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To the editor:

Ferric chloride thrombosis model: unraveling the vascular effects of a highly corrosive oxidant

The topical application of ferric chloride ($FeCl_3$) to the vasculature is one of the most commonly used experimental approaches to induce thrombosis. The method was first described by Kurz and colleagues[1] and has subsequently been proven to be a highly effective and reliable approach to elucidate the role of platelet receptors, ligands, and activation pathways in promoting thrombosis.[2-7] It has also shed new light on the role of coagulation proteases in regulating thrombin generation and thrombus growth[8-11] and has been used in the context of thrombolysis,[12-17] although the model appears to have significant limitations in this regard. Despite its widespread use, the precise mechanism by which $FeCl_3$ induces thrombosis remains controversial. A recent study in *Blood* by Ciciliano and colleagues has provided further insight into the molecular mechanisms underlying $FeCl_3$-induced thrombosis, suggesting an important role for charge-dependent aggregation effects of $FeCl_3$ on blood cells and plasma proteins.[18]

It has long been assumed that the major effects of $FeCl_3$ are limited to the vasculature. $FeCl_3$ ions have been localized to the endothelium with uptake of iron through endothelial pinocytic processes. Several studies have described ferric ion-rich membrane-enclosed bodies which transmigrate into the endothelium, followed by exocytosis into the vessel lumen.[19,20] It was assumed that this accumulation of iron and generation of reactive oxygen species produce endothelial toxicity and denudation, leading to the exposure of subendothelial elements that promote thrombus formation.[1,21] However, a number of studies have revealed minor endothelial denudation and collagen exposure following $FeCl_3$ treatment,[20,22,23] raising the possibility that $FeCl_3$ has effects beyond the vessel wall.

The first demonstration that red blood cells (RBCs) may play an important role in promoting $FeCl_3$-induced thrombosis was derived from our in vitro studies using isolated blood cell components perfused through mouse aortae ex vivo.[23] Surprisingly, endothelial cells exposed to $FeCl_3$ alone exhibited minor levels of injury. However, in the presence of whole blood or isolated RBCs, $FeCl_3$-induced red cell hemolysis and hemoglobin oxidation promoted extensive vascular injury and thrombosis.[23] Elegant electron microscopy studies by Barr and colleagues confirmed extensive red cell accumulation on the endothelium following $FeCl_3$ exposure in vivo, with platelets rapidly recruited to accumulated red cell-derived structures.[22] Eckly and colleagues revealed surface expression of tissue factor on the ferric ion-rich spherical bodies, which they attributed as the primary mediator of platelet adhesion and fibrin formation.[20]

The plot thickens further. Ciciliano and colleagues used microfluidic devices coated with endothelial cells to dissect the effects of $FeCl_3$ on individual blood cell and plasma components.[18] These studies demonstrated concentration-dependent effects of $FeCl_3$ on protein and blood cell aggregation, independent of effects on the endothelium. This aggregation effect was principally attributed to colloidal chemistry, whereby cells and proteins adhere and aggregate as a result of their charge. The authors have proposed that this physiochemical effect of $FeCl_3$ on blood cells is the primary instigator driving blood cell adhesion to the endothelium. They argue that this mechanism then facilitates the "secondary" phase of $FeCl_3$ injury, with red cell aggregates and damaged endothelium providing a reactive surface for the accumulation of platelets and initiation of blood coagulation, necessary for stable thrombus formation.[19-21] However, it remains to be determined to what extent oxidative damage vs physicochemical effects predominate to induce RBC aggregation and thrombosis.[23] In this context, aluminum chloride ($AlCl_3$), which carries a similar charge to Fe^{3+} and was used as an additional means of evidence to support the role of colloidal chemistry in cellular and protein aggregation, is also well known to cause oxidative damage, including lipid peroxidation and RBC hemolysis.[24] This is in contrast to chromium chloride ($CrCl_3$), which was also used in this study as a negative control; however, this molecule has antioxidant properties.[25] Whether the effects of $AlCl_3$ can be offset by antioxidants, as was demonstrated for $FeCl_3$ in our own ex vivo studies using isolated aorta,[23] will be important to determine. It is also interesting to note that the oxidative effects of $FeCl_3$ we observed in isolated aorta were initiated with concentrations of $FeCl_3$ lower than that observed to induce macroscopic precipitation of plasma proteins.[23]

Collectively, the studies highlighted above unequivocally demonstrate that the effects of $FeCl_3$ on vascular injury and

thrombosis are multifaceted and far more complex than originally envisioned. It will be a challenge to define a precise, unified mechanism of $FeCl_3$-induced thrombosis because both the physicochemical and pro-oxidant effects of $FeCl_3$ on blood cells and the vasculature are highly dependent on $FeCl_3$ concentration and exposure time. Whether simplified in vitro models that use microfluidics and 3-dimensional printing approaches can accurately recapitulate the complex changes operating in the vasculature of a living animal remains to be seen. The dynamic interface between blood cells, subendothelial elements, and vascular reactivity is central to the thrombotic response, and therefore it will be important to demonstrate that the thrombosis mechanisms operating in microfluidic devices are similar to those occurring in isolated vessel segments. Nonetheless, it is likely that $FeCl_3$ as an inducer of experimental thrombosis is here to stay, due to its simplicity, widespread availability, and ease of use. However, as noted by numerous authors,[13,18,20-23] caution should be used when interpreting data from such a model, and particularly when attempting to draw generalizing conclusions about the proposed mechanisms regulating coagulation and blood cell interactions with the vessel wall during thrombus initiation.

It is notable that considerable effort has been made to avoid experimental bias in in vivo studies, by limiting genetic variability, sex differences, and the impact of diet, pathogens, and age-related changes in the mouse. However, it could be argued that we do not apply the same level of rigor to our experimental thrombosis models, all too regularly accepting the findings from a single in vivo thrombosis model. This, of course, is not unique to our field. However, an improved understanding of the molecular mechanisms promoting thrombosis in specific models and increased recognition of the pitfalls and limitations of each of our models, coupled with a requirement to report data using several distinct thrombosis models, should help enhance the veracity of our experimental findings and reduce future controversy. Only time will tell.

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