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REVIEW

# Refinement, reduction and replacement approaches to *in vivo* cardiovascular research

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In this review, the justification and benefits of refinement, reduction and replacement (3Rs) approaches to cardiovascular research are examined using the field of platelet biology and arterial thrombosis as an example. Arterial thrombosis is a platelet-driven condition and platelets are regulated by autologous signals, but also by external factors such as the vascular endothelium. *In vitro* assays using isolated platelets therefore poorly reflect *in vivo* platelet function and human disease. As a consequence, animal models, including mouse models, are frequently used. In particular, models of thromboembolic mortality have been successfully employed to determine the role of the vascular endothelium in regulating platelet function and thrombosis *in vivo*. Such models raise both scientific and ethical concerns and have recently been refined permitting the use of fewer mice at a lower severity level. These refinements have been scientifically beneficial in permitting analysis of the development and progression of thrombotic diseases and in improving our understanding of the role of the vascular endothelium in regulating platelet function and thrombosis. For many, the ultimate goal in 3Rs-driven science is replacement of animal models with non-animal alternatives; this is exemplified, in the platelet field, by the development of *in vitro* flow systems. The development of 3Rs approaches to cardiovascular research is shown to have led to improved scientific models. Further characterization and use of these models will likely contribute to increased understanding of thrombotic disease processes and facilitate drug development in the cardiovascular field.

## LINKED ARTICLES

This review is the second in a series on the topic of refinement, reduction and replacement approaches to research; the first was Holmes *et al.*, http://dx.doi.org/10.1111/j.1476-5381.2009.00176.x and has an accompanying commentary by Robinson, http://dx.doi.org/10.1111/j.1476-5381.2009.00280.x

### Abbreviations

ITAM, immunoreceptor tyrosine-based activation motif; L-NAME, Nω-nitro-L-arginine methyl ester; NC3Rs, National Centre for the Replacement, Refinement and Reduction of Animals in Research; NOS-3, nitric oxide synthase-3

## Introduction

Arterial thrombosis is driven by inappropriate activation of platelets. The platelet surface contains numerous receptor molecules that mediate adhesion to exposed subendothelial surfaces following vascular injury as a result of either vessel trauma or cardiovascular disease. Inappropriate platelet activation drives arterial thrombotic events such as myocardial infarction and platelets are cornerstones of pharmacological anti-platelet therapy: aspirin selectively targets platelet cycloxygenase and clopidogrel prevents platelet activation by adenosine diphosphate. Both compounds are effective in improving outcome and recurrence of thrombotic events, but therapeutic resistance and complications, such as gastric bleeding, mean that new pharmacological approaches are required.

Platelets are regulated by an array of autologous signals, but the vascular endothelium is also a key determinant of their function. It provides a barrier between the circulating platelet and vascular collagen and generates nitric oxide and prostacyclin, which inhibit platelet activation. The role of the vascular endothelium is also key to the pharmacological profile of anti-platelet agents. For example,

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aspirin may inhibit cycloxygenase activity in both platelets and the vascular endothelium and, thus, has the potential to inhibit the production of compounds that both inhibit (prostacyclin) and enhance (thromboxane $A_2$) platelet activity. Chronic aspirin administration produces an anti-thrombotic effect due to the anucleate platelet's inability to regenerate cycloxygenase protein so that the effects of prostacyclin override those of thromboxane $A_2$ during aspirin therapy (Mitchell and Warner, 2006). Thus, many groups work *in vivo* to investigate the interplay between platelets, the vascular endothelium and other cell types in determining the pharmacological profile of aspirin.

The gold standard measurement of platelet functional responsiveness is Born aggregometry involving detection of increased light transmission through translucent platelet suspension during aggregation (Born, 1962). Platelet aggregation can also be assessed in whole blood by measuring electrical impedance. A major limitation of *in vitro* aggregometry is that the platelet, or blood sample, is isolated from its physiological environment of the circulating blood stream and thus stripped of the influence of endothelial mediators. Thus, *in vitro* platelet aggregation poorly predicts the platelet functional response *in vivo* (Morley and Page, 1984). This can be exemplified by considering the platelet response to loss of endogenous NO *in vitro* and *in vivo*. Nitric oxide synthase (NOS) inhibitors have no effect of platelet function *in vitro,* but *in vivo* produce a profound potentiation of platelet-driven responses due to the suppression of NO production by the vascular endothelium (Radomski *et al.*, 1987; Tymvios *et al.*, 2009; Moore *et al.*, 2010). Similarly, *in vitro* platelet responses to collagen and other agonists are readily inhibited by acute exposure to aspirin due to inhibition of pro-thrombotic thromboxane $A_2$ generation by platelet cycloxygenase (Evans *et al.*, 1968; Armstrong *et al.*, 2008). *In vivo*, however, the situation is less clear-cut as the pharmacology of aspirin with respect to the vascular endothelium and prostacyclin production remains unclear (Patrono, 2001; Mitchell and Warner, 2006).

Modelling the interaction between endothelial activity and platelet function and thrombosis is therefore critical to our understanding of thrombotic mechanisms, the pharmacology of current therapies and the development of novel anti-platelet strategies. As in many other areas of pharmacological research, the interplay between distinct tissues justifies the use of animal models in experimental research and the genetically malleable mouse has become a cornerstone of thrombosis research.

## Experimental mouse models

Mouse models of thrombosis invariably involve damage of the vascular endothelium by the application of chemicals, laser injury or mechanical trauma to replicate elements of the thrombotic disease process such as vascular injury and dysfunction, reviewed in Bodary and Eitzman (2009). These models are driven by platelet activation but have additional haemostatic, vascular and neurological determinants and do not functionally isolate the platelet (Nieswandt *et al.*, 2005). Furthermore, these models have proven inconclusive when used to ascertain the effects of mediators derived from the vascular endothelium in regulating platelet thrombotic events. For example, the vascular endothelium constitutively expresses NOS-3. This enzyme regulates vascular tone and NOS-3-deficient mice are hypertensive (Huang *et al.*, 1995). NO negatively regulates platelets and so NOS-3 ablation would be predicted to enhance platelet-mediated events such as haemostasis and thrombosis. Haemostasis (as bleeding time) is enhanced in NOS-$3^{-/-}$ mice (Freedman *et al.*, 1999) but data from mouse models of thrombosis are contradictory (Heeringa *et al.*, 2000; Iafrati *et al.*, 2005; Marjanovic *et al.*, 2005; Ozuyaman *et al.*, 2005; Dayal *et al.*, 2006). There are reports indicating both a lack of thrombotic phenotype following vessel injury (Ozuyaman *et al.*, 2005; Dayal *et al.*, 2006) and an anti-thrombotic phenotype (Iafrati *et al.*, 2005; Marjanovic *et al.*, 2005) possibly due to up-regulated fibrinolysis (Iafrati *et al.*, 2005). There are also models in which NOS-3 ablation enhances thrombosis (Heeringa *et al.*, 2000). What is the reason for these contradictory data? The explanation may lie partly in the use of different experimental models where thrombi are induced through different mechanisms and in varying anatomical locations, although there are disparities between comparable data sets: carotid artery injury models have revealed both anti-thrombotic and undetectable consequences of NOS-3 ablation (Ozuyaman *et al.*, 2005; Dayal *et al.*, 2006). Recently, it has been suggested that the use of conventional vascular injury models of thrombosis to ascertain the functional consequences of loss of endothelial mediators is fundamentally flawed (Moore *et al.*, 2010). Determining the role of endothelial components involves functional assessment of the consequences of pharmacological (or genetic) inhibition of active constituents of the endothelium, such as NOS, relative to appropriate controls. An accurate assessment requires that the control element of the experiment contains a fully functioning version of the component of interest. Where thrombosis models are used this is not the case as the vascular endothelium, and

hence the component under assessment, is absent or damaged as part of the process of thrombus induction. Vascular injury models therefore cannot be used to reliably investigate the function of endothelial components such as NOS, or the complex pharmacology of aspirin, which may explain the contradictory reports outlined above.

There is a need therefore for mouse models of thrombosis with an intact, undamaged vascular endothelium that can be manipulated pharmacologically or genetically to determine the role of the vascular endothelium in regulating platelet responsiveness. Thromboembolic mortality models involving the injection of agents including collagen, adrenaline and adenosine diphosphate into the tail vein of conscious mice to induce fatal thromboembolism have been, and continue to be, employed to assess platelet functionality in the presence of an intact vascular endothelium (DiMinno and Silver, 1983; Crikis *et al.*, 2010; Martin *et al.*, 2010). Such models have been useful in determining the role of endothelial mediators such as endogenous NO as negative regulators of platelets and thrombotic events *in vivo* (Emerson *et al.*, 1999). The use of mortality as an end point raises scientific as well as ethical concerns. Thrombotic diseases are invariably progressive in nature and symptoms range from asymptomatic to fatal. Good animal models should provide a means of replicating elements of the treatable phases of disease to drive forward new therapeutic options. By inducing mortality, one models one extreme end point of diseases with a broad spectrum and fails to assess the development and progression of the disease process. The ethical concerns associated with the injection of clotting agents directly into the tail veins of conscious mice are obvious: in their paper describing the methodology, DiMinno and Silver describe 'bulging eyes', 'gasping for breath' and mice 'unable to walk, even in response to prodding' as end points for ascertaining a fatal thrombotic event.

## Refinement, reduction and replacement

The refinement, reduction and replacement (3Rs) principal relating to animal research was introduced by Russell and Burch in their work 'The principles of humane experimental technique', published in 1959, see Balls and Straughan (1996). The foundation of the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) in May 2004 following the recommendation of a House of Lords Select Committee report on Animals in Scientific Procedures, published in July 2002, and their funding of 3Rs focused scientific research through project grants and PhD studentships, provides a mechanism for driving forward humane and scientifically improved research involving, or avoiding the use of, animal models (see http://www.nc3rs.org.uk).

*British Journal of Pharmacology* has shown support for this initiative by publishing guidelines for the reporting of experiments on animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The current article is the second in a series on refinement, reduction and replacement, the first concerning opportunities for the replacement of animals in the study of nausea and vomiting (Holmes *et al.*, 2009, with a commentary by Robinson, 2009).

## Refinement

Refinement of animal procedures refers to improvement of scientific techniques involving animals to minimize pain and suffering over the lifetime of the animal. In 2008, a paper was published describing refinements to mouse thromboembolic mortality procedures that permitted the assessment of non-lethal platelet thromboembolism under general anaesthesia (Tymvios *et al.*, 2008). This paper described methods for assessing the thromboembolic responses of radiolabelled platelets *in vivo* via external scintillation probes. The refinement arises from the fact that the entire process is conducted under general anaesthesia and fatal responses are not induced, rather reversible thromboembolism is recorded and painful procedures are avoided. Thus, the benefits of the original thromboembolic model, which allowed assessment of platelets *in situ*, are retained and the experience of the experimental animals enhanced, by the introduction of general anaesthesia. In addition, refinement produces the additional benefit of permitting assessment of non-lethal thromboembolism to better model the non-fatal stages of the disease process and, in that respect, produces a more relevant scientific model.

## Reduction

Reduction involves the acquisition of comparable, or improved, scientific data sets from fewer animals. In refining mortality models, Tymvios *et al*. were able to introduce considerable elements of reduction by demonstrating multiple and reproducible responses within an individual mouse (Tymvios *et al.*, 2008). To assess the effectiveness of this approach, in terms of reduced animal use, one needs, according to the original definition of 'Reduction', to compare

equivalent data sets. In 1999, Emerson *et al*. demonstrated the essential role of endogenous NO in protecting against thromboembolic mortality, using the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (Emerson *et al*., 1999). The study involved groups of 20 mice, injected with varying doses of collagen such that 200 mice were used to acquire the published data set. In 2009, Tymvios *et al*. demonstrated a similar effect in their refined model whereby L-NAME reduced the concentration of thrombin required to elicit dose-dependent thromboembolism under general anaesthesia (Tymvios *et al*., 2009). The ability to analyse meaned parameters and record multiple responses within individual mice permitted the acquisition of the data set published in 2009 using only 30 mice, a reduction in mouse use of some 85% compared with the earlier paper.

## Replacement

For many, the ultimate goal in 3Rs research is the replacement of animal models with human or virtual model systems. This is a huge challenge for the cardiovascular field, but one that is being driven forward by a number of research groups. In the platelet field, a number of investigators are working with parallel flow chambers to study human platelet function in whole blood and real time (Ruggeri, 2009). Critically, such *in vitro* model systems permit modelling of arterial and venous shear rates and have been used to investigate the role of extracellular matrix proteins, endothelial cells and microorganisms upon human platelet and vascular function without the use of animals (Colgan *et al*., 2007; Kerrigan *et al*., 2008). Further characterization and development of these human *in vitro* systems is warranted from a 3Rs perspective but also from a scientific view point. Mouse platelets are poor models of their human counterparts; for example, the ITAM (immunoreceptor tyrosine-based activation motif) bearing receptor FcgammaRIIa has recently been shown to mediate αIIbβ3 outside-in integrin signalling in human platelets (Boylan *et al*., 2008) but is not expressed in mice explaining the differential spreading responses of human and mouse platelets at sites of vascular injury. In addition, the use of human platelet systems facilitates investigations of patients with platelet disorders and other cardiovascular diseases, reducing our reliance upon animal models, including genetically modified mice.

*In vitro* models are also driving progress in other areas of cardiovascular science. Research into cardiac function and disease, for example, is highly dependent upon animals as sources of active primary cardiomyocytes, as human tissue is difficult to acquire. Recently, however, an NC3Rs-funded project has validated human embryonic stem cell-derived cardiomyocytes as models of their adult counterparts thus reducing the reliance of this area of research on animal models, energizing the search for improved therapies and, potentially, creating a source of cardiomyocytes for transplantation to the failing heart (Brito-Martins *et al*., 2008; Abdul Kadir *et al*., 2009). This again demonstrates the ability of projects with a 3Rs justification to drive forward our understanding of cardiovascular diseases and the development of new therapies.

## Cardiovascular research and the 3Rs: the future

Animal models have been, and continue to be, invaluable in enhancing our understanding of cardiovascular diseases and in developing new pharmacological therapies. The UK legislates that practitioners of animal research must consider and implement 3Rs approaches to their work so that procedures are conducted at the minimal severity level and the minimum number of animals is used. Engagement of the 3Rs principles is being driven by a number of research groups and funding organizations such as NC3Rs. In this review, examples have been given of improvements to animal models and opportunities in the cardiovascular field that have arisen from projects with a 3Rs justification. Thus, appropriate consideration of 3RS in the field of animal research can drive forward our understanding of cardiovascular diseases, such as arterial thrombosis, and increase the likelihood of scientific breakthroughs in this, and other, areas of science.

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## Conflict of interest

None to declare.

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