Warfarin-related nephropathy is the tip of the iceberg: direct thrombin inhibitor dabigatran induces glomerular hemorrhage with acute kidney injury in rats

Margaret Ryan¹, Kyle Ware¹, Zahida Qamri¹, Anjali Satoskar¹, Haifeng Wu¹, Gyongyi Nadasdy¹, Brad Rovin², Lee Hebert², Tibor Nadasdy¹ and Sergey V. Brodsky¹

¹Department of Pathology, The Ohio State University, Columbus, OH, USA and ²Department of Medicine, The Ohio State University, Columbus, OH, USA

Correspondence and offprint requests to: Sergey V. Brodsky; E-mail: sergey.brodsky@osumc.edu

ABSTRACT

Background. Excessive anticoagulation with warfarin can result in acute kidney injury (AKI) by causing glomerular hemorrhage and renal tubular obstruction by red blood cell (RBC) casts in some patients, especially in those with chronic kidney disease (CKD). This condition was described as warfarin-related nephropathy (WRN). Recent evidence suggests that WRN-like syndromes are not confined to anticoagulation with warfarin, but may be seen with other anticoagulants, such as dabigatran. The aim of this study was to investigate dabigatran effects on kidney function in an animal model of CKD and possible pathogenic mechanisms of AKI.

Methods. Control and 5/6 nephrectomy rats were treated with different doses of dabigatran and protease-activated receptor 1 (PAR-1) inhibitor SCH79797.

Results. Dabigatran resulted in changes in coagulation in rats similar to those in humans at 50 mg/kg/day. Dabigatran resulted in a dose-dependent increase in serum creatinine (Scr) and hematuria in both control and 5/6 nephrectomy rats. SCH79797 also increased Scr and hematuria, more prominent in animals with CKD. Morphologically, numerous RBC tubular casts were seen in 5/6 nephrectomy rats treated with either dabigatran or SCH79797 and only occasional RBC casts in control rats.

Conclusions. Our data indicate that WRN is remarkably common in high-risk patients who develop an acutely increased INR > 3.0 [3]. We later developed an animal model to study WRN using the 5/6 nephrectomy model of CKD in rats [4, 5]. The present work tests the hypothesis that the AKI associated with warfarin coagulopathy is not specific for warfarin but may be seen with other classes of systemic anticoagulants.

We were the first to describe a novel renal complication of warfarin therapy. We found that in a significant number of patients treated with warfarin, an increased international normalized ratio (INR) > 3.0 was associated with acute kidney injury (AKI). Kidney biopsy in a subset of these patients showed that there was extensive tubular obstruction by red blood cell (RBC) casts, indicating that these patients had developed severe glomerular hematuria. Remarkably, there were only minor glomerular changes [1]. We termed this condition warfarin-related nephropathy (WRN) [1–3]. WRN can have dire consequences, particularly in chronic kidney disease (CKD) patients [1–3].

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Recently, several novel oral anticoagulants were approved for use in the USA. These oral anticoagulants include the direct thrombin inhibitor (dabigatran etexilate), and the factor Xa inhibitor (rivaroxaban). Little is known about the effects of these oral anticoagulants on kidney function. However, a recent case report [6] described AKI in a patient with dabigatran-induced coagulopathy. Kidney biopsy done after resolution of the coagulopathy showed extensive tubular obstruction by RBC casts. This WRN is part of a broader condition involving any one of the several classes of systemic anticoagulants. The present work makes the case that there is a new category of AKI, anticoagulant-related nephropathy (ARN), of which vitamin K and direct

INTRODUCTION

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thrombin inhibitors are members. We suggest that the clinical implications of these findings are substantial.

**MATERIALS AND METHODS**

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals [7]. Male Sprague-Dawley rats (140–160 g) were allowed food and water ad libitum.

Ablative nephropathy (5/6 nephrectomy) surgery

The 5/6 nephrectomy was performed under a ketamine/xylazine (60 mg/7.7 mg/kg) anesthesia by a nephrectomy of the right kidney and resection of two-thirds of the left kidney as was described earlier [4, 5]. Animals were allowed to recover from the surgery for 3 weeks before the start of dabigatran treatment.

Experimental design

Dabigatran was given per os in drinking water in the concentrations described below. Proteinase-activated receptor 1 (PAR-1) inhibitor SCH79797 was injected intraperitoneally in 20% DMSO twice a day (one-half of the daily dose in each injection) [8, 9]. Serum creatinine (Scr) and hematuria were measured daily.

Assessment of serum creatinine, coagulation and hematuria

Scr was measured using a creatinine reagent assay (Raiche, San Marcos, CA) according to the manufacturer protocol as we described earlier [4, 5]. Briefly, 30 μL of serum was mixed with 300 μL of working reagent at 37°C in a 96-well plate and the absorbance was read at 510 nm at 40 and 100 s using a Molecular Devices Versa Max plate reader (Molecular Devices, Sunnyvale, CA).

Activated partial thromboplastin (aPTT) and ecarin clotting time (ECT) were measured using an Electra 750 coagulation analyzer (Medical Laboratory Automation, Pleasantville, NY) according to the manufacturer protocol as described earlier [10]. Briefly, blood was collected into a tube containing 3.8% sodium citrate in a ratio of 9:1. The blood was centrifuged at 1000 RCF for 15 min and 0.1 mL of plasma was placed in the incubation station with 0.1 mL of the aPTT reagent (Thermo Scientific, Waltham, MA). Then, after 5 min, 0.1 mL of 0.025-M calcium chloride was added. Clotting time was recorded in seconds. For ECT, 0.1-mL freshly centrifuged citrated plasma was then incubated for 2 min and then the addition of 6 units of Ecarin (Sigma-Aldrich, St. Louis, MO) solution was used to initiate coagulation. ECT was recorded in seconds.

Hematuria was measured using DiaScreen (Chronimed, Inc., Minnetonka, MN) reagent strips in the urine. Hematuria was graded using a semi-quantitative scale from 0 to 3+ arbitrary units (a.u.). Score 0 was designated for negative hematuria, score 1+ for mild hematuria, score 2+ for moderate hematuria and score 3+ for large hematuria [1, 5].

Renal pathology assessment

Animals were sacrificed under the ketamine/xylazine anesthesia and the kidney was removed and fixed in 10% buffered formalin. Tissue was embedded in paraffin and cut at 3-μm sections. In each animal, the entire area of a longitudinal section of one kidney was evaluated. The areas related to the 5/6 nephrectomy (scarred areas) were excluded from the evaluation in 5/6 nephrectomy rats. Each kidney section contained >60 glomeruli and >800 tubules.

Statistical analysis

Results are presented as mean ± standard deviation if not otherwise specified. Differences between groups were analyzed by the two-paired t-test or ANOVA test, where applicable. Dunnnett’s multiple comparison post test was performed to analyze the differences between groups in conjunction with ANOVA. Differences at individual time points between two groups were analyzed using a two-tailed Student’s t-test.

**RESULTS**

Dabigatran increases aPTT in a dose-dependent manner in both control and 5/6 nephrectomy rats

Dabigatran was given in drinking water in different doses. The dose selection was based on (i) literature data for use in rats [11, 12] and (ii) dose recommended for humans [13–15]. Treatment with dabigatran increased aPTT and ECT [10, 16, 17] in a dose-dependent manner in both control and 5/6 nephrectomy rats (Figure 1). Of note, a 2-fold increase in aPTT, that is recommended for humans [13], was achieved with 50 mg/kg/day dabigatran by Day 8 after initiation of treatment. Treatment with either 3 mg/kg/day or 10 mg/kg/day of dabigatran by Day 8 after initiation of treatment. Treatment with either 3 mg/kg/day or 10 mg/kg/day of dabigatran (the doses closer to that recommended for humans, which is ∼4 mg/kg/day, considering the Food and Drug Administration-approved treatment with 150 mg of dabigatran twice daily [18]), did not result in significant changes in aPTT in neither control nor 5/6 nephrectomy rats (Figure 1A and B). When 150 mg/kg/day of dabigatran was used (3-fold higher than the therapeutic dose in rats), there was an ∼3-fold increase in aPTT when compared with the pre-treatment values. These are also the levels of aPTT seen in patients with dabigatran overdose [19]. The ECT assay showed similar dose-dependent increase in ECT in animals treated with dabigatran, both control and 5/6 nephrectomy (Figure 1C and D).

Dabigatran results in a dose-dependent increase in serum creatinine (Scr) in both control and 5/6 nephrectomy rats

Treatment with dabigatran increased Scr in both control and 5/6 nephrectomy rats (Figure 2A and B). The Scr increase in control animals was significantly higher with the 150 mg/kg/day dabigatran when compared with 3 mg/kg/day (Scr 1.28 ± 0.03 versus 0.79 ± 0.02 mg/dL at Day 8 of treatment, P ≤ 0.0001, respectively, Figure 2A). The Scr also increased significantly in 5/6 nephrectomy rats treated with...
Dabigatran 3 weeks after the ablative surgery. Treatment with 150 mg/kg/day resulted in a significant Scr elevation after 8 days of treatment, when compared with the 5/6 nephrectomy rats treated with 3 mg/kg/day (Scr 1.46 ± 0.16 versus 0.87 ± 0.01 mg/dL, P ≤ 0.0001, respectively, Figure 2B). In 5/6 nephrectomy Scr increase was noted earlier than in control rats, e.g. 50 mg/kg/day of dabigatran resulted in a significant elevation in Scr in 5/6 nephrectomy rats by Day 6, whereas in control animals a significant elevation in Scr was noted by Day 7 only (Figure 2A and B).

Dabigatran results in progressive hematuria in experimental animals

Animals treated with dabigatran had increasing hematuria associated with the treatment. Hematuria was noted in control animals treated with any of the dabigatran dose used, but animals treated with 3 and 10 mg/kg/day had only a small increase in hematuria by Days 7–8 of treatment. Animals treated with 150 mg/kg/day showed increased hematuria already after the first day of dabigatran treatment (Figure 2C). Intermediate dabigatran doses showed a dose-dependent effect on the hematuria induction.

The 5/6 nephrectomy rats were more sensitive to the ability of dabigatran to induce hematuria. Thus, dabigatran treatment with lower doses (10 mg/kg/day) resulted in hematuria by Days 3–4 of dabigatran treatment. Other dabigatran doses also resulted in an earlier onset of hematuria when compared with control rats (Figure 2D).

Dabigatran results in RBC tubular cast formation in 5/6 nephrectomy rats

Treatment with dabigatran resulted in a dose-dependent increase in RBC tubular cast formation in 5/6 nephrectomy rats (Figure 3B and C). Interestingly, RBC tubular casts were found only in one control rat and only in a single area of the kidney. This rat received 150 mg/kg/day of dabigatran for 8 days (Figure 3A). The other control rats dosed at 150 mg/kg/day (in total, kidneys from 32 control rats were analyzed) showed no evidence of RBC tubular casts.

Selective PAR-1 antagonist SCH79797 increases serum creatinine and hematuria in rats

In order to elucidate the role of PAR-1 receptors in the pathogenesis of dabigatran-induced glomerular hematuria and AKI, 5/6 nephrectomy and control rats were treated with the selective PAR-1 inhibitor SCH79797. Treatment with SCH79797 slightly increased aPTT in both control and 5/6 nephrectomy rats (40.5 ± 0.6 and 42.2 ± 0.6 s in control and 40.7 ± 0.6 and 43.6 ± 1.2 s in 5/6 nephrectomy rats before and by Day 5 of treatment with 1 mg/kg/day of SCH79797, respectively). However, SCH79797 in a dose-dependent manner increased Scr and

**FIGURE 1:** Coagulation effects of dabigatran in control and 5/6 nephrectomy rats. Control and 5/6 nephrectomy rats were treated with dabigatran per os in drinking water (the duration of treatment is shown on the X axis in days). 5/6 NE rats were treated 3 weeks after the ablative surgery (black arrow). BL, baseline data before the ablative surgery. (A and B) Activated partial thromboplastin time (aPTT) was measured daily in control (A) and 5/6 NE (B) rats. (C and D) ECT was measured in selected experimental groups in control (C) and 5/6 NE (D) rats. n = 5 in each experimental group. Dabigatran doses are shown in the figure legends.

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hematuria in 5/6 nephrectomy and control rats (Figure 4). Effects in 5/6 nephrectomy rats were more pronounced than in control rats. Thus, hematuria increased more rapidly and the amount of hematuria was larger in 5/6 nephrectomy than in control rats (Figure 4C and D). Morphologically, occasional RBC casts were seen in the tubules in 5/6 nephrectomy, but not control rats (Figure 3D).

**DISCUSSION**

To the best of our knowledge, this is the first and only report that describes in detail the kidney injury associated with the novel thrombin inhibitor dabigatran in an animal model.

We had previously described WRN as AKI with tubular RBC casts associated with the use of vitamin K antagonists in both humans [1] and rats [4, 5]. At that time there was no compelling reason to suspect that other classes of anticoagulants could also induce AKI. However, a recent case report described an anticoagulant-related AKI in a patient who had a coagulopathy induced by the thrombin inhibitor dabigatran [6]. The kidney biopsy findings were consistent with that of WRN in that renal tubular obstruction by RBC casts was pervasive. Also, the glomerular changes could not plausibly explain the extensive glomerular hemorrhage.

Dabigatran is being prescribed with increasing frequency. For example, at our institution 2105 patients were started on warfarin therapy and 185 patients were started on dabigatran therapy in 2011. Dabigatran is widely used for the prevention of venous thromboembolism after hip and knee replacement surgery and for the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation [20].

The main advantage of dabigatran is that it does not require routine coagulation monitoring. However, intentional and accidental dabigatran coagulopathy is being reported with increasing frequency [19, 21–24]. A recent case report describes a patient who developed fatal hemorrhage while he was on dabigatran treatment. This patient also developed AKI [21]. The authors suggested that the AKI was the result of dabigatran excessive anticoagulation. In light of the previously cited case report [6] and our findings in rats, we may argue that the AKI in this patient was also the result of dabigatran nephrotoxicity.

Our data indicate that dabigatran increases Scr in a dose-dependent manner in 5/6 nephrectomy rats, similar to the effects of warfarin in this experimental model, as we reported
earlier [4]. Unexpectedly, similar effects of dabigatran on Scr were seen in control, non-operated rats. Based on our previous data, excessive anticoagulation with vitamin K antagonists is not associated with Scr increase in control rats [4, 5]. The 5/6 nephrectomy rats were more sensitive to dabigatran and developed hematuria earlier and after lower doses of dabigatran when compared with control rats (Figure 2C and D). Thus, the 50 mg/kg/day of dabigatran resulted in a 2-fold increase in aPTT (as recommended in humans [13]) by Days 6–7 after the treatment, and both serum creatinine and hematuria were significantly increased by the same time (Figure 2). The 100 and 150 mg/kg/day of dabigatran resulted in accelerated and more significant increase in serum creatinine and proteinuria, indicating dose-dependent effects. Morphologically, the findings in 5/6 nephrectomy rats treated with dabigatran were similar to those found in animals with WRN, with respect to RBC tubular casts and acute tubular injury [4, 5]. Interestingly, we found a single area in 1 out of 30 kidneys from control animals treated with dabigatran, which also had RBC tubular casts (Figure 3A). This phenomenon was not seen in control rats treated with vitamin K antagonists even after fatal excessive anticoagulation [5].

We believe that this is a very important observation for clinical practice, indicating that constant monitoring of kidney function may be necessary in patients on dabigatran treatment. The necessity of constant kidney function monitoring may outweigh the benefits of not monitoring coagulation parameters in these patients [25].

Therefore, based on our animal study and recent observation in patients, we believe that WRN is just the tip of the iceberg and it represents only part of a much broader condition, anticoagulant-related nephropathy (ARN). Novel anticoagulants, such as direct thrombin inhibitors and Factor Xa inhibitors, are just entering into clinical practice, and little is known about their effect on the kidneys.

Our findings, that dabigatran affects kidney function, change our view on the pathogenesis of ARN. After the first publications of our works based on vitamin K antagonists [1, 4, 5], there was no compelling evidence that other anticoagulants might affect the kidney. Taking our findings with vitamin K antagonists and dabigatran together, we currently hypothesize that there is a common pathway in the pathogenesis of ARN and this pathway does not include vitamin K-dependent proteins. The common result of both vitamin K antagonists and direct thrombin inhibitors is diminished thrombin activity. By acting on thrombomodulin, which is expressed on endothelial cells, thrombin activates protein C and modulates the anticoagulation cascade. Another important
receptor for thrombin, which is also expressed on endothelial
cells, is PAR-1. PAR-1 is a G protein-coupled receptor and it
participates in the regulation of the endothelial functions, vas-
cular permeability, leukocyte migration and adhesion [26, 27].

In vitro studies indicate that PAR-1 activation changes endo-
thelial monolayer integrity [28]. We propose that thrombin
plays an important role in the glomerular filtration barrier
function, and its decreased activity (secondary to anticoagula-
tion) results in glomerular filtration barrier abnormalities.
Indeed, our data indicate that treatment with selective PAR-1
inhibitor SCH79797 results in increased SCr, hematuria and
tubular RBC casts, findings similar to those in animals with
WRN or treated with dabigatran. Interestingly, these findings
were more pronounced in 5/6 nephrectomy than in control
rats, indicating that ablative nephropathy by itself makes
kidneys more sensitive to changes in coagulation disturbances
and it is probably related to changes in PAR-1. In fact,
decreased PAR-1 protein expression was found in several
human kidney diseases, such as crescentic glomerulonephritis
and thrombotic microangiopathy [29, 30]. Supportive of our
hypothesis is that hematuria increases shortly after treatment
with dabigatran in control rats (albeit mild and only at the highest doses), and
only 1 of 30 control animals developed very focal RBC casts, it
appears unlikely that tubular obstruction plays more than a
very minor role in AKI in control rats. Of note, despite that
changes in coagulation parameters were similar to those that
are recommended for patients [13, 31], dabigatran affected
renal function and induced hematuria in concentrations sig-
ificantly higher than those used for humans [13]. These
differences may be explained by different pharmacodynamics
and pharmacokinetics of dabigatran in rats, which require a

**FIGURE 4:** Selective PAR-1 inhibitor SCH79797 results in increased serum creatinine and hematuria in rats. SCH7979 increases serum cre-
atinine in both control (A) and 5/6 nephrectomy (B) rats. Treatment with SCH79797 results in rapid increase in hematuria in both control (C)
and 5/6 nephrectomy (D) rats. Hematuria was semi-quantitated as described above in Figure 2. n = 6 in each experimental group. *P < 0.05 com-
pared with 100 µg/kg/day SCH79797.
significantly higher dose of this drug than humans to achieve the same degree of anticoagulation [11, 12]. This raises the possibility of direct nephrotoxic effects of dabigatran.

In conclusion, the novel thrombin inhibitor dabigatran causes AKI when dosed sufficiently to cause a coagulopathy. These effects are similar to those of WRN. However, in contrast to WRN, where kidney injury was seen only in animals, the effects of dabigatran were prominent in control rats as well. These findings suggest that the risk to the kidney by dabigatran may be greater than that of warfarin.

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CONFLICT OF INTEREST STATEMENT
The results presented in this paper have not been published previously in whole or part, except in abstract format.

REFERENCES