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Tail vein transection bleeding model in fully anaesthetized haemophilia A mice

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Corresponding Author:	Carsten Dan Ley, PhD Novo Nordisk A/S Måløv, Zeeland DENMARK
Corresponding Author's Institution:	Novo Nordisk A/S
Corresponding Author E-Mail:	csdl@novonordisk.com
Order of Authors:	Ariadna Carol Illa
	Sarah Baumgarten
	Dennis Danielsen
	Karin Larsen
	Torben Elm
	Peter B. Johansen
	Tom Knudsen
	Brian Lauritzen
	Mikael Tranholm
	Carsten Dan Ley, PhD
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TITLE:

Tail Vein Transection Bleeding Model in Fully Anesthetized Hemophilia A Mice

AUTHORS AND AFFILIATIONS:

Ariadna Carol Illa[1,2], Sarah Baumgarten[1], Dennis Danielsen[1], Karin Larsen[3], Torben Elm[1], Peter B. Johansen[4], Tom Knudsen[5], Brian Lauritzen[1], Mikael Tranholm[6], Carsten D. Ley[1]

[1]Global Drug Discovery, Novo Nordisk A/S, Maløv, Denmark
[2]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark
[3]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, København N, Denmark
[4]Independent consultant
[5]Catalyst Biosciences, South San Francisco, CA, USA.
[6]Værløse Dyreklinik, Furesø, Denmark

Email addresses of co-authors:

Ariadna Carol Illa	(ezai@novonordisk.com)
Sarah Baumgarten	(sbum@novonordisk.com)
Dennis Danielsen	(ddnl@novonordisk.com)
Karin Larsen	(karin.larsen@sund.ku.dk)
Torben Elm	(telm@novonordisk.com)
Peter B. Johansen	(peterbygballe@yahoo.dk)
Tom Knudsen	(tknudsen@catbio.com)
Brian Lauritzen	(blz@novonordisk.com)
Mikael Tranholm	(mikael.tranholm@gmail.com)
Carsten D. Ley	(csdl@novonordisk.com)

Corresponding authors:

Carsten D. Ley	(csdl@novonordisk.com)

KEYWORDS:

tail vein transection model, tail bleeding, animal model, pharmacology intervention, haemophilia, factor FVIII knock out mice.

SUMMARY:

The refined tail vein transection (TVT) bleeding model in anesthetized mice is a sensitive *in vivo* method for the assessment of hemophilic bleeding. This optimized TVT bleeding model uses blood loss and bleeding time as endpoints, refining other models and avoiding death as an endpoint.

ABSTRACT:

Tail bleeding models are important tools in hemophilia research, specifically for the assessment of procoagulant effects. The tail vein transection (TVT) survival model has been preferred in many settings due to sensitivity to clinically relevant doses of FVIII, whereas other established models, such as the tail clip model, require higher levels of procoagulant compounds. To avoid using survival as an endpoint, we developed a TVT establishing blood

loss and bleeding time as endpoints and full anesthesia during the entire experiment. Briefly, anesthetized mice are positioned with the tail submerged in temperate saline (37°C) and dosed with the test compound in the right lateral tail vein. After 5 min, the left lateral tail vein is transected using a template guide, the tail is returned to the saline, and all bleeding episodes are monitored and recorded for 40 min while collecting the blood. If no bleeding occurs at 10 min, 20 min, or 30 min post-injury, the clot is challenged gently by wiping the cut twice with a wet gauze swab. After 40 min, blood loss is quantified by the amount of hemoglobin bled into the saline. This fast and relatively simple procedure results in consistent and reproducible bleeds. Compared to the TVT survival model, it uses a more humane procedure without compromising sensitivity to pharmacological intervention. Furthermore, it is possible to use both genders, reducing the total number of animals that need to be bred, in adherence with the principles of 3R's. A potential limitation in bleeding models is the stochastic nature of hemostasis, which can reduce the reproducibility of the model. To counter this, manual clot disruption ensures that the clot is challenged during monitoring, preventing primary (platelet) hemostasis from stopping bleeding. This addition to the catalog of bleeding injury models provides an option to characterize procoagulant effects in a standardized and humane manner.

INTRODUCTION:

Animal models are essential for understanding the pathogenesis of hemophilia and developing and testing treatment regimens and therapies. The Factor VIII knock-out mice (F8-KO) is a widely used model for the study of hemophilia A[1,2]. These mice recapitulate key features of the disease and have been widely used for treatment development, such as recombinant FVIII products[3–5] and gene therapy strategies[6,7].

There are various bleeding injury models for evaluating the pharmacological effects of different hemostatic compounds *in vivo*. One of these coagulation models is the tail vein transection survival model in mice[8–14], measuring the ability of hemophilic mice to survive exsanguination after tail transection. This method was introduced more than four decades ago[15] and is still used[9,16,17]. However, the model utilizes survival as an endpoint and requires observation of the animals over a period of up to 24 h, during which the animals are conscious and hence can experience pain and distress.

Bleeding models of shorter duration and under full anesthesia have been described previously, such as the tail clip model (also known as the tail tip)[8,18–28]. Nevertheless, for a complete normalization of blood loss after the bleeding challenge, these models require doses of procoagulant compounds (e.g., FVIII) far higher than those administered clinically[29]. A different injury model under anesthesia, the vena saphena bleeding method, is sensitive to lower doses of procoagulant compounds[30] but requires a high experimenter intervention level since the clots must be disrupted almost continuously (as opposed to 3 times in the presented model).

Standardization towards a common protocol to test new procoagulant compounds is needed to facilitate data comparison between laboratories[31–33]. In TVT models, there is not yet a common agreement on studied endpoints (blood loss[7,26], bleeding time[9,34], and survival rate[35,36]), and experimental length varies between studies[13].

The primary objective is to describe and characterize an optimized model with high reproducibility, the possibility of studying of on-demand as well as a prophylactic treatment, sensitivity to pharmacological intervention equivalent to the survival model, yet not using death or near-death as endpoints, and where animals would not be conscious during bleeding. Thus, reducing pain and distress in the animals involving a more ethical endpoint[37].

Tail clip models are generally conducted in one of two variants, either amputating the tip of the tail, e.g., amputation of 1–5 mm[18–21,23,24] or, in a more severe variant, transected at a tail diameter around 1–3 mm[8,22,25]. This causes a combined arteriovenous bleed, as the lateral and dorsal veins and ventral artery are usually severed, and in general, the larger the amputation, the lower the sensitivity to a procoagulant compound. Furthermore, since the tail tip is amputated, the arteriovenous injury is exposed without any opposing tissue; thus, at least in theory, it is dissimilar to the most common hemophilic bleeds.

As the name implies, only the vein is injured in tail vein transection models such as described in this paper, thus resulting in a venous bleed only. Since the vessel is not fully severed, the injury is expected to be smaller than in the amputation models, and the tissue around the cut, which a clot may adhere to, is retained. In addition, there is lower blood pressure in the vein as opposed to the artery. These factors contribute to an increased sensitivity relative to amputation models, such that normalization of bleeding can be achieved with clinically relevant doses of replacement therapy, e.g., with rFVIII in hemophilia A, which is useful for evaluating the magnitude and durability of effects of procoagulant treatment[26,38,39].

PROTOCOL:

All procedures described in this protocol have been approved by the Animal Welfare Body at Novo Nordisk A/S, and the Danish Animal Experiments Inspectorate, The Danish Ministry of Food, Agriculture, and Fisheries. The optimized 40 min method includes anesthesia and dosing time in the design (**Figure 1**). Hemophilic mice of both genders between 10–16 weeks of age are required for this procedure.

1. Preparations before the study

1.1 Prepare the test solutions in the correct concentrations.

1.2 Start the water bath and heat to 37 °C. Fill the 15 mL centrifuge tubes for blood collection with saline (0.9% NaCl).

1.3 Place the 15 mL saline tubes in the holes in the warmed base plate at least 15 min prior to the start of the experiment.

1.4 Identify the mice and record their weight. Avoid handling mice more than necessary as this can cause stress and affect the study.

1.5 Prepare the workstation in the fume hood before proceeding so that everything is within reach: napkins, tail holder, gauze, syringes, scalpels, stopwatches, and blood flow notation paper.

1.6 Place the tail mark and cutting blocks on the heating plate – cold blocks will make the veins contract and thereby affect the bleeding.

2. Anesthesia

2.1 Conduct the isoflurane anesthetic procedure inside a fume hood.

2.2 Set the gas vaporizer to initially 5% isoflurane in 70% O_2/30% N_2O in the anesthesia chamber with 1 L/min flow. Allow sufficient time for the anesthesia chamber to fill (about 5 min depending on chamber volume and gas flow rate).

2.3 Place the mice in the anesthesia chamber until they lose consciousness.

NOTE: This should occur within a minute or less if the chamber is sufficiently filled.

2.4 Ensure proper anesthetization by the absence of painful response to pedal reflex (firm toe pinch).

2.5 Place the mice on the heating plate, making sure that the nose is in the nose cone.

2.6 Reduce the anesthesia to a maintenance level of 2 % isoflurane in 70% O_2/30% N_2O and place a plastic cover above the mice to reduce the loss of heat. Apply a suitable eye ointment to prevent dryness while under anesthesia.

2.7 Mark the tail at a diameter of 2.5 mm using the tail mark block. Do not force the tail into the slit in the block – it must fit snugly (**Supplemental Figure 1**)

2.8 Place the tail in the saline tube for at least 5 min to ensure a warm tail vein that is optimal for intravenous (i.v.) dosing.

3. Dosing of test solution

3.1 Place one mouse in the tail holder with the nose in an anesthesia mask.

3.2 Dose the animal with the compound of interest (in this case, rFVIII) and immediately start the stopwatch (t = 0).

3.3 Place the mouse back on the heating plate with the tail in the saline tube. Repeat the procedure with the other mice.

4. **Performing tail vein transection**

4.1 Perform the tail vein transection exactly 5 min after dosing. Place the tail in the cutting block and turn 90° to expose the vein (**Supplemental Figure 2**).

4.2 Perform the cut on the opposite side/vein from where the test solution is dosed.

4.3 Draw the #11 scalpel blade through the slit of the cutting block holding the tail to create bleeding. Reset the stopwatch and return the tail immediately to the saline.

5. Observation time and challenges

5.1 Observe the bleeding and annotate the start and stop of the bleeding throughout 40 min; annotate it on the raw datasheet.

NOTE: This visual assessment may vary slightly due to subjectivity.

5.2 The primary bleeding must stop within 3 min after the cut is made. If this is not the case, disqualify the mouse, euthanize, and replace (failure to stop the primary bleed can indicate a too severe injury or lacking primary hemostasis, as in vWF KO mice).

5.3 If there is no bleeding at 10 min, 20 min, and 30 min post-injury, challenge the tail cut.

5.4 Use a gauze swab soaked in warm saline from a separate tube kept in the water bath. Lift the tail out of the saline and softly wipe twice with the wet gauze in a distal direction over the tail cut.

5.5 After each challenge, immediately re-submerge the tail into the saline tube again.

6. Blood sampling

6.1 At t = 40 min, obtain blood samples from the supraorbital vein.

7. Euthanasia

7.1. Euthanize the mice by cervical dislocation while still under full anesthesia.

8. Treatment of samples

8.1. Centrifuge the 15 mL blood collection tubes with saline at 4000 x *g* for 5 min at room temperature.

8.2. Discard the supernatant from the 15 mL tubes, resuspend the pellet in 2–14 mL of erythrocytes (RBC) lysing solution, and then dilute it until it reaches a light coffee color.

8.3. Note the total volume (volume of blood + volume of erythrocytes (RBC) lysing solution added using the graduation marks on the tube).

8.4. Transfer 2 mL of the dilution to a hemoglobin tube and refrigerate it until the hemoglobin analysis.

8.5. Determine the blood loss by measuring the hemoglobin concentration in the saline. Measure the absorbance at 550 nm on a microplate reader (**Table of Materials)**.

8.6. Convert the absorbance to nmol hemoglobin using a standard curve prepared from human hemoglobin (**Table of Materials**).

9. Statistical analyses

9.1. Analyze the data using any appropriate software. Here Prism software was used. Over a range of studies, the following statistical methods were found to perform well.

NOTE: To analyze blood loss, bleeding time, exposure, platelet counts, and hematocrit; Brown-Forsythe and Welch ANOVA test was used, as the data were continuous but without variance homogeneity of the residuals applying Dunnett's test to adjust for multiple comparisons. A Pearson's test was used to test for correlations between bleeding time, blood loss, and doses. To determine ED_{50} values, a four-parameter inverse log (dose) response equation was fitted to bleeding- and blood loss data. To analyze the gender effect, a two-way ANOVA test was used, applying the Bonferroni correction to adjust for multiple comparisons. The significance level was defined as $P < 0.05$. Data are displayed as means ± SEM.

REPRESENTATIVE RESULTS:

To assess the applicability of the optimized model, a study was performed in F8-KO (C57BL) mice administered with a commercially available recombinant factor VIII replacement therapy (rFVIII); four different doses were tested: 1 IU/kg, 5 IU/kg, 10 IU/kg, and 20 IU/kg. Furthermore, we tested the corresponding vehicle (negative) control in F8-KO mice and wild-type (WT) group using C57BL mice as a positive control group to assess the response range in the model.

Following the optimized protocol, there was a significant reduction in blood loss for rFVIII treatment groups compared to the vehicle group. Additionally, a reduction in bleeding time was observed in the 5 IU/kg, 10 IU/kg, and 20 IU/kg treatment groups compared to the vehicle group (**Figure 2**). In WT mice, total blood loss ranged from 201.8–841.9 nmol Hgb (95% CI). Following the approximate equivalence of 1000 nmol Hgb ~125 µL of whole blood, the median bleeding in vehicle-treated mice was 780.25 µL, while on the 20 IU/kg group was 89.95 µL. To completely normalize the bleeding, a dose of 20 IU/kg was needed, and administration of 10 IU/kg caused a significant effect on reducing blood loss nearly to the upper limit of the WT range (**Figure 3**). The bleeding time of WT mice ranged from 0.98–9.16 min (95% CI), and dose levels of 10 IU/kg and 20 IU/kg reduced bleeding time to within this range. A strong correlation between blood loss and bleeding time was observed in the combined data ($r = 0.9357$, $P < 0.0001$) (**Figure 4**).

To evaluate the sensitivity of the model, a four-parameter inverse log(dose) response equation was fitted to blood loss and bleeding time observations, and a clear dose-dependent effect of rFVIII administration on blood loss and for the bleeding time was observed (**Figure 3**). The estimated ED_{50} values for blood loss and bleeding time were 2.41 ± 1.69 IU/kg and 2.55 ± 2.80 IU/kg, respectively.

To illustrate how bleedings occur in the model, all recorded bleeding episodes have been plotted to provide a visualization of the length and number of bleeds experienced by each

mouse (**Figure 5**). The primary bleeding is very similar in all the groups. Most of the hemophilic vehicle group starts to re-bleed even before the second challenge at 20 min, and for about half of these animals, the bleeding does not stop after the first challenge. Finally, as described, rFVIII treatment reduced the length of the bleeding episodes in treated F8-KO mice compared to vehicle, with already an observable change at the lowest dose. At the highest dose levels, most of the mice only bled briefly after being challenged.

Plasma rFVIII concentration was measured by Luminescent oxygen channeling assay (LOCI) detecting hFVIII and analogs to verify that the effect observed in reducing blood loss and bleeding time was concentration-dependent (**Figure 6**). There is notable variability within each group (mean CV 46%), but still, significant differences between groups can be observed, thus corroborating that the effect observed in blood loss and bleeding time is dependent on the plasma concentration of FVIII. All vehicle-treated mice measured below the lower limit of quantitation for the assay (2 U/L) and are represented with this value. WT mice were not measured since the applied FVIII assay is specific for detecting human FVIII.

Platelet counts and hematocrit were determined for all groups using the blood samples collected post-bleeding (**Figure 7** and measured with a hematological analyzer (**Table of Materials**). There was no variation between groups in platelet counts, indicating that platelet numbers are not affected, and the mice, therefore, remain capable of primary hemostasis. For hematocrit measurements, normal levels were observed in animals receiving moderate and higher FVIII doses (5 IU/kg, 10 IU/kg, and 20 IU/kg), whereas significantly lower levels were observed in the vehicle and 1 IU/kg treated groups (compared to WT animals). This is a frequent observation in hemophilic animals after heavy bleeding.

Classically, only one gender of animals (typically male) has been used in animal studies, and this is also described for TVT survival models[8,9,11,12]. Striving to reduce the total numbers of haemophilic mice needed in future studies (for breeding and the study), both mice genders were used. To evaluate the effect of gender in this optimized model (**Figure 8**), both blood loss and bleeding time results were subjected to two-way ANOVA analysis with gender and dose as factors. In this analysis, the effect of rFVIII dose was statistically significant ($P < 0.0001$), but mouse gender did not significantly affect the results ($P \approx 0.35$), and no significant interaction was found between the parameters, meaning that responses to treatment did not differ between genders.

FIGURE AND TABLE LEGENDS:

Figure 1: Temporal design of the experimental setup. The pre tail vein transection (TVT) phase includes anesthesia induction, warming the tail in saline, and dosing. After the TVT injury, a 40 min monitoring of the bleed under anesthesia, with subsequent challenges every 10 min, is performed. The experimental procedure is concluded by the collection of blood samples and euthanasia.

Figure 2: Effect of rFVIII after intravenous administration. Total blood loss (left panel) and total bleeding time (right panel) of F8-KO mice. Each mouse is shown as individual observations and mean ± SEM. The different test doses of rFVIII are represented in the x-axis. Data were analyzed by Brown-Forsythe and Welch ANOVA applying Dunnett's test to adjust for multiple comparisons. **** $P < 0.0001$.

Figure 3: Dose-response curves for blood loss and bleeding time. Blood loss (left panel) and bleeding time (right panel). The grey area represents the 95% CI from the values from 6 untreated wild-type C57BL/6 mice.

Figure 4: Correlation plot for blood loss and bleeding time. $R^2 = 0.8755$, p-value < 0.0001.

Figure 5: Individual bleeding profiles in wild-type, vehicle, 1 IU/kg, 5 IU/kg, 10 IU/kg, and 20 IU/kg rFVIII treated mice. Each line represents the bleeding profile of a single mouse, whereas each dotted bar represents a bleeding episode.

Figure 6: Exposure to rFVIII. rFVIII concentration in plasma was measured by a Luminescent oxygen channeling assay (LOCI) for detection of human FVIII. Vehicle mice were under the lower limit of quantitation (LLOQ) and were therefore plotted with the LLOQ value (2 U/L). WT animals were not measured since the applied FVIII assay is specific for detecting human FVIII. The different test doses of rFVIII and a WT control are represented in the x-axis. ** P < 0.01, *** P < 0.001 and **** P < 0.0001.

Figure 7: Hematological data. Platelet count (left panel) and hematocrit (right panel) of F8-KO mice are shown as individual observations mean with SEM. The different test doses of rFVIII and a WT control are represented in the x-axis. Data were analyzed by Brown-Forsythe and Welch ANOVA applying Dunnett's test to adjust for multiple comparisons. ** P < 0.01 and *** P < 0.001.

Figure 8. Effect of gender. Blood loss (left panel) and bleeding time (right panel) classified by treatment are shown as individual observations with mean ± SEM. Data were analyzed by two-way ANOVA applying the Bonferroni correction to adjust for multiple comparisons.

Table 1: ED_{50} values. Comparison of ED_{50} values of different available bleeding models for hemophilia studies in mice. Data is extracted from cited articles [superscripted] and presented as ED_{50} (IU/kg)

Supplemental Figure 1: Measuring template. Produced in aluminum. Size specifications: 20 mm x 40 mm x 10 mm (L x W x H). Groove: 2.5 mm depth and 2.5 mm width; radius 1.25 mm.

Supplemental Figure 2: Cutting template. Produced in stainless steel. Size specifications: 20 mm x 40 mm x 10 mm (L x W x H). Groove: 3 mm depth and 3 mm width; radius 1.5 mm.

DISCUSSION:

This optimized method of tail vein transection (TVT) has several advantages compared to the TVT survival method. The animals are fully anesthetized for the entire study duration, which makes mouse handling easier and increases animal wellbeing. Further, unlike the TVT survival model, overnight observation is not required, and this optimized model offers the possibility of measuring blood loss and observing the exact bleeding time over 40 min. Also, longer periods of bleeding in conscious animals can cause death by exsanguination, leading to pain and distress in the animals and probably stress, potentially resulting in increased variation due to a less controlled environment to perform the experiments[14]. Replacing time to death

or near-death, bleeding time, and blood loss have been successfully characterized and validated as endpoints. The actual tail injury is well defined and standardized since it uses a cutting block guide to perform the TVT, securing a reproducible cut, reducing the difficulty of the procedure and the technical variability. Hence, higher model robustness can be achieved, reducing the number of animals needed. Furthermore, data has demonstrated that it is possible to use both male and female mice, thus reducing the total number of animals to be bred, in accordance with the 3R principles. Along with these observations, there is a trend that female mice bleed slightly less and vary slightly more in high-bleeding, but not in low-bleeding groups. Lower blood loss measures could be associated with females having a smaller size, and therefore less blood volume compared to same-aged males.

Spontaneous bleeds in hemophilic patients are usually internal, specifically involving musculoskeletal, soft tissue, and mucocutaneous bleeding, while external injuries (such as minor cuts) are not the most common cause of extended bleeds, although more severe cuts and trauma can be life-threatening[40,41]. This optimized model induces venous-only bleeding, while other models, such as the tail clip, induce a mix of arterio-venous bleeding. Since the described model is a venous-only model, the procoagulant dose-effect in this TVT model might not reflect the effect in a more severe arterial injury; thus, other bleeding models should be used.

As shown by the individual bleeding profile, the primary bleeding is very similar in all the groups since primary hemostasis, i.e., aggregation of activated platelets, is intact in the hemophilia A setting[42]. In an immobilized injury, even under severe hemophilia, platelet aggregation can be sufficient to attenuate bleeding. For that reason, through empirical studies, we have found that inducing a bleeding challenge every 10 min is a necessary step in the protocol in order to disrupt aggregates of activated platelets, which can be strong enough to prevent bleeding even without fibrin generation from functional coagulation. Since fully anesthetized mice do not move and cannot physically challenge the injury as it happens in the non-anesthetized TVT survival model, it was necessary to introduce the challenge steps to prevent platelet aggregation alone from attenuating bleeding. Some untreated hemophilic mice re-bleed spontaneously prior to the challenge, but after the first challenge, spontaneous re-bleeds occur in most untreated hemophilic mice, and after the second challenge, many bleed continuously until the end of the experiment. As expected, there was increased bleeding in vehicle F8-KO mice compared to WT mice. Treated mice with increased doses of rFVIII showed a dose-dependent reduction in both blood loss and bleeding time. Significant effect on bleeding was found at doses 5 IU/kg and above (compared with vehicle-treated mice). The two highest doses reduced bleeding to levels quite close to the wild-type bleeding response, indicating normalization or near-normalization of bleeding. Dosing takes place at time t = 0, but it can be modified, for example, to dose the animals prior to the experiment.

In (**Table 1**), we present a comparison of different ED_{50} values for several bleeding models, classified by the studied endpoints. In this optimized model, we observed comparable ED_{50} values for blood loss and bleeding time (2.4 ± 1.7 IU/kg and 2.6 ± 2.8 IU/kg, respectively). On the same model studying rFVIIa, the ED_{50} values for blood loss bleeding time were (0.42 mg/kg and 0.39 mg/kg, respectively)[38]. This is a greater sensitivity to pharmacological intervention than previously described bleeding models under anesthesia, such as the tail clip model, with ED_{50} for blood loss of 4.6 ± 0.5 IU/kg[27], 28 IU/kg[28], and 53 IU/kg[20]. Furthermore, there is a high

variability of ED_{50} values in the different tail clip studies[20,27,28]. Another model under anesthesia is the severe tail clip model. It is a faster method since the observation period is only 20 min, but it is less sensitive to procoagulant activity. Doses higher than 200 IU/kg of FVIII were necessary to achieve a statistically significant reduction of blood loss compared to vehicle-treated animals[25]. In the vena saphena model[30], the ED_{50} value for bleeding time was 8.1 ± 2.2 IU/kg, and ED_{50} for average blood loss was 5.1 ± 2.1 IU/kg, also less sensitive than our refined model in regards to both parameters. Furthermore, this model requires delicate work to perform the vessel injury and multiple repeated interventions which, if not performed reproducibly, could influence the outcome.

In the traditional TVT survival model, survival is evaluated for a period of 24 h after dosing, during which bleeding or re-bleeding can occur at any time. Thus, efficacy in the TVT survival model requires the procoagulant effect of treatment to persist for at least the majority of the 24-h observation period. In the method presented here, we assess acute effects between 5 min and 40 min after dosing; thus, a direct comparison of ED_{50} values is difficult to establish since higher doses will be needed in the survival models in order to maintain hemostatic coverage during the latter part of the observation period. However, if so desired, the optimized TVT model can be used to evaluate the duration of hemostatic coverage by introducing a delay between dosing and the bleeding procedure. This has been described for a previous version of our optimized TVT model where performing the TVT 24 h after dosing resulted in an ED_{50} of roughly 10–15 IU/kg for unmodified rFVIII[26]. As noted in **Table 1**, in tail-transection models with a 24-h survival as an endpoint, ED_{50} values of 21 IU/kg [9] and 58 IU/kg[36] of rFVIII have been reported. Similarly, tail clip survival models[8] also require higher procoagulant doses than their acute counterparts.

In perspective, several different coagulation factors (FVIII, FVIIa, FIX) and derivatives, some of which are now marketed, have been evaluated with the optimized model using different hemophilic mouse strains[26,38,43–48]. We have also been able to adapt the model to the study of on-demand interventions by dosing after the transection, just prior to the first challenge. Furthermore, we have successfully used this model to evaluate bispecific antibodies with procoagulant activity (Østergaard et al., accepted for publication in Blood), overcoming species cross-reactivity challenges by dosing human FIX and human FX. This demonstrates the versatility and translational value of the model development of new medicines in the hemophilia field, such as platelet-targeted strategies[49]. AAV-based or genome editing strategies can also be evaluated in cases where there is a surrogate that is pharmacologically active in mice. Therefore, this optimized TVT bleeding model is an alternative to tail vein transection and tail clip survival models, as well as a valuable alternative to other bleeding models under anesthesia. This model is more humane compared to the survival model and an example of Refinement as the animals do not experience pain and suffering. In our view, the sensitivity to clinically relevant dose levels, relative technical simplicity, and avoiding death/near-death as endpoints are significant advantages.

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DISCLOSURES:

The authors are or were employees and/or shareholders of Novo Nordisk A/S at the time this research was carried out.

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601

Figure 1

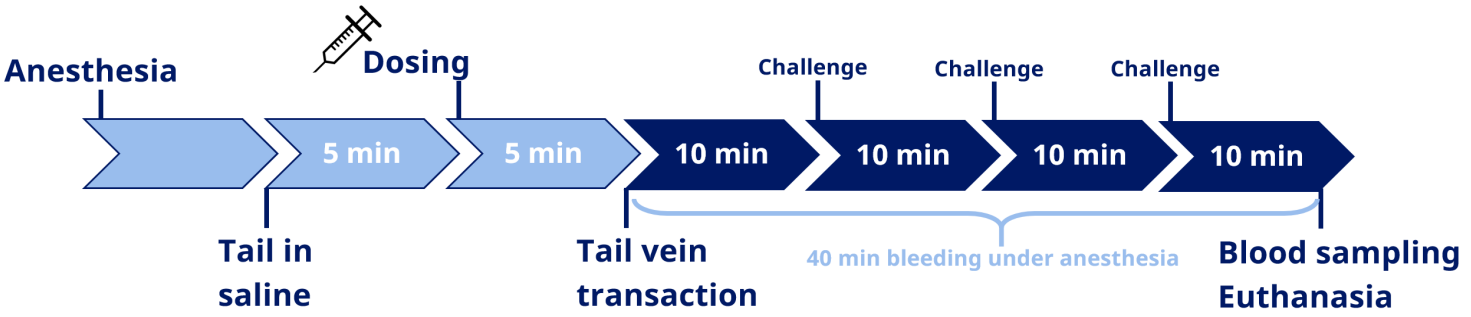
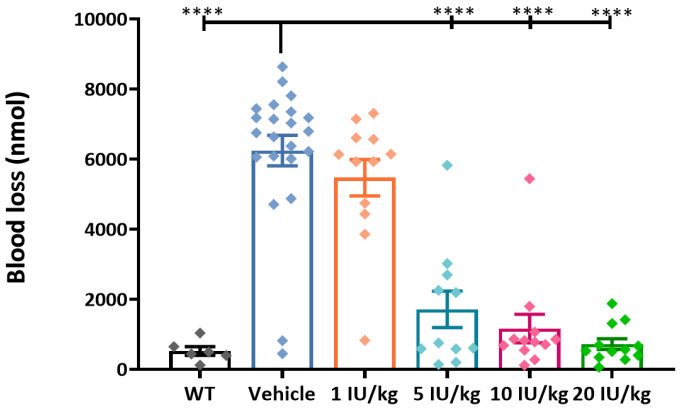


Figure 2



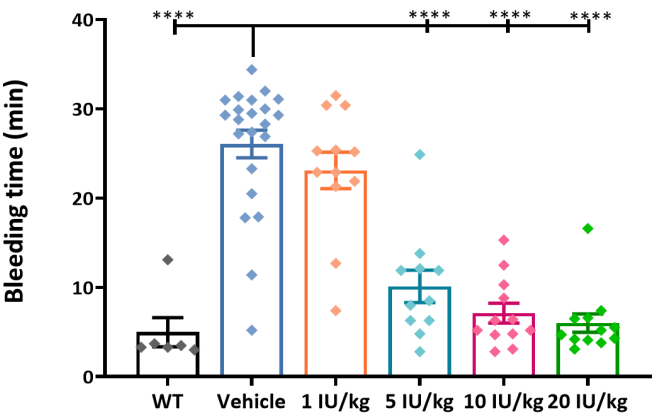
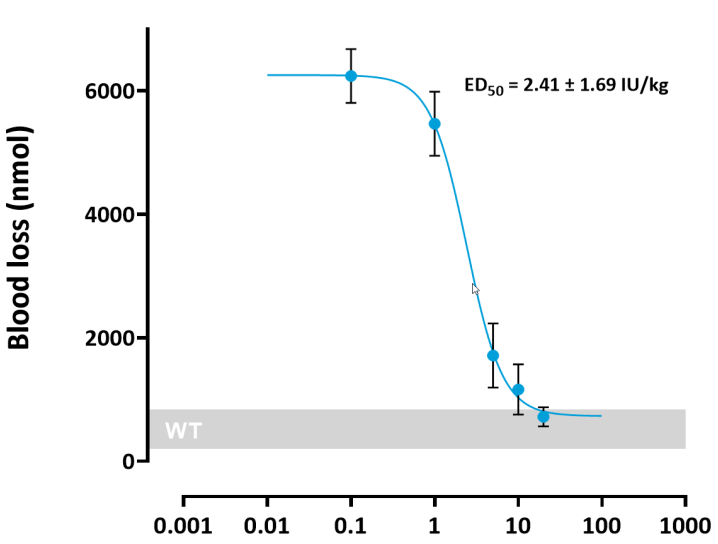
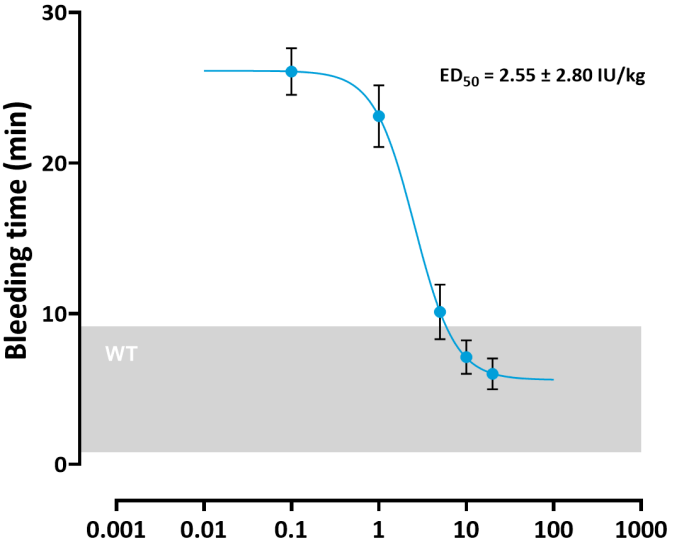


Figure 3





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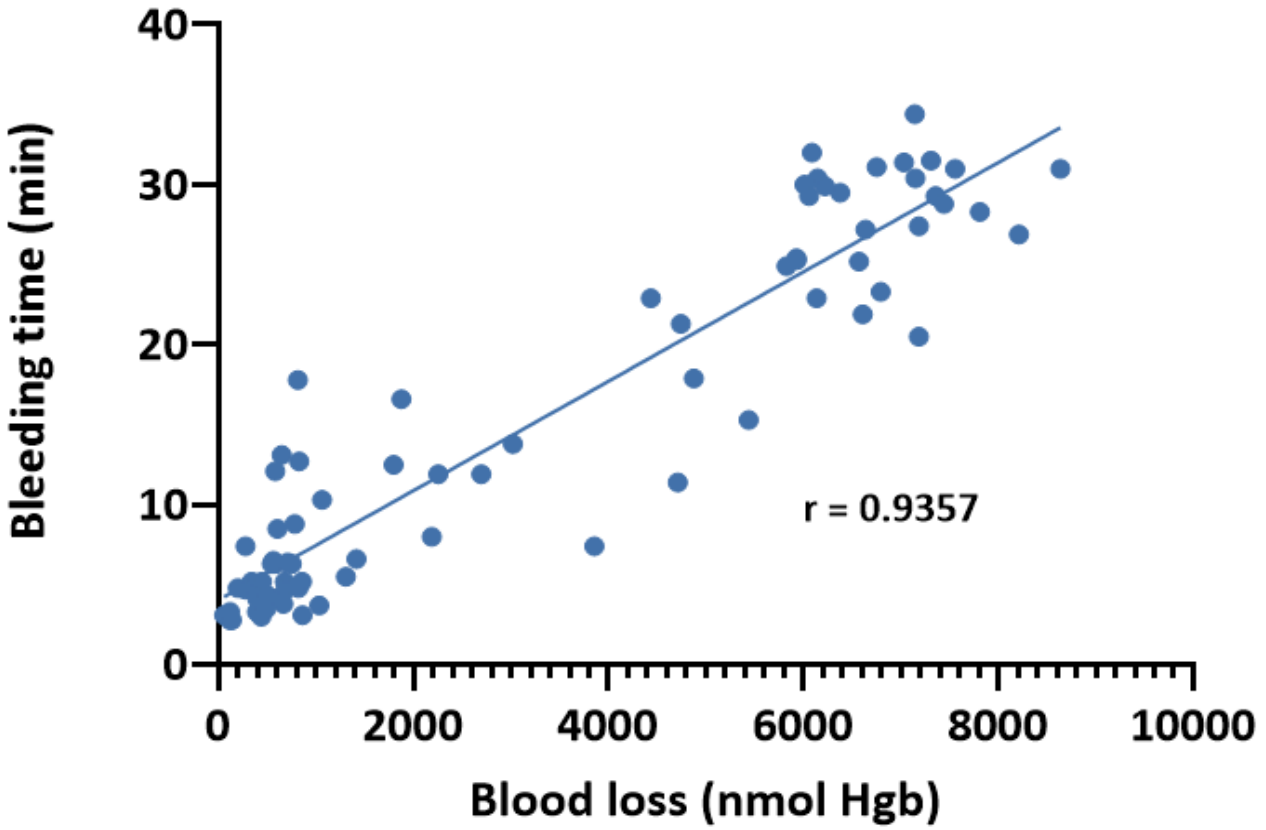


Figure 5

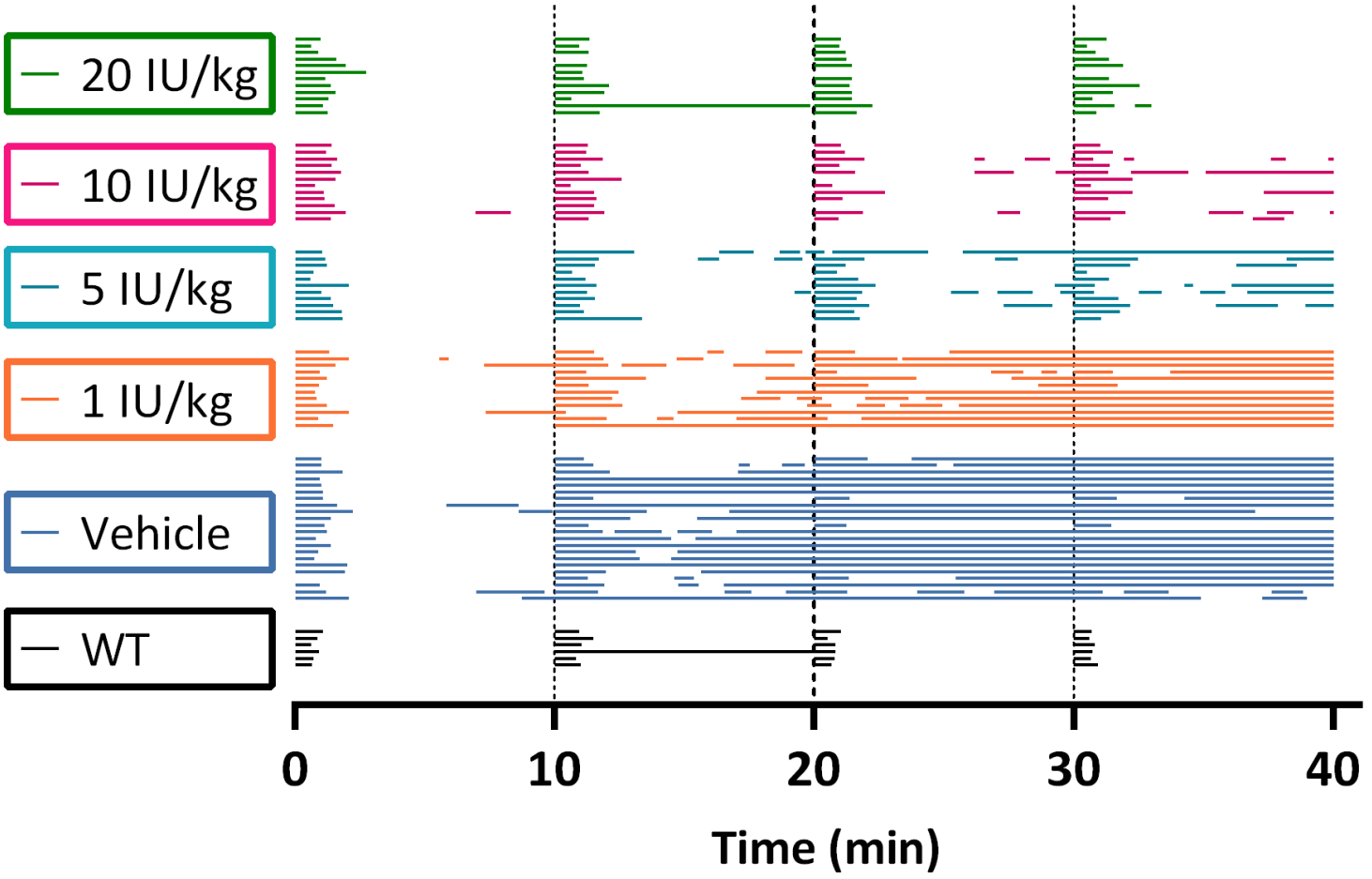


Figure 6

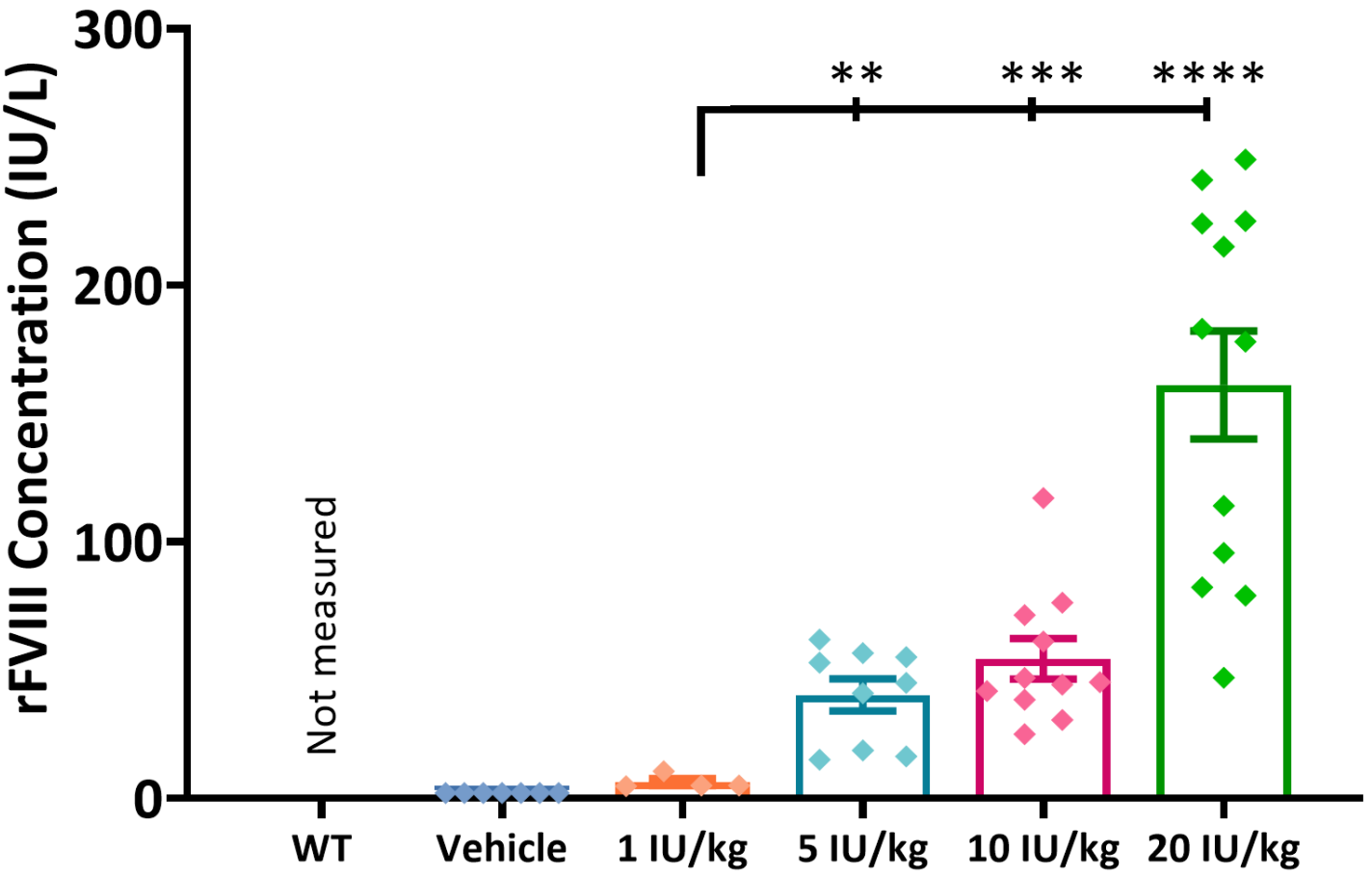


Figure 7

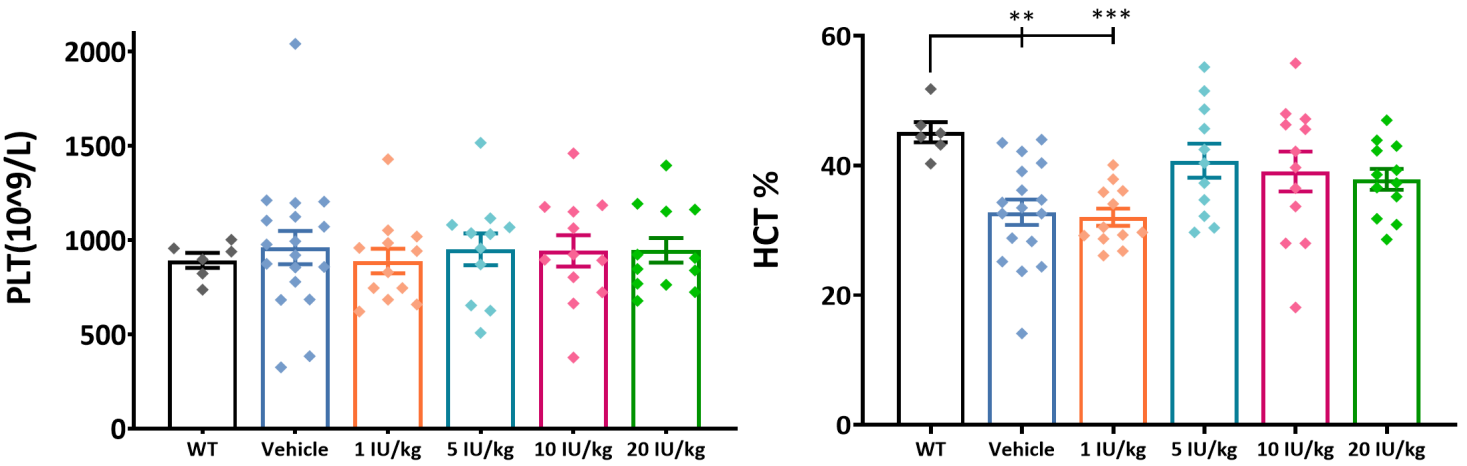
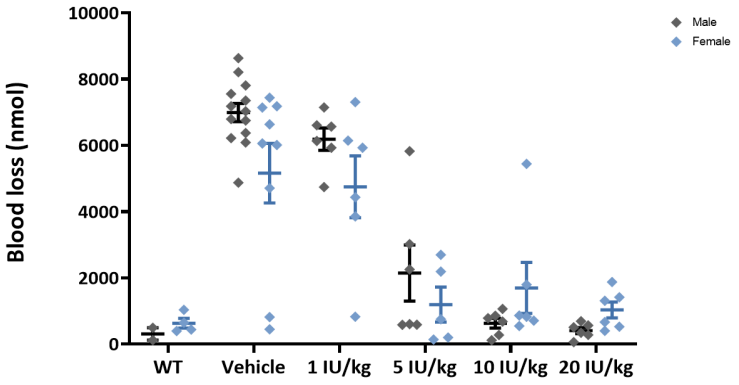


Figure 8

Click here to access/download;Figure;Figure 8.pdf



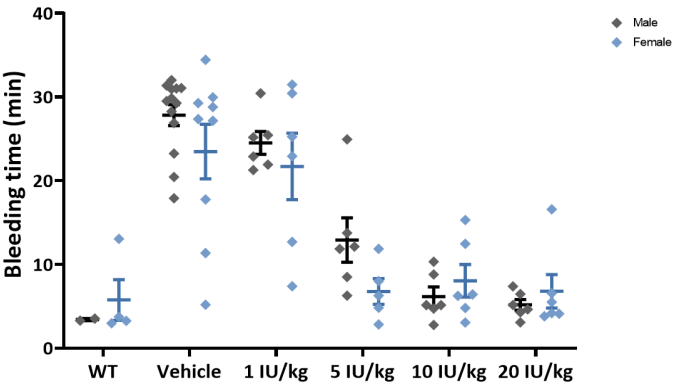


Table 1

		ED50 bleeding time (IU/kg
Models under anesthesia	Optimized tail vein transection model	2.6 ± 2.8
	Vena saphena model[30]	8.1 ± 2.2
	Moderate tail clip (L=3mm)[27]	not reported
	Moderate tail clip (L=4mm)[28]	39
	Moderate tail clip (L=4mm)[20]	not reported
Survival models	Tail vein transection survival model[36]	not reported
	Tail vein transection survival model[10]	not reported

ED50 blood loss (IU/kg)	ED50 survival (IU/kg)
2.4 ± 1.7	not relevant
5.1 ± 2.1	not relevant
4.6 ± 0.5	not relevant
28	not relevant
53	not relevant
not reported	58
not reported	21

Dear editor and reviewers,

Thank you for the relevant feedback. Below, are all the comments from the editorial and the peer reviewers addressed individually.

Best regards,

Ariadna Carol Illa

REBUTTAL LETTER

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

A general revision of the manuscript has taken place.

2. Please revise your title; usually anaesthesia, although important in many animal studies, is not focused on in the video and especially in the title.

It is an interesting point. Traditionally, survival bleeding models in haemophilia have been conducted without anaesthesia, and even though there is a switch to using models under anaesthesia; it cannot be assumed that it is always under those conditions. Therefore, the title should emphasize the fact that the model is conducted under anaesthesia.

3. Please use the period to indicate decimal and not comma, e.g., 0.9% NaCl (Table of Materials) not 0,9% NaCl.:

The change has been made in the Table of Materials and reviewed in the article.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Lysebio etc

The changes have been made and as well properly referenced in the Table of Materials.

5. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please mention how proper anesthetization is confirmed.

b) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

The changes have been applied on protocol sections 2.4. and 2.5.

6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The protocol has been revised for personal pronouns.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described

in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The protocol has been reviewed and the respective changes annotated.

8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol (e.g., section 1) to the introduction or Discussion.

The first section of the protocol has been moved to the introduction.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Further details in the protocol has been added, specifically regarding the treatment of samples.

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

The mentioned format has been changed into the manuscript.

11. Please add any limitations of this method to your discussion section.

The model limitations have been further described in the discussion.

On one hand, the TVT model produces a venous-only bleeding, and therefore the procoagulant doses needed might not be reflective of a severe arterial injury, in which a higher dose would be needed. Therefore, another bleeding model should be used, as for example the tail clip model.

On the other hand, this model requires reproducible technical skills. The initial cut and the challenge to prevent platelet aggregation on the injury side can vary depending on the operator, therefore it is very important to standardize the methodology so there is less uncontrolled variability.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

All the references have been updated following these guidelines.

13. Please sort the Materials Table alphabetically by the name of the material.

Changes has been made.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Ariadna et al refined tail vein transection (TVT) bleeding model in anaesthetized hemophilia mice, and proved the assay as a sensitive in vivo method for assessment of haemophilic bleeding in a standardized and humane manner. What's more, this optimized TVT bleeding model used blood loss and bleeding time as endpoints, refining other models and avoiding death as an endpoint. This manuscript describe a protocol that will be of interest to other scientists, the efficacy of the protocol had be fully demonstrated. There are a few concerns under below should be settled before acceptance.

Major Concerns:

1. They need to discuss differences among various treatment such as AAV based/genome editing/platelet-targeted strategy when using TVT model for therapeutic evaluation, just like "evaluate bispecific antibodies with procoagulant activity" in the manuscript.

Thank you for the suggestion, we have added the discussion of these different strategies onto the manuscript.

Minor Concerns:

1. The authors could show the exact time point for injection of rFVIII in the PROTOCOL so that it may avoid misunderstanding of readers.

It is now specified in the protocol section 3.1, and further mentioned in the discussion section. Since the dosing time it is not specific for this protocol, but rather for the compound or experimental conditions that are intended to be assessed, for example dosing 1h before the TVT.

2. The journal style (cited journal name) should be consistent across the references, for example, #12/#24/#25 and so on.

All the references have been updated following the JoVE guidelines and the journal names homogenized.

Reviewer #2:

Manuscript Summary:

In this manuscript, Carol Illa and colleagues reported a tail vein transection bleeding model to evaluate the effect of procoagulant treatment in haemophilia A mice. This is a well-designed model, which will be a beneficial tool in haemophilia research. There are some concerns in the current version of this protocol.

Major Concerns:

1. They should describe how the volume of blood loss from the TVT test was determined or measured in the method section "8.0 Treatment of samples". This section is poorly written, and unclear how they determine the amount of blood loss from each animal in the test.

The blood loss is determined by measuring the haemoglobin concentration in the saline measured by the absorbance. The absorbance is then converted to nmol haemoglobin by the use of a standard curve prepared from human haemoglobin. Section 8.0 has been expanded to clarify the sample treatment.

2. In the result section, lines 190-193, the authors used "nmol Hgb" to define the blood loss, and they defined 1000 nmol Hbg equivalence to around 125 µl. This is not reflecting the volume of blood lost. They should comment on what hemoglobin level in that 125 µl blood they referred to. Using "nmol Hgb" to quantify blood loss could have a potential problem if an animal has anemia before the test. One way to quantify the blood lost from each animal is to test the hemoglobin level in each animal before the TVT test and then, based on the amount of the hemoglobin lost in the test to calculate how many microliters of blood loss from the animal during the test.

Since the blood loss is measured in Hgb, we added the conversion factor so the reader could convert the amount of Hgb to volume in mL if interested. The conversion factor corresponds to 8 mmol/L which equals 12.89 g/dL. In this paper [1] the haemoglobin level in C57Bl/6 mice is reported which falls into the mentioned values.

Of course, if the mouse has a lower haematocrit, the parameter will be higher than measured; but Hbg gives a good impression of functional blood although the total volume might deviate. Besides, in the haematological analysis, these animals don't have significant differences in the haematocrit (HCT%) measurements between groups (*see figure below)*.

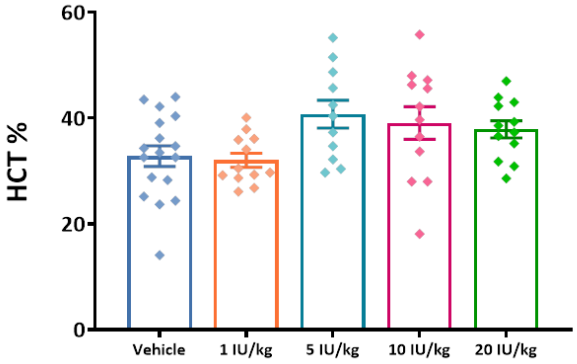


Figure 1. HCT% values for the Factor VIII knock-out mice (F8-KO).

As you mention, for example a 5µl capillary tube could be obtained at baseline from the retrobulbar plexus to calculate the blood volume lost. But an invasive blood sampling right before the experiment could alter the bleeding capacity of the animals.

3. While the TVT assay described in this manuscript has its advantage, e.g., sensitivity to low levels of plasma FVIII and animals are fully anesthetized during the test, only the vein is injured. In patients, artery injury may lead to more severe bleeding and requires higher doses of FVIII infusions. From this point of view, the dose-effect in TVT might not reflect the effect in artery injury. Each assay has its advantages and disadvantages. They should comment on the limitation of the assay in the discussion.

Thank you for the feedback; the model limitations on arterial injury have been added to the discussion.

Minor concerns:

1. It is unclear what they meant by "plasma" in line 168 since blood loss was collected in a tube with a large volume of saline.

This protocol section has been re-written to ensure a more clear and understandable procedure.

2. Lines 217-218 and Figure legend for Figure 6: if FVIII levels were determined by ELISA, they should specify the assay instead of saying antibody-based method (line 251). Instead of saying the assay is not sensitive to murine FVIII, they may want to rephrase "assay is specific for detecting human FVIII" (lines 217-218).

The assay type has been specified; in fact it is a Luminescent oxygen channelling assay (LOCI).

Reviewer #3:

Manuscript Summary:

In this work Illa and colleagues provide a detailed characterization of a tail vein transection model for the study of hemophilia A treatments in mice. This work builds on a previous publication by a number of the co-authors

that initially characterized the fully anaesthetized hemophilia A mouse tail vein transection bleeding model. As the authors highlight a standardized technique has been lacking in this field and the authors protocol provides a useful appropriate technique to improve standardization. The authors provide complementary data to their previously published work on the effect of rFVII treatment on bleeding in this model.

Major Concerns:

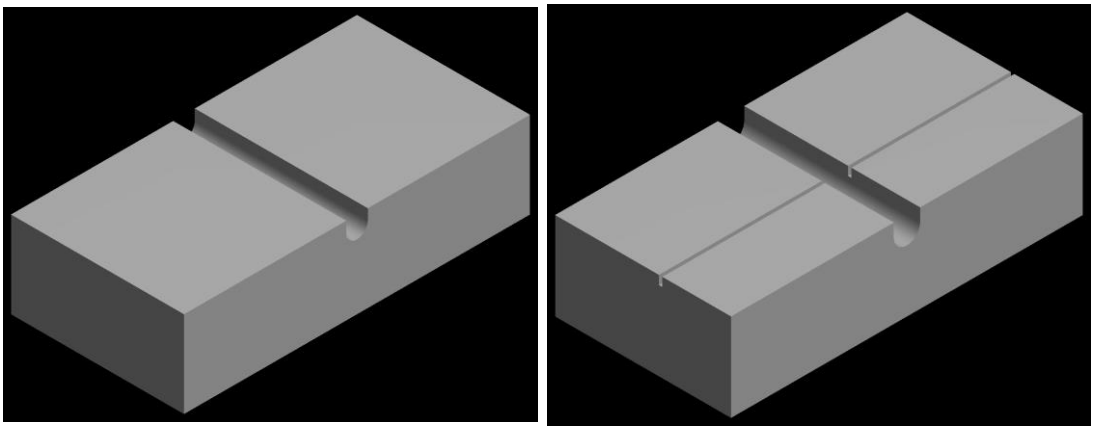
1. The reproducibility of this technique by other investigators hinges on the ability to replicate the tail vein transection described by the authors. The authors refer to a cutting block in the protocol and reference this in the materials section. However, further information would be of benefit to enable replication. Could the others provide images and schematics (or even a CAD model) for the cutting block so that other investigators might be able to make this device.

Thanks for the suggestions, below the specifications of the blocks are mentioned. The general dimension of both blocks are 20Lx40Wx10H mm.

The measuring template *(left picture)* is made in aluminium: the groove has 2.5mm depth and 2.5 width; with a radius of 1.25.

The cutting template *(right picture)* is made in stainless steel, more resistant to the use with the scalpel. The groove has a 3mm depth and 3mm width; with a radius of 1.5.

Furthermore, the specific dimensions are also described in the Materials table.



2. Some of the data presented appears to be non-normally distributed. The authors are encouraged to conduct and provide details of normality tests used. Non-normal data is better represented as median and interquartile range as opposed to mean and standard error of the mean and should be analysed using a non-parametric statistic such as Kruskal-Wallis. Appropriate data handling and processing is important when providing representative data on a technique that is hoped to become a standard approach.

Data should always be analysed by appropriate methods. We encourage use of normality test and adequate statistics. Over a range of larger studies, we have found the mentioned statistical analysis to perform well. Therefore, we suggest that groups are compared in terms of differences between means.

We agree that for the description of the sample values, median and interquartile range are useful alternatives to average and standard deviations. Our purpose to include means and SEM on the plot of sample values was to help visualizing the outcome of the statistical analysis. The mean of a set of sample values will be better described by a normal distribution than the set of sample values, and therefore a confidence interval based on the assumption of a normal distribution of the mean will be reasonable.

The Kruskal-Wallis statistic is useful when the compared groups have identical forms, but different position. This is not what we observe: in general we observe that differences in means go together with differences in variance (which is the reason for applying Welch ANOVA).

Minor Concerns:

1. The figure legends provide limited details of the data represented. For example, in figure 2 details of the number of mice per group, the values referred to on the x-axis (presumably rFVII) would be beneficial. Similar details for the other figures would be beneficial.

Further data describing the figures have been added. Since the different observations are plotted as one point for every animal and all the animals are plotted in all the graphs, the number of mice per group is not described in the legend.

2. In figure 4 it appears that the authors have conflated the R2 value of goodness of fit of the trend-line with the r value of the pearson correlation. Could the authors check these values. Further the data also looks non-normal and may be more appropriately assessed using the non-parametric spearmans rank test.

Thank you for the observation, the r value of the Pearson correlation has been added now to the graphs.

3. Could the authors detail how blood count data were generated in the protocol and provide details of equipment in the materials?

The blood count data was generated on a Sysmex XT 200IV haematological analyser. This information has been added both in the protocol as well as in the Table of Materials.

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1 Santos, E. W. *et al.* Hematological and biochemical reference values for C57BL/6, Swiss Webster and BALB/c mice. *Brazilian Journal of Veterinary Research and Animal Science.* **53** (2), 138-145, doi:10.11606/issn.1678-4456.v53i2p138-145, (2016).