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Towards standardization of *in vivo* thrombosis studies in mice

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BIORHEOLOGY SUBCOMMITTEE OF THE SSC OF THE ISTH

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Introduction

The last two decades have seen a tremendous growth in the availability of murine models to study the process of thrombus formation *in vivo*. Some of these models allow direct visualization of platelet-platelet and platelet-vessel wall interactions, permitting a real-time analysis of these events, while others measure changes in blood flow only. Several reviews have provided comprehensive comparisons of existing experimental approaches to induce arterial, venous or microvascular thrombosis in mice [1–3]. The use of different injury and vascular models permits the analysis of different aspects of the complex mechanisms that contribute to the process of thrombus formation. Indeed, despite the notion that all of the methods that are used to induce thrombus formation are artificial, the formed thrombi mimic the morphology of thrombi found in human acute coronary syndromes [4], underscoring the critical role of these models in studying the mechanism of thrombus formation *in vivo* and evaluating novel therapeutic leads. However, analysis of the literature concerning *in vivo* thrombus formation reveals a wide variation in protocols for any given model. It seems conceivable that these protocol differences may affect the outcome of experiments and may therefore compromise comparisons between independent studies.

Given the notion that *in vivo* thrombosis formation represents a highly rheology-determined process, the Biorheology Subcommittee felt it their task to take the initiative in order to get to more coherent protocols. As a first step, a questionnaire was sent out to different laboratories worldwide known to be experienced in using murine models for thrombosis. A

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survey (Table S1) was sent to email addresses of authors that were selected following analysis of PubMed abstracts (keywords: mouse, thrombosis, *in vivo*), and responses from 36 different laboratories (out of 78 mails sent) were received. The primary responders were all principal investigators experienced in the use of murine thrombosis models. Of note, some of the returned questionnaires were only partially completed, and several questions allowed multiple answers, explaining total percentages higher than 100% in several instances. Laboratories were located in 11 different countries: Australia (2), Austria (1), Belgium (2), Canada (4), France (5), Germany (1), Japan (2), the Netherlands (2), Switzerland (1), the United Kingdom (1) and the United States of America (15); numbers represent responding laboratories per country.

Types of thrombosis model used

An overview of the answers to this questionnaire is given in Table S2. All 36 responding laboratories indicate the use of murine models for thrombosis, with 24 laboratories (67%) using at least two different models and 12 (33%) using one single model. The four most commonly used methods to induce vascular damage are: ferric-chloride (32 laboratories; 89%), laser (13; 36%), mechanical (nine; 25%) and photochemical (eight; 22%) injury. Other injury methods are used to a lesser extent by 25% of the teams (electrical or electrolytic injury, ultrasound rupture, microvascular anastomosis, etc.) The majority of models are used to study arterial thrombosis rather than venous thrombosis. With regard to the four main models, most users indicate that they are satisfied with their model (83–100% satisfaction) and consider it reproducible (80–100%). According to 67–73% of the users, these thrombosis models are fairly easy to set up in the laboratory, with the exception of the laser-induced injury model, which is considered by only 20% to be easy to introduce. Consistent with this view, users agree that the laser model requires a high level of expertise. Interestingly, despite the opinion that other models can be easily developed, users still emphasize the need for a high level of expertise of the experimenter. Along the same lines, the experimental skills of the operator are rated as crucial for a reliable performance for each model, and about half of the laboratories are of the opinion that the outcome of *in vivo* thrombosis experiments is operator dependent.

Motivation for choosing a particular injury and vessel model

Participants were asked for their motivation for choosing a particular model. Motivations include: vascular bed to be studied (14 laboratories), molecule to be studied (14), genetic background of the mice (five), experimental question (four), and level of expertise required by the experimenter (four). With regard to the vascular bed, two injury models are primarily used under specific circulation conditions: laser-induced injury to the microcirculation in 87% of the cases and mechanical injury to the macrocirculation in 93% of the cases. In contrast, both photochemical and ferric-chloride-induced injuries are not perceived as vascular-bed specific, because they are used in both micro- and macrovasculature (38% vs. 62% for photochemical and 52% vs. 48% for ferric-chloride). For all models, the type of anesthetic is not judged as being critical, with pentobarbital and ketamine/xylasine being the most commonly used.

Ferric-chloride-induced injury

When asked for critical issues regarding the ferric-chloride injury model, two groups of parameters were frequently mentioned: (i) parameters that are independent of the particular model and hence common to all models, such as genetic background of the mice, shear and diameter of the vessels, thermal body regulation, skill of the operator; and (ii) parameters specific to the ferric-chloride injury model, including age of the animal (in view of the amount of fat tissue around vessels), extent of contact between filter paper and vessel, duration of contact between filter paper and vessel (between 1 min and never removed, most common time 2–5 min) and concentrations of anhydrous ferric-chloride used (2.5% up to 50%, with the most common used concentration being 10%). Importantly, the use of anhydrous $FeCl_3$ (162.2 g mol^{-1}) or $FeCl_3 \cdot 6H_2O$ (270.3 g mol^{-1}) should be taken into account when defining a working concentration due to the very different molecular weights of these two forms. A solution of 10% (w/v) anhydrous $FeCl_3$ thus corresponds to 617 mM and a 10% (w/v) $FeCl_3 \cdot 6H_2O$ solution to 370 mM. It should further be noted that optimal ferric-chloride concentration and duration of filter contact can be different when injury is induced in small vessels (e.g. mesenteric vessels) or large vessels (e.g. the carotid artery). Of note, methods to quantify vessel occlusion are different when using micro- vs. macrovascular beds. Visualization of platelet accumulation via video-assisted intravital microscopy is often used for microvascular beds, whereas measurement of blood flow by the Doppler system is more common for macrovascular beds.

Taken together, we have identified a number of parameters that can potentially be standardized (Table 1): concentration of freshly prepared ferric-chloride (an acceptable range of 5–15% (0.3–0.9 M) when using anhydrous $FeCl_3$ (lower or higher concentrations can be used for specific needs); duration of filter paper contact for a minimum of 2 min; selection of microvessels not covered by fat tissue; use of vessels with appropriate blood flow (avoid vessels with irregular pulsative flow or flow that is too slow); and general condition of the animal (such as normal body temperature and breathing). Nevertheless, a number of parameters remain difficult to standardize (if possible at all): skill of the experimenter, genetic background of the animal, diet, etc.

Photochemical method

According to the users, critical issues specific to the photochemical-thrombosis model are: age of the mice, Rose Bengal concentration (concentrations used are 10–50 mg kg^{-1}, use of another dye has been reported by one group, i.e. FITC-Dextran); and duration and intensity of dye illumination (from 20–40 s to continuous). In particular, the dye illumination conditions (choice of duration and intensity of excitation, and insertion of neutral density filters) may profoundly influence the thrombotic process.

Based on these responses, a number of parameters seem eligible for standardization, given a particular light source and optics (Table 1): concentration of Rose Bengal and mode of Rose Bengal administration (e.g. bolus + infusion to maintain a stable circulating concentration of the dye). Less prone to standardization are: skill of the experimenter; mice (genetic background, diet, etc.); intensity of the illumination lamp (old vs. new; use of intensity

reducing filter or not); and exposure time. Standardization of illumination intensity is difficult given the different types of equipment that are used between laboratories.

Laser-induced injury

When asked for critical issues specific to the laser-induced microvascular thrombosis model, a number of parameters were frequently mentioned: type of ablative laser; wavelength of ablative laser (range of 337–647 nm, 440 nm nitrogen laser most commonly used); type of microscope (inverted, four teams; upright, seven teams); laser application manually or software-driven; time protocol of laser illumination and focal plane; and analysis of collected data. An issue that deserves particular attention with regard to the laser model is the intensity of the laser beam. Depending on the system set up in the laboratories, different degrees of severity are reached after a laser-induced injury. Obviously, the extent of injury may modulate the process of thrombus formation.

In summary, we identify a number of parameters that deserve standardization (Table 1): wavelength of ablative laser, perfusion of tissue, and use of vessels of similar shear and diameter. More difficult to standardize are: skill of experimenter; equipment (inverted vs. uptight microscope, and type of laser); mice (genetic background, diet, etc.).

Mechanical-induced injury

Common critical issues concerning mechanical vascular injury are difficult to define, because a variety of vascular beds (aorta, carotid artery) are studied. In addition, most of these models are used in one laboratory where they were developed and are well characterized. To damage the vessel wall, various techniques are used involving ligation, balloon, flexible wire, pinching, perforation, ultrasound application, etc. However, no inter-laboratory comparison is feasible in most cases. Of note in most instances, teams using mechanical models often study thrombosis in vessels in pathological settings such as diabetes mellitus, atherosclerosis, neointimal stenosis and hyperhomocysteinemia.

Conclusion and recommendations

The technical complexity of the various thrombosis models and the different types of equipment being used, restrict to some extent a full standardization of *in vivo* thrombosis models. Nevertheless, a certain degree of harmonization of protocols can be reached, and a number of relevant parameters have been summarized in Table 1. As equipment for studying *in vivo* thrombosis varies widely between laboratories, similar settings cannot always be used (this seems particularly true for the laser-induced and the photochemical-induced injury models). Rather, we recommend that each laboratory should define and report on their own settings to reach standard results for a given model. These preferentially include the listing of relevant parameters, such as time to first occlusion, percentage of occlusion after a fixed time, number of embolic events, time to maximal thrombus size and/or thrombus size after a fixed time. A negative control (e.g. inhibition of occlusion by using a pharmaceutical inhibitor) should be included as well.

The current survey has further revealed that a better characterization of models is needed in terms of endothelial damage, exposure of subendothelial and other vessel components and pharmacological dependency. We believe that advanced discussions between laboratories using the same model are critical to defining standardized boundaries. Meetings of the Subcommittees for Biorheology and/or Animal Models may provide the appropriate platforms to initiate these discussions, and if necessary working parties on the separate injury models could be installed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Recommendations for standardization

Model	Issue	Recommendation
Model independent	Mouse	Control body temperature of mouse
		Adjust anesthesia to regular breathing and heart beat during whole procedure
	Vessels	Ensure undisturbed blood flow (use equipment to measure blood velocity)
		Select vessels not covered by fat (microcirculation)
		Use vessels of similar shear and diameter
		Dissect free the tissue and vessel without luminal damage (e.g. increased leukocyte rolling represents untimely endothelial activation)
		Define and control the extent and size of vascular damage ((sub)-endothelial)*
	Measurement parameters†	Define for specific vascular beds a time window in which a certain parameter should be reached. Also define a negative control
		Parameters could include: time to first occlusion, percentage of occlusion after a fixed time, number of embolic events, time to maximal thrombus size, thrombus size after fixed time
$FeCl_3$‡	$FeCl_3$ preparation	Freshly prepared
		5–13.5% (0.3–0.8 M) anhydrous $FeCl_3$ (for microcirculation)
		10–15%, (0.6–0.9 M) anhydrous $FeCl_3$ (for macrocirculation)
	Duration filter contact	≥ 2 min (for microcirculation)
		2–3 min (macrocirculation)
	Vessels	Not covered by fat tissue
Laser	Wavelength	440 nm
	Laser power and duration	Depending on optics and thickness of tissue and vessel wall
	Perfusion of the tissue	Constant perfusion of the cremaster to avoid inflammation of the tissue
	Analysis of the data	Calculation of the median of integrated fluorescent intensity
Photochemical‡	Rose Bengal preparation	Freshly prepared to avoid light-induced degradation
		10–50 mg kg^{-1}
	Administration	bolus + infusion to maintain a stable circulating concentration of the dye

*Please refer to references 5–11 of the Data S1 for further reading about how the vascular wall is affected by different conditions, and how this may help to control the extent of vessel wall damage. Additional supplementary references relate to thrombosis models in general (references 1–4), dose-response studies when using ferric-chloride (references 9–11), and the influence of mouse strains on the hemostatic potential (references 12).

†Dependent on what aspect of thrombus formation is investigated (adhesion, thrombus growth, thrombus stability/embolism, etc.), one or more appropriate parameters can be chosen.

‡Both ferric-chloride and Rose Bengal have the potential to induce radical formation, which contributes to the vessel wall injury. Simultaneously, these radicals may affect the redox equilibrium of the system, inducing secondary effects as well (see references 1 and 11 of Data S1 for further reading).