsequent analysis by linear regression. Values represent the average of three individual experiments ± 1 S.D.

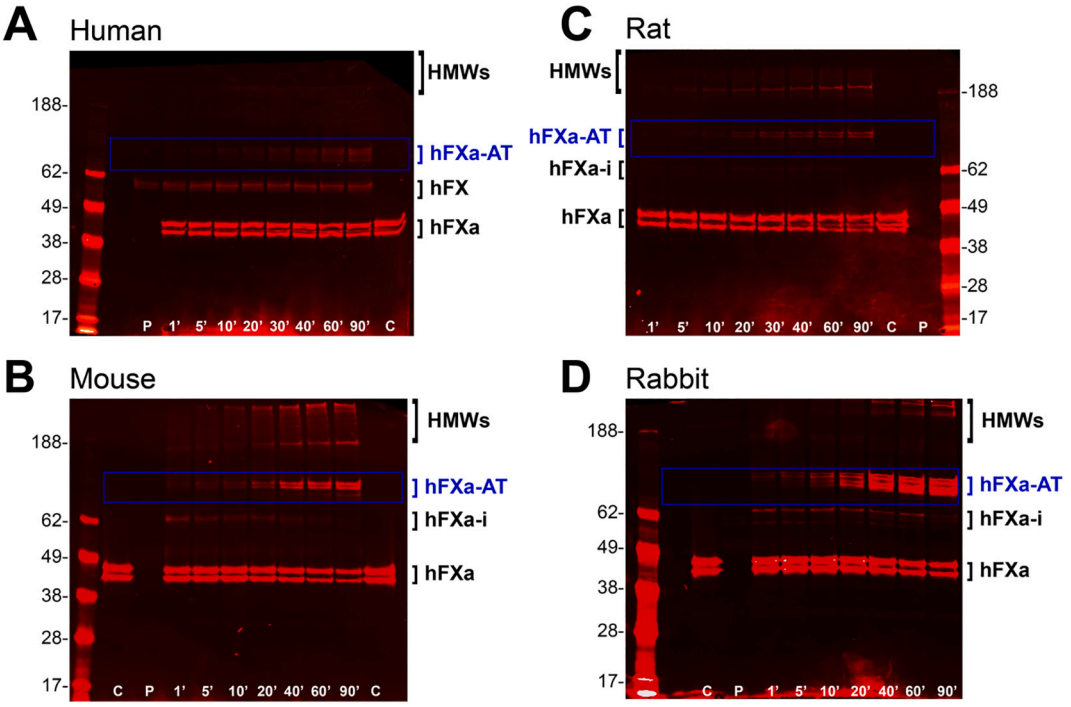

Fig. 4. Immunoblotting of hFXa incubated in human, mouse, rat, or rabbit plasma. (A) hFXa (5 nM) was incubated in 12-fold diluted human plasma for 1–90 min at ambient temperature. Samples were taken at various time points, subjected to reducing SDS-PAGE analysis, blotted to a nitrocellulose membrane, and probed using a polyclonal goat-anti-human FX antibody as detailed in 'Materials and methods'. The incubation time of hFXa (in minutes) is indicated at the bottom of the images; control lanes containing hFXa-αβ (hFXa, C) or plasma (P) only are indicated. (B–D) Similar to panel A, except hFXa was incubated in either 12-fold diluted mouse (B), rat (C) or rabbit (D) plasma. The protein bands corresponding to the hFXa-AT complexes (blue, boxed), High Molecular Weight species (HMWs), endogenous plasma derived human FX (hFX), hFXa intermediate (hFXa-i), recombinant human FXa (hFXa), and the apparent molecular weights (kDa) of the standards are indicated. Representative immunoblots of two independent experiments per species are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

40.7 ± 1.8 s) (Fig. 3D). In addition, we studied the relative contribution of AT to hFXa inhibition in more detail in pooled plasma from C57BL/6 mice with AT plasma levels of 0.8–2% resulting from siRNA treatment that leads to knockdown of AT expression [13]. Remarkably, the rate of hFXa inhibition in AT-low plasma (k_2 78 ± 5) was only 1.5-fold lower relative to that of wild-type plasma (Fig. 3D), which suggests that AT only partially accounts for the higher rates of hFXa inhibition in mouse plasma.

3.5. *Mouse, rat, and rabbit plasma display distinct profiles of human FXa-antithrombin complex formation*

The elevated rates of hFXa inhibition in rabbit and rodent plasma were investigated in more detail via immunoblotting of time-dependent hFXa incubations in diluted plasma using a polyclonal anti-human FX antibody. In addition, the relative rate of complex formation between hFXa and endogenous AT, thereby generating hFXa-AT, was assessed. As expected, incubation of hFXa in human plasma predominantly resulted in the formation of hFXa-AT complexes which differed in size according to FXa species (FXa-α 46 kDa, FXa-β 42 kDa) [28] and AT glycosylation status (ATα kDa 58, ATβ 55 kDa) (Fig. 4A) [29]. Furthermore, prolonged incubation (>10 min) also resulted in the appearance of minor amounts of high molecular weight species (HMWs, Mr >188 kDa) that are yet to be identified.

In line with the observations for human plasma, incubation of hFXa in mouse plasma led to the formation of hFXa-AT complexes with a similar distribution in apparent molecular weight (Fig. 4B). However, the relative rate of hFXa-AT complex formation was 2.8-fold higher in mouse plasma relative to human plasma (Fig. 5A, B), and consumption of hFXa was clearly observed (Fig. 4B). Moreover, extensive formation of multiple HMWs over time was observed in mouse plasma. Furthermore, a hFXa-intermediate (hFXa-i) migrating at 62 kDa was apparent at the early time points and disappeared upon prolonged incubations of hFXa (Fig. 4B). In contrast, the generation of hFXa-i and HWMs was rather limited upon incubation of hFXa in rat plasma (Fig. 4C), while the relative rate of hFXa-AT complex formation was 2.1-fold higher relative to human plasma (Fig. 5C). These findings highlight that the hFXa-AT complexation profile in rat plasma was more similar to that of human plasma. Finally, incubation of hFXa over time in rabbit plasma resulted in a persistent appearance of hFXa-i, notable consumption of hFXa, and a HMWs banding pattern that was distinct from mouse and rat plasma. In addition, the relative rate of hFXa-AT complex formation was 3.4-fold higher in rabbit plasma as compared to human plasma. Importantly, cross reactivity between the anti-human FX antibody and plasma FX native to mouse, rat, or rabbit was not observed. In summary, our observations indicate that the elevated rates of hFXa inhibition in rabbit and rodent plasma are partly mediated by enhanced hFXa-AT complex formation. In addition, the observed species-specific differences in the hFXa plasma absorption profile suggest the presence of unidentified hFXa binding components that are unique to each plasma.

4. Discussion

Studies that serve to evaluate the procoagulant activity of human coagulation proteins build upon animal models and coagulation assays originally intended for human plasma. A relevant animal model will allow for reliable preclinical assessment and potential to extrapolate findings to the human system. As mouse, rat, rabbit, pig, and goat are frequently used in preclinical hemostasis ad thrombosis research [30,31], we evaluated the coagulant characteristics of pooled plasmas from these species by means of extrinsic and intrinsic clotting analyses

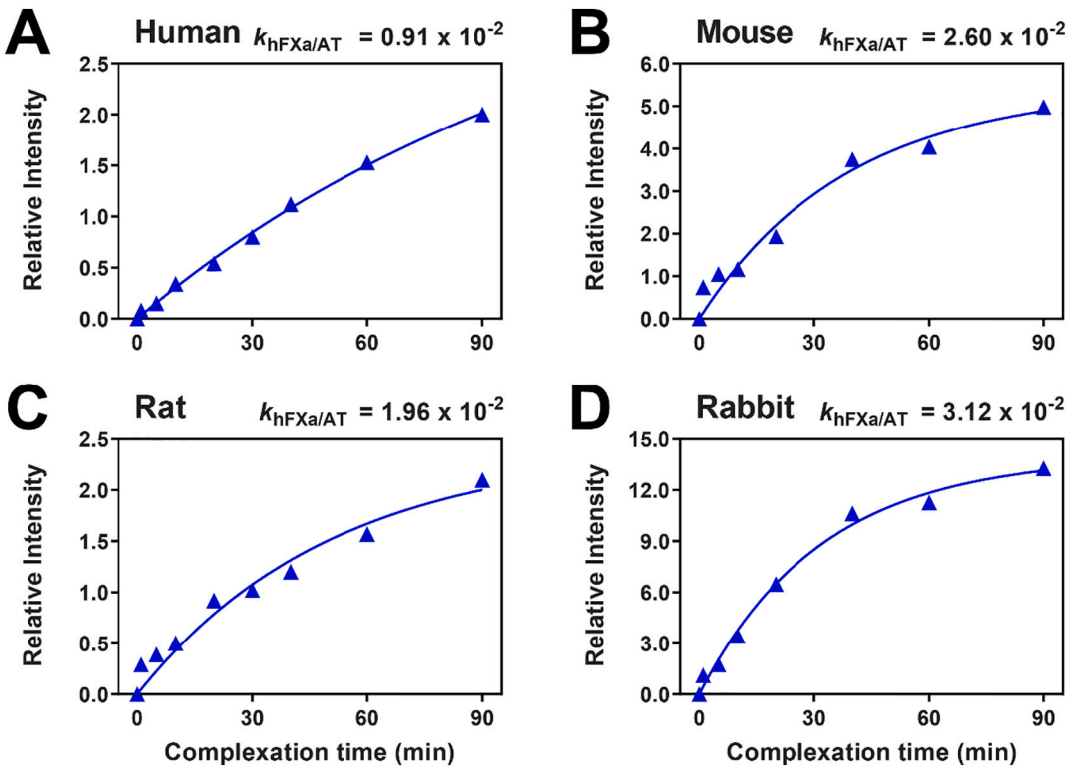

Fig. 5. Rate of human FXa-antithrombin complex formation in human, mouse, rat, or rabbit plasma. The rate of hFXa-AT complex formation was assessed under pseudo-first order conditions. (A–D) hFXa (5 nM) was incubated over time in 12-fold diluted human, rabbit, rat, or mouse plasma, and hFXa-AT complex relative band intensities were quantified and analyzed via non-linear regression as described in 'Materials and methods'. The rate of hFXa-AT complex formation ($k_{hFXa/AT}$, relative intensity per minute) is indicated. Datapoints represent averages of two independent experiments.

and CAT measurements. Side-by-side evaluation of standard PT and APTT clotting parameters revealed that of all species studied, goat plasma displayed a clotting profile comparable to human plasma, hallmarked by similarly prolonged clotting times following plasma dilution (Fig. 1). CAT parameters obtained using human TF as extrinsic trigger demonstrated porcine plasma to behave in a manner analogous to human plasma, indicated by a diminished thrombin generating potential of diluted plasma (Table 1). In addition, both goat and rat plasma were able to effectively generate thrombin in this system. As such, these data indicate that thrombin formation in goat, porcine, and rat plasma is sensitive to human TF, an essential reagent specific to human FX.

Interestingly, plasma from mice, rat, and rabbit was characterized by an enhanced potential to generate thrombin after plasma dilution, whereas the opposite was observed for human and porcine plasma (Table 1). In line with these findings, plasma dilutions trended towards a potentiation of fibrin clot formation for smaller animals vs. a mostly dampened clot formation for larger animals (Fig. 1). These differences in coagulation profile may in part be related to variations in the levels and/or inhibition kinetics of naturally occurring plasma inhibitors (e.g. AT, TFPI) that tightly control the procoagulant activity of serine proteases such as FXa in order to prevent runaway coagulation. Evidence of a role for interspecies differences in FXa inhibition that may be at the basis of this finding comes from our observation that mouse, rat, and rabbit plasma demonstrated fundamentally higher rates of hFXa inhibition (Fig. 3). In contrast, the rate of hFXa inhibition in goat, porcine, and human plasma was nearly identical. Detailed plasma analysis by means of immunoblotting further revealed higher rates of hFXa-AT complex formation in rabbit, rat, and mouse plasma relative to human plasma (Fig. 5). As such, we speculate that the distinctive coagulant profile observed for rabbit, rat, and mouse plasma results, at least in part, from enhanced FXa inhibition by AT. This may be the consequence of increased circulating levels of AT. Support for the latter comes from our observation that inhibition of thrombin generation by supra-physiological AT levels was mitigated upon dilution of human plasma (Fig. 2). However, it is important to mention that information on AT levels in rabbit, rat, and mouse is scarce [32]. Summarizing, dilution of rabbit, rat, and mouse plasma increases the clotting potential as a result, in part, of reduced endogenous AT plasma levels. As dilution is not expected to significantly impact the nanomolar amounts of AT-targeted coagulation factors generated in coagulation assays, lowering of the AT concentration will therefore impair efficient protease inactivation due to their intrinsically slow target inhibition rates in the absence of heparin-like saccharides [17]. As the rate of hFXa inhibition was only partially reduced in plasma from AT-low C57BL/6 mice (Fig. 3), it seems likely that additional inhibitors contribute to the inhibition of FXa and as such to reduced clotting. TFPI may not play a substantial role to overall inhibition of hFXa, since under the conditions employed the rate of hFXa inhibition was comparable for plasma of wild-type and TFPI-low mice (Fig. 3). Other FXa inhibitors endogenous to mouse plasma include protein Z-dependent protease inhibitor (ZPI) and alpha-2-macroglobulin [33–35]. The serpin ZPI circulates in complex with its cofactor protein Z, and this complex inhibits FXa in a calcium- and phospholipid-dependent manner [36]. Therefore, ZPI is not expected to contribute the kinetics of FXa inhibition under the conditions employed. The inhibitor α2-macroglobulin targets a broad spectrum of proteases in the circulation. Subsequent to binding and cleavage by the target protease, α2-macroglobulin undergoes an irreversible conformational change which traps the protein within a cage-like structure without directly blocking the protease active site [37,38]. As such, α2-macroglobulin is not expected to impact the observed rate of FXa-inhibition. Finally, as knowledge on the spectrum and functionality of serpins implicated in murine hemostasis is limited, the involvement of an expanded set of serpin species including serpins from other clades, such as α1-antitrypsin variants or ovalbumin-like serpins [39,40], cannot be ruled out. Whether the range

and functional characteristics of naturally occurring plasma inhibitors is homologous for rabbit, rat, and mouse is unclear at this point.

The notion that multiple FXa-targeting inhibitors may circulate in mouse plasma agrees with our observation that hFXa interacted with several mouse proteins (Fig. 4). Furthermore, Parng and colleagues corroborated our findings as they previously reported high plasma protein binding of catalytically active hFXa-I16L in mouse plasma, resulting in rapid plasma elimination of hFXa-I16L [41]. Moreover, they demonstrated that spiking of hFXa-I16L in mouse plasma leads to hFXa-I16L-HMW complexes, which were suggested to correspond to hFXa-I16L in complex with either a serpin or alpha-2-macroglobulin. An alternative explanation for the hFXa-HMW species observed may lie in the polymerization/aggregation of hFXa-AT complexes as described for human AT [42,43]. While identification of the hFXa binding proteins was beyond the scope of this study, our immunoblotting strategy clearly underscores the intricate complexity of studying the procoagulant effects of human FXa in a non-human plasma environment.

A limitation of the current study is that additional data is required in order to substantiate claims towards our premise of elevated levels of circulating inhibitory proteins in rabbit, rat, and mouse plasma that would underpin some of the findings obtained here. More specifically, knowledge on the exact levels of coagulation proteins including factors X and VII, prothrombin, and AT, are necessary for a better understanding of the noted species differences. Unfortunately, evaluation of these parameters is hampered by the lack of species-specific reagents that would allow for these types of analyses. Other limitations are that the inhibition of species-specific FXa variants was not assessed, and the initiation of coagulation by rabbit- or rodent-derived proteins was not evaluated, nor that of the contribution of platelets. Examination of the above-mentioned elements is necessary to draw definitive conclusions regarding relative endogenous pro- or anticoagulant activity in these animals.

In conclusion, the current body of work serves as an informative standardization study in which selected species were compared with respect to coagulation assays and human FX(a). While it is difficult to find an animal model that fully mimics human physiology, we identified the rat plasma clotting system to be more relevant to preclinical studies that incorporate CAT, human TF, and human FXa as compared to murine plasma. The plasma clotting system of larger animals, such as goats or pigs, was however more similar to that of humans. Furthermore, it was uncovered that these differences are, in part, related to a fundamentally higher rate of FXa inhibition and FXa-inhibitor complex formation in rabbit, rat, and mouse plasma. These novel findings will help to further our understanding of non-human coagulation systems and will contribute to the extrapolation of experimental data during preclinical assessment of novel therapeutic strategies.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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