Published in final edited form as:

Thromb Haemost. 2010 August 2; 104(2): 366–375. doi:10.1160/TH09-09-0672.

Thrombogenesis with continuous blood flow in the inferior vena cava: A novel mouse model

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Summary

Several rodent models have been used to study deep venous thrombosis (DVT). However, a model that generates consistent venous thrombi in the presence of continuous blood flow, to evaluate therapeutic agents for DVT, is not available. Mice used in the present study were wild-type C57BL/6 (WT), plasminogen activator inhibitor-1 (PAI-1) knock out (KO) and Delta Cytoplasmic Tail (Δ CT). An electrolytic inferior vena cava (IVC) model (EIM) was used. A 25G stainless-steel needle, attached to a silver coated copper wire electrode (anode), was inserted into the exposed caudal IVC. Another electrode (cathode) was placed subcutaneously. A current of 250 μ Amps over 15 minutes was applied. Ultrasound imaging was used to demonstrate the presence of IVC blood flow. Analyses included measurement of plasma soluble P-selectin (sP-Sel), thrombus weight (TW), vein wall morphometrics, P-selectin and Von Willebrand factor (vWF) staining, transmission electron microscopy (TEM), scanning electron microscopy (SEM); and the effect of enoxaparin on TW was evaluated. A current of 250 μ Amps over 15 minutes consistently promoted thrombus formation in the IVC. Plasma sP-Sel was decreased in PAI-1 KO and increased in Δ CT vs. WT (WT/PAI-1: $p=0.003$, WT/ Δ CT: $p=0.0002$). Endothelial activation was demonstrated by SEM, TEM, P-selection and vWF immunohistochemistry and confirmed by inflammatory cell counts. Ultrasound imaging demonstrated thrombus formation in the presence of blood flow. Enoxaparin significantly reduced the thrombus size by 61% in this model. This EIM closely mimics clinical venous disease and can be used to study endothelial cell activation, leukocyte migration, thrombogenesis and therapeutic applications in the presence of blood flow.

Keywords

Endothelial dysfunction; thrombosis; electrolytic injury; inflammation; animal model

Introduction

Animal models serve a vital role in deep venous thrombosis (DVT) research in order to study thrombus formation/ resolution and to test potential therapeutic compounds (1-7).

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New compounds to be utilised in the treatment and prevention of DVT are currently being developed. Specifically, those being investigated as potential target molecules include P-selectin, plasminogen activator inhibitor-1 (PAI-1) and von Willebrand factor (vWF) (8-12). The delivery of potential therapeutic antagonist compounds to an effected thrombosed vein has been problematic. In the context of therapeutic applications, a model that uses partial stasis and consistently generates thrombi within a major vein has not been established. The ideal DVT model would permit blood to flow past the developing thrombus and therapeutic agents to enter the systemic circulation. Here, we present a new mouse model, which allows thrombus formation to occur in the presence of blood flow.

Materials and methods

Mice

Strains of male mice used for this study included: wild-type C57BL/6 (WT, n=52) (Charles River Laboratories, Wilmington, MA, USA), PAI-1 knock out (PAI-1 KO, n=8) with increased fibrinolytic properties (Daniel Lawrence, Ann Arbor, MI, USA) and hypercoagulable Delta Cytoplasmic Tails (Δ CT, n=8) (Denisa Wagner, Cambridge, MA, USA). Δ CT mice produce P-selection protein lacking its cytoplasmic domain that results in high circulating levels of plasma soluble P-Selection (sP-Sel). Mice distribution is shown in (►Table 1).

Animal model

Mice, weighing 20 to 25 grams (g), were anaesthetised with isoflurane (2%), placed in dorsal recumbency and the inferior vena cava (IVC) was approached directly via a midline laparotomy utilising an aseptic technique. Venous side branches were ligated using 7–0 Prolene suture (Ethicon, Inc, Somerville, NJ, USA). Back branches remained patent.

In the electrolytic IVC model (EIM), a 25G stainless-steel needle, attached to a 30G silver coated copper wire (KY-30–1-GRN, Electrospec, Dover, NJ, USA) is inserted into the exposed caudal IVC (►Fig. 1), and positioned against the anterior wall (anode). Another wire is implanted subcutaneously completing the circuit (cathode). In order to establish the optimal parameters for consistent thrombus formation, current intensities ranging from 100 to 300 micro-amperes (μ Amp) applied for 10 to 45 minutes (min) were tested (data not shown). A current of 250 μ Amp over 15 min was applied using a Grass S48 square wave stimulator and a constant current unit (Grass Technologies, An Astro-Med, Inc., West Warwick, RI, USA). The direct current results in the formation of toxic products of electrolysis that erode the endothelial surface of the IVC promoting a thrombogenic environment and subsequent thrombus formation (13,14). In sham animals, the needle was placed into the IVC for 15 min without application of the current.

Ultrasound (US) imaging was done of the IVC, at baseline and two days post EIM, to demonstrate the presence of a blood flow channel. At euthanasia, blood was collected via cardiac puncture for measurement of plasma sP-Sel and the IVC/thrombus was harvested for thrombus weight (TW), morphometrics, P-selectin and vWF staining, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

Thrombus weight (TW)

In brief, groups of mice were analysed for (wet) thrombus weight (TW). At the time of euthanasia, the IVC and the associated thrombus was removed and weighed (4).

Mouse soluble P-selectin

Mouse plasma samples from all strains (n=8 per group) were evaluated for circulating P-selectin levels two days post thrombosis. EDTA anti-coagulated blood was processed according to the manufacturer's ELISA kit for plasma sP-Sel (R&D Systems Minneapolis, MN, USA). All samples were run in duplicate and the results were normalised to total protein using the standard BCA assay (Pierce Rockford, IL, USA).

IVC thrombus and flow channel measurement

The area of venous thrombi and their associated flow channels from H&E stained specimens (200X) were analysed using SCION IMAGE Beta 4.0.2 imaging software (Scion Corporation, Frederick, MD, USA). Mouse IVC US double blinded studies were done before and two days post thrombus induction using a Siemens Sonoline Antares (Siemens Medical Systems Inc, Issaquah, WA, USA) and a linear multi-hertz (12–15 MHz) transducer. A minimum of five US images per mouse were performed and the measurements were averaged.

Scanning electron microscopy (SEM)

To visualise the vein wall intimal surface three dimensionally, IVC samples (n=5, Table 1) were examined under SEM (15). Briefly, after fixation, the IVC was opened and fixed to cover slips with the endothelium facing up. Specimens were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated using graded ethanol concentrations, mounted on stubs, sputter coated with gold, and then subjected to SEM using a JEOL JSM-840 Scanning Electron Microscope (Tokyo, Japan).

Transmission electron microscopy (TEM)

Mice IVC (n=10) were perfused with 4% paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M Sorensen's buffer (Table 1). IVCs were excised and then emersion fixed in the same fixative overnight at 4°C. Tissues were dehydrated in graded ethanol concentrations, treated with propylene oxide, and embedded in Epon epoxy resin. Semi-thin sections were stained with toluidine blue for tissue identification. Selected 70 nm thickness sections were post stained with uranyl acetate and lead citrate. They were examined using a Philips CM100 electron microscope at 60 KV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system, using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA, USA).

Vein wall morphometrics

Mouse IVCs (n=5) were sectioned two days post thrombosis and stained with hematoxylin and eosin (H&E) and examined under oil immersion light microscopy. Five representative high-power fields (HPFs) were examined around the vein wall, and the cell counts analysed (Cells/5HPFs). Cells were identified as neutrophils (NEU), monocyte/macrophages (MON), or lymphocytes (LYM) on the basis of standard histological criteria in a blinded fashion by a board-certified veterinary pathologist (4,5) (Table 1).

P-selectin and vWF staining

Mouse IVCs samples were harvested immediately after electrolytic injury and were fixed, embedded in paraffin, cut into 4 µm sections and stained for P-selectin and vWF. Primary antibodies were diluted for P-selectin, Goat-Anti-Mouse P-selectin (1:800) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and for vWF, Rabbit anti-mouse VWB (1:25) (Abcam Inc. Cambridge, MA, USA). Secondary antibody and ABC step used reagents from commercial kits (P-selectin – Goat Elite kit and vWF – Vector Rabbit Elite – Vector Labs,

Burlingame, CA, USA). Colour development occurred with DAB substrate and slides were counterstained with Hematoxylin QS (Vector Labs).

Low-molecular-weight heparin (LMWH) treatment protocol

To evaluate the therapeutic effects of enoxaparin in the mouse thrombosis model, wild-type mice (n=10) were administered saline or enoxaparin (Aventis-Pharma, Bridgewater, NJ, USA), 6 mg/kg/ day subcutaneously, starting immediately post procedure until two days post thrombus induction. TWs from these animals were determined and the results were compared with previous data from the IVC ligation model using the same treatment (n=10) (Fig. 5).

Temperature test

In order to prove that the electrolytic procedure does not cause thermal injury to the venous endothelium, a tissue implantable thermocouple microprobe, IT-23 (Physitemp Instruments Inc, Clifton, NJ, USA) was introduced into the IVC via a side branch associated with the study area (n=2). *In vivo* temperature measurements were recorded within the IVC before, during and after induction of electrolytic injury (Fig. 6).

Statistical analysis and animal use

Statistical analysis included mean $\pm$ standard error of mean (SEM). Statistical significance was calculated using an unpaired t-test with Welch's correction (GraphPad Software, Inc., La Jolla, CA, USA). Significance was defined as $p \leq 0.05$. Direct comparisons between the groups were made for thrombus weight, plasma soluble P-selectin, vein wall morphometrics, and LMWH therapeutic groups. A Pearson correlation coefficient with regression was done to analyse the relationship between thrombus weight and plasma soluble P-selectin. All work was approved by the University of Michigan, University Committee on Use and Care of Animals and was performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (16).

Results

EIM and thrombus formation

From the day 2 analysis the EIM consistently generated IVC thrombosis in all mice (100%) for this study (n=30). On the contrary no thrombi were observed in the IVC of mice undergoing sham operation (n=9) at same time point (Table 1).

Thrombus weight and plasma soluble P-selectin

There was a direct correlation found between TW and plasma sP-sel. The mean TW in WT mice (n=5) was 0.0177 ± 0.0028 g; in PAI-1 KO mice (n=5) it was 0.0087 ± 0.0007 g and in Δ CT mice (n=5) the TW was 0.0268 ± 0.0018 g (►Fig. 2A). Plasma sP-sel was decreased in PAI-1 KO mice (5.15 ± 2.2 ng/ml) and increased in Δ CT mice (30.99 ± 1.4 ng/ml), compared to WT (9.29 ± 4.6 ng/ml) (WT/PAI-1: $p=0.003$; WT/ Δ CT: $p=0.0002$) (Fig. 2B). This data shows a direct correlation between TW and plasma sP-sel in all three strains ($r^2=0.74$) in this animal model (Fig. 2C).

Thrombus/ flow channel measurements

The evaluation of venous thrombi on day 2 from all mice in this study showed that each thrombus had a downstream (towards the feet) head and an upstream tail (towards the heart) at harvest (►Fig. 3A). This shape suggested laminar thrombus formation in the presence of blood flow. The thrombus area, calculated from H&E stained specimens, was 74% and the

blood flow channel was 26% (Fig. 3B). Ultrasound performed before EIM demonstrated patency in all IVCs (n=5). The mean IVC thrombus stenosis on day 2 was 71% (Fig. 3C-D).

Endothelial cell activation and neutrophil migration

Vein wall samples that were evaluated by SEM immediately after electrical stimulation showed small areas of endothelium denudation with erythrocytes and fibrin deposition, mainly near the needle implantation site (►Fig. 4 A-B). The remainder of the IVC interface showed intact endothelium. Leukocyte migration within the first hour after electrical stimulation was documented by TEM for the entire IVC (Fig. 4 C-F). In addition, mice had significantly higher numbers of vein wall neutrophils (NEU) on day 2 when compared to monocytes (MON) [22.9 ± 7.1 vs. 1.0 ± 0.4 cells/5 HPs, $p=0.01$], and lymphocytes (LYM) [22.9 ± 7.1 vs. 1.8 ± 0.4 cells/5 HPs, $p=0.01$] (Fig. 2). An increase in positive P-selectin and vWF protein staining was detected immediately after electrical stimulation in the IVC vein wall by light microscopy.

LMWH therapy

TW in the enoxaparin group was reduced 61% compared to controls (0.0069 ± 0.001 g, n=5 vs. 0.0178 ± 0.003 g, n=5, $p=0.0153$). Of interest, preliminary studies using the same therapeutic approach in our complete stasis model of thrombosis showed a 37.1% reduction in TW (0.0207 ± 0.002 g, n=5 vs. 0.0329 ± 0.001 g, n=5, $p=0.0153$) (►Fig. 5).

EIM does not use heat to generate thrombosis

In the temperature test, no significant variation from normal mice reference body temperature ($36.5 - 38.0^{\circ}\text{C}$ [17, 18]) was observed before, during or after electrical stimulation as measured in the IVC (►Fig. 6).

Discussion

Currently, in the literature, there is no mouse strain that spontaneously develops a DVT. However, the mouse offers some unique physiological and genetic characteristics that make it an extremely useful tool to evaluate venous thrombosis. None of the existing mouse DVT models provide both a consistent thrombi size and a flow channel for therapeutic application in the IVC.

Several mice models have been used for DVT research including: photochemical (19), stasis (4-6), electrolytic stasis (2), IVC stenosis (7), and mechanical trauma (20,21). Day et al. have reviewed the current options (22). The IVC *photochemical injury model* uses Rose Bengal dye activated by a green light laser (540 nm) for 15 min. This technique, modified from Eitzman et al. (22,23), produces a subtle endothelial injury that activates the vascular endothelium, but produces inconsistent thrombosis (personal communication, 9–1–09 DDM).

The *stasis mouse model of DVT, or ligation model*, places a non-reactive suture ligation around the IVC just below the renal veins to produce complete blood stasis (3-5,12,22). In this model, the IVC yields quantifiable amounts of vein wall tissue and thrombus. It has proven useful for evaluating interactions between the vein wall and the occlusive thrombus and for assessing the progression from acute to chronic inflammation. The major disadvantage with the stasis model is that the lack of blood flow inhibits the maximum effect of administered systemic therapeutic agents on the thrombus and vein wall.

Another *murine model of DVT* forms a femoral vein thrombus in a temporal fashion after electrical stimulation (2). This thrombosis model is useful for evaluating venous

thrombogenesis. Thrombosis is induced at the site of the electrode and grows in a sequential fashion. The main limitation of this model is the small yield of thrombus and vein wall mass for protein and gene expression analysis, in comparison to the IVC thrombosis models. The IVC thrombosis model presented in our study overcomes this limitation, allowing an appropriate size sample that can be subjected to further analysis. Cooley et al. speculated that thrombus formation in their model is the result of either direct electrical injury (resistance heating) or a free radical induced injury to the vein wall. However, Jackson et al. (14) demonstrated that injury due to free radicals is the main mechanism leading to thrombus formation. Importantly, we have demonstrated that heat does not participate in thrombus formation in our model. This is important information because heat applied to the vein wall will inevitably lead to protein denaturation. Thus, while Cooley's model induces DVT in the femoral vein, our model induces DVT formation in the IVC with the expected larger thrombus size, which ultimately provides a larger sample (vein wall and thrombus) for research assays minimising the number of animals required per study.

In the *mouse stenosis model* a silk ligature is placed around the IVC tied down on a 5/0 Prolene which is then removed to reduce blood flow. This, reduction in blood flow combined with temporary endothelial compression with haemoclips, produces a laminar thrombus allowing the study of cellular kinetics and effects of therapeutic agents (1,7). It is technically simple, easily reproducible and delivers a large sample. However, the degree of stenosis is inconsistent leading to a variability in thrombi sizes. Consistency in thrombus formation is a critical aspect of research aimed to investigate drug effect on thrombogenesis. EIM ensures consistency in thrombus size required in this setting.

Finally, the *mechanical injury model* measures the kinetics of temporal thrombus growth and resolution in the femoral vein. This model uses an external mechanical force to damage the endothelium. Fiber optic technology is used to trans-illuminate the vein to visualise and record thrombosis. One disadvantage is that this model only allows studying early stages of thrombosis. Also, expensive and specialised optical equipment is required to visualise thrombus generation and resolution in this model (20,21,24).

The EIM formed a laminar thrombus in the presence of continuous blood flow in all mice, as demonstrated by histology and ultrasound. Plasma sP-sel is currently being studied as a marker for DVT and a correlation between plasma sP-Sel and TW was found using EIM, which was similar to that observed in the mouse ligation model (data unpublished) (Fig. 3 A-B).

Small areas of endothelial denudation were observed at the area of the needle introduction which can contribute to thrombus formation. However, IVC endothelial activation with neutrophil migration was demonstrated by the TEM, cell counts and confirmed by P-selectin and vWF immunohistochemistry staining. The TEM images obtained during the first hour after EIM demonstrate intact endothelial lining with neutrophil migration into the vein wall (Fig. 4). In parallel, the P-selectin immunohistochemistry further demonstrated intact endothelial cells and an increase of P-selectin at the level of the endothelial cells and also in the lumen. In the lumen, it can be hypothesised that P-selectin is provided by activated blood components due to free radicals liberated into the blood flow (i.e. platelets). Taken together all of these findings highly support a venous-type thrombogenesis.

Of great interest, this new mouse thrombosis model generates consistent partially occlusive venous thrombi with the presence of a flow channel. In addition, IVC thrombus was observed in 100% of the mice (n=30), using 250 μ Amp for 15 min, which makes this model reproducible. The fact that no thrombus was observed in the sham surgeries suggests that the

combination of electric current and endothelial activation were critical to develop the thrombus in the IVC.

The development of this partial blood flow model is important because it allows the evaluation of current and new therapies targeting venous thrombosis. The physiological importance of this concept was demonstrated when direct comparisons were made between our electrolytic model and a total occlusion thrombosis model. Mice given LMWH and undergoing EIM showed a 61% decrease in thrombus size compared to a 37.1% decrease in the complete occlusion thrombosis model also using LMWH (Fig. 5). This indicates that direct access of a therapeutic agent to the entire thrombus is important in promoting thrombus resolution.

We addressed the question if a direct electrical current (i.e. resistance heating) is in part responsible for vein wall injury and thrombus formation. The intravascular temperature probe was placed in the vein lumen, millimeters away from the anodal electrode, which emitted a continuous square wave. In this experiment, a progressive increase in lumen temperature was not detected during the procedure. The results demonstrate that the EIM does not involve heat to induce venous thrombosis (Fig. 6), a significant observation because a thermal injury could denature proteins and alter gene expression. Ongoing experiments in this laboratory include quantifying inflammatory mediators in both the vein wall and blood post electrolytic injury.

In conclusion, the EIM for mice is an exciting new model option for studying biological changes during venous thrombogenesis. The electrolytic induction of injury to the intimal surface of the IVC results in endothelial activation and partial blood stasis, two components of Virchow's triad, in order to generate venous thrombosis. This novel model closely simulates clinical situations of thrombus formation and would be ideal to study venous endothelial cell activation, leukocyte migration, venous thrombogenesis and therapeutic applications. The EIM model is technically simple, easily reproducible, produces consistent thrombus sizes and allows a large sample (i.e. thrombus and vein wall) which is required for analytical purposes. In addition, the cost of the necessary equipment for the EIM is reasonable. This animal model of venous thrombosis meets criteria established by The National Research Council (NRC) (25).

Acknowledgments

We would like to acknowledge Dorothy R. Sorenson for the TEM, Sasha Meshinchi for SEM image analysis, Dr. Robert E. Sigler for performing the histopathology evaluation and Denisa D. Wagner for her comments.

Financial support: This study was supported by NIH 1P01HL089407-01A1 (Lawrence, PI), Animal Core A, NIH 1 K01 HL080962-01A2 (Myers, PI).

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Figure 1. Surgical technique

A) Anatomy of mouse at the needle entry point (X). The right iliac artery (RIA) crosses over the IVC and a lymph node (LN) is located in the area, to the right side of the IVC (mouse perspective). Note that the thrombus forms in the presence of flow and extends upstream (towards the heart) within the IVC. B) The needle placed in the recommended IVC area. C) Needle inserted into the IVC. Before (top) and after (bottom) the procedure, 15 minutes of 250 μ Amp of current. D) IVC anatomical variation in the same strain. The insertion of lateral branches into the IVC occurs at different levels and affects thrombus formation. To be consistent, ligation of the side branches is mandatory.

Figure 2. Thrombus weight (TW), soluble P-selectin (ELISA), correlation between soluble P-selectin and TW, leukocyte counting (H&E stain) and P-selectin immunohistochemistry

A) TW was measured two days post EIM in PAI-1 KO (n=5), WT (n=5) and Δ CT mice. For shams, a needle was placed into the IVC without turning on the current (n=9). Significantly lower TW were observed in PAI-1 KO mice ($p=0.0364$) and significantly higher TW in Δ CT mice compared to WT ($p=0.0340$). No thrombi were observed in the shams group. The mean TW in PAI-1 KO mice was 0.0087 ± 0.0007 g; in WT mice it was 0.0177 ± 0.0028 g; and in the Δ CT the mean TW was 0.0268 ± 0.0018 g. B) soluble P-selectin (sP-sel) was measured on day 2 post EIM in PAI-1 KO, WT and Δ CT mice. The trend shows lower levels of sP-sel in PAI-1 KO mice and higher levels in Δ CT mice. sP-sel was decreased in PAI-1 KO mice (mean 5.15 ± 2.2 ng/ml) and increased in Δ CT mice (mean 30.99 ± 1.4 ng/ml), compared to WT (mean 9.29 ± 4.6 ng/ml) [WT/PAI-1: $p=0.003$; WT/ Δ CT: $p=0.0002$]. C) Correlation between sP-sel and TW: These data show a direct correlation between TW and sP-sel in all three strains ($r^2=0.74$) when we performed EIM. (D) White blood cell counts from H&E stained slides. NEU were the most frequently observed cells in the vein wall two days after EIM. (NEU/MON: $p=0.0038$; NEU/LYM: $p=0.0052$). E) Staining for P-selectin showed an increase of P-selectin in the vein wall immediately after EIM (2) vs. control (1). Abbreviations: TW, thrombus weight; EIM, electrolytic IVC model; PAI-1, PAI-1 KO mice; WT, c57BL/6 mice; Δ CT, hypercoagulable Δ CT mice; sP-sel, soluble P-selectin; TC, true control (no treatment); NEU, neutrophil; MON, monocyte; LYM, lymphocyte; Total, NEU + MON + LYM.

Figure 3. Representative pictures of mouse IVC harvested two days after EIM and ultrasonography study at the same time point (n=5)

A) IVC and thrombus (T) specimen at harvest. B) H&E stain: The thrombus area corresponds to 73.7% of the total IVC area. The remaining 26.3% correspond to free flow area, confirming that this method forms thrombi in the presence of flow. C) Ultrasound (US) duplex colour showing patent IVC (blue). D) US measurement of IVC (D1) and the flow channel (D2) at the level of thrombi. Note that 71% of the IVC is occupied by the thrombus.

Figure 4. Scanning electron microscopy (SEM) (n=5) and transmission electron microscopy (TEM) (n=10)

A) SEM showing accumulation of red blood cells and fibrin during thrombus formation. B) Needle (n) within the IVC specimen prepared for SEM. C-F) Representative pictures from TEM immediately after EIM (C), showing leukocyte transmigration 30 minutes (D) and 1 hour after EIM (E, F). The pictures are oriented with the lumen (L) on the right and the vein wall on the left. R, red blood cells; en, endothelial cell.

Figure 5. EIM as a model for therapeutic approach

An experiment was conducted to demonstrate the model efficiency for therapeutic approach. WT (n=5) receiving enoxaparin and WT (n=5) receiving saline were used. Significant reduction in TW was observed in the group treated with enoxaparin (A, B) ($p=0.0177$). The percentage of TW reduction in the EIM in mice treated with enoxaparin was 61%, almost twice the percentage of reduction observed in the IVC ligation model when using enoxaparin (C, D) (37.1%, $p=0.0034$).

Figure 6. Temperature test *in vivo* performed in order to demonstrate that heat is not the stimulus that initiates thrombus formation using EIM

A) Needle in IVC and temperature detector inserted through a side branch to record the temperature. B) The temperature was recorded before, during and after EIM. No significant variation was observed.

Table 1

Mice distribution

Type	n	Procedure	Experiment objective	Harvest
WT	5	EIM (250 μ Amp – 15')	Demonstrate presence of IVC flow: ultrasound study and H&E	2 days post EIM
WT	5	EIM (250 μ Amp – 15')	Characterising biological changes in blood, Sol P-selectin + thrombus weight	2 days post EIM
	3	Sham (no current – 15')	vein wall and thrombus undergoing EIM	2 days post EIM
pat1	5	EIM (250 μ Amp–15')		2 days post EIM
	3	Sham (no current – 15')		2 days post EIM
Δ CT	5	EIM (250 μ Amp – 15')		2 days post EIM
	3	Sham (no current – 15')		2 days post EIM
WT	2	EIM (250 μ Amp – 15')	Transmission electron microscopy (TEM)	Immediately after EIM
	2	EIM (250 μ Amp – 15')		30 minutes after EIM
	2	EIM (250 μ Amp – 15')		1 hour after EIM
	2	EIM (250 μ Amp – 15')		3 hour after EIM
	2	EIM (250 μ Amp – 15')		6 hour after EIM
WT	5	EIM (250 μ Amp – 15')	Scanning electron microscopy (SEM)	Immediately after EIM
WT	10	EIM (250 μ Amp – 15')		Immediately after EIM
WT	2	EIM (250 μ Amp – 15')	P-selectin and vWF immunohistochemistry	Immediately after EIM
WT	10	EIM (250 μ Amp – 15')	Temperature test	Immediately after EIM
			LMWH therapy	
WT	10	IVC ligation model	LMWH therapy – Data from previous experiments	2 days post EIM
Total		76		2 days post EIM