Assessing the anticoagulant effect of dabigatran in children: An in vitro study

Kevin Dietrich, Linda Stang, Joanne van Ryn, Lesley G. Mitchell

Introduction

Dabigatran etexilate is the orally available prodrug of the direct thrombin inhibitor, dabigatran. It is currently used in adults for the prevention of venous thromboembolic events after total hip or knee replacement [2–4], for treatment of deep venous thrombosis (DVT) and pulmonary embolism (PE) after use of a parenteral anticoagulant replacement [2–3]. Treatment of venous thromboembolic events after total hip or knee replacement [4], for treatment of deep venous thrombosis (DVT) and pulmonary embolism (PE) after use of a parenteral anticoagulant replacement [2–3] or a requirement for parenteral administration [11–13]. Differences in anticoagulant effects that necessitate routine monitoring and dose adjustment or a requirement for parenteral administration [11–13]. Factors such as central venous catheters, hospitalization, infection/sepsis, or cardiac disorders are susceptible to DVT and require anticoagulant therapy [8–10].

The most commonly used anticoagulants in children are low-molecular-weight heparins (LMWHs), unfractionated heparin (UFH), and vitamin K antagonists (VKAs). These conventional anticoagulants have general drawbacks, such as variable pharmacokinetic and anticoagulant effects that necessitate routine monitoring and dose adjustment or a requirement for parenteral administration [11–13]. Factors such as central venous catheters, hospitalization, infection/sepsis, or cardiac disorders are susceptible to DVT and require anticoagulant therapy [8–10].

There are well-described physiological, age-related variations in the hemostatic system occurring from birth and throughout childhood that profoundly impact anticoagulation in children [14–16]. Differences in the hemostatic system result in variation in response to anticoagulants and coagulation assays over childhood. This study used in vitro methods to determine i) optimum coagulation assays for dabigatran in children and ii) anticoagulant effect of dabigatran across pediatric age groups.

Materials and Methods: Pooled plasma samples from healthy children aged 0 to <1, 1 to <5, 5 to <10, 10 to <17 years and adults were spiked with increasing concentrations of dabigatran and the effect was assessed in five coagulation assays. The samples were also assessed for overall hemostasis potential using a fibrin clot formation and lysis assay.

Results: In all five coagulation assays, there were no differences in responses to dabigatran over all pediatric age groups. The international normalized ratio was the least sensitive measure. Activated partial thromboplastin time showed moderate sensitivity and a nonlinear response curve. Thrombin time (TT), dilute TT (dTT) and ecarin clotting time were linearly correlated with dabigatran concentrations; however, the ecarin time and TT were overly sensitive. In the overall hemostasis potential assay, increasing dabigatran concentrations delayed the initiation of clot formation and reduced the time to 50% clot lysis. The responses to initiation of clot formation and clot lysis were consistent across all pediatric groups and comparable to responses in adults.

Conclusion: The dTT is the most suitable assay for measuring dabigatran concentrations in children. Fibrin clot generation and lysis assay responses to dabigatran in children over all ages were consistent and comparable to those of adults.

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developed for monitoring anticoagulation in adults show a varying response to anticoagulation in children. Indeed, the in vitro response of standard clotting assays to UFH and the ex vivo response to warfarin differ when performed in plasma from children compared with plasma from adults [17–20].

Dabigatran exetilate acts independently of endogenous antithrombin levels, and has the advantage of oral administration, dose-proportional pharmacokinetics and pharmacodynamics, and few drug interactions, obviating routine coagulation monitoring in adults [21]. Several assays have been evaluated for the qualitative or quantitative determination of dabigatran in plasma [22]. To date, no study has assessed dabigatran in pediatric patients. Determining the optimum coagulation assay for assessing plasma levels in children and characterizing the anticoagulant response to dabigatran across different age groups is important. We therefore assessed the in vitro effect of dabigatran in plasma of children of differing ages, compared with adults, using standard clotting assays. In addition, we determined the anticoagulant response to dabigatran over childhood using an overall hemostasis potential assay that assesses the capacity for fibrin clot formation and fibrin clot lysis.

Methods

Plasma Samples

Venous blood samples were pooled from at least 9 to 11 subjects in each pediatric age group and 20 adults. The protocol was approved by University of Alberta Ethics Review Board. Normal healthy children having clinical blood work done for routine elective surgery at Stollery Children’s Hospital, Edmonton, Canada were eligible for inclusion. Children/legal guardians gave written assent/consent.

Platelet-poor plasma was prepared from citrated whole blood by centrifugation and was stored at –80 °C.

Dabigatran

Dabigatran (Boehringer Ingelheim, Biberach, Germany) dissolved in 0.1 N hydrochloric acid to 10 mg/ml was frozen in single-use aliquots (–80 °C). These were diluted for use with HEPES-buffered saline (pH 7.05) to three stock concentrations. Pooled platelet-poor plasma from each age group was divided into five aliquots and dabigatran (or vehicle) was added: final concentrations 0, 50, 250, and 450 ng/ml.

Coagulation Assays

Assays were performed for each dabigatran concentration using an automated coagulation analyzer (ACL 300+, Instrumentation Laboratory, Milan, Italy) following standard procedures [23].

Thrombin Time (TT)

Sample plasma and reagent (1.5 National Institutes of Health [NIH] units/ml human thrombin [Stago, Toronto, Canada] containing calcium) were prewarmed (37 °C, 180 seconds) and mixed.

Dilute Thrombin Time (dTT)

Sample plasma, diluted 1:8 with saline, was incubated with standard plasma pool (Hemoclot Thrombin Inhibitor Kit, Hyphen Biomed, Neuville-sur-Oise, France) (37 °C, 60 seconds). Prewarmed human calcium thrombin from the kit was added [24].

Ecarin Clotting Time (ECT)

Plasma was mixed with ecarin reagent containing calcium (Sigma-Aldrich E0504, Oakland, Canada) at a final concentration of 2 units/ml after prewarming (37 °C, 120 seconds) [25].

Activated Partial Thromboplastin Time (aPTT)

Plasma was incubated (37 °C, 270 seconds) with Synthasil (Instrumentation Laboratory, Bedford, MA, USA), then prewarmed calcium (0.2 M) was added.

Prothrombin Time (PT)

Prewarmed (37 °C, 180 seconds) plasma and Hemoliance RecombiPlasTin (Instrumentation Laboratory) containing recombinant human tissue factor and calcium were mixed. PT values were used to estimate international normalized ratio (INR; ratio of test to control PT).

Overall Hemostasis Potential Assay

The assay [26] was performed in microtiter plates (Corning Inc, Corning, NY, USA). Change in turbidity at 405 nm was followed using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Assays were initiated by adding 60-μl aliquots of plasma sample (35 μl) in 0.02 M HEPES/0.15 M sodium chloride/0.01% Tween 80 (25 μl) to 40-μl aliquots containing 5 mM calcium chloride, 1/800 dilution tissue factor final concentration 2.5 PM, (RecombiPlasTin, Instrumentation Laboratory, Richmond Hill, ON, Canada), 15 μM L-α-phosphatidylcholine/L-α-phosphatidylethanolamine/L-α-phosphatidyserine vesicles (Avanti Polar Lipids, Alabaster, AL, USA), and 3 nM tissue plasminogen activator (Alteplase, Cedarlane Laboratories, Burlington, Canada).

Lag time to initiation of clot formation is the time from the initial reading to an increase in absorbance from baseline. Time to 50% of clot lysis (t50, clot lysis) is the interval from maximum absorbance at 405 nm to a 50% reduction in turbidity.

Statistical Analyses

The coagulation assays and overall hemostasis potential assay parameters were compared between age groups and dabigatran concentrations using the Kruskal-Wallis test; P < 0.05 considered significant.

Results

Patients

Median ages of the pediatric groups were: 0 to < 1 year, 5 months (n = 9); 1 to <5 years, 2 years (n = 9); 5 to <10 years, 6 years (n = 11); 10 to <17 years, 12 years (n = 12). The adult group consisted of 20 subjects.

Coagulation Assays

There was no difference in response to increasing dabigatran concentrations in plasma from each of the pediatric age groups in the five coagulation assays. However, the clotting times, with increasing concentration of dabigatran, for the aPTT, ECT, and TT were longer in children compared with adults. Baseline aPTT and ECT were significantly longer across the pediatric groups compared with the adult group. Therefore, the aPTT and ECT were calculated as a ratio of the test to the baseline clotting time. These ratios of aPTT and ECT response to increasing dabigatran concentrations were similar in adults and children. Baseline TT did not differ significantly between adult and pediatric groups. Compared with the adult group, the pediatric groups showed increased sensitivity to dabigatran using the TT. The dTT showed no significant difference at baseline between children and adults. The response to increasing dabigatran concentrations was similar in the pediatric and adult plasma. However, there were differences in the sensitivity and linearity among assays (Fig. 1).
**aPTT**

The aPTT was moderately sensitive to increasing dabigatran concentrations with an ~3-fold prolongation over baseline at the highest concentration, though the response was not linear. The pediatric groups had a prolonged baseline aPTT; however, when baseline was corrected for, by calculating the data as a ratio to baseline, it was apparent that there was no difference in the sensitivity to dabigatran when compared with the adult plasma (data not shown). The aPTT results were reproducible at dabigatran concentrations up to 450 ng/ml (Fig. 1A).

**ECT**

The ECT assay showed an extremely sensitive linear response to dabigatran concentrations in all pediatric groups. ECT was consistent over all pediatric age groups, but significantly different from the adult group. The baseline ECTs were prolonged compared with adults; when baseline was corrected for by calculating as a ratio over baseline, there was no difference in the sensitivity to dabigatran when compared with adults (data not shown). Clotting times were ~6.5-fold over baseline at 450 ng/ml dabigatran (Fig. 1B). Reproducibility of the assay was poor at increased dabigatran concentrations.

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**Fig. 1.** Relationship between dabigatran concentration and response using five different coagulation assays in plasma of children and adults.
**PT (INR)**

The PT values represented as an INR were relatively insensitive, but displayed a linear response to increasing dabigatran concentrations. The pediatric groups displayed comparable results to the adults. Even with concentrations up to 450 ng/ml, a 2-fold prolongation over baseline was not achieved (Fig. 1C). Reproducibility of assay measurements was good.

**TT**

The TT assay was extremely sensitive to increasing dabigatran concentrations in pediatric and adult plasma (Fig. 1D). Mean baseline TT was 18.9 to 19.5 seconds across all ages. There were no differences in response to dabigatran across pediatric groups. The sensitivity across pediatric groups was somewhat increased compared with adult plasma, with an ~15–18-fold prolongation in adult plasma, versus a 13.1-fold elevation in adult plasma. The assay was not reproducible for prolonged clotting times.

**dTT**

The dTT assay was sensitive to increasing dabigatran concentrations in pediatric plasma and displayed a linear relationship between clotting time and dabigatran concentration. The results were similar between pediatric and adult groups, with a ~3-fold prolongation over baseline measured at the highest dabigatran concentration of 450 ng/ml in pediatric plasma and a 3.36-fold prolongation in adult plasma (Fig. 1E). The dTT was very reproducible between measurements.

**Overall Hemostasis Potential** (Fig. 2)

In children and adults, increasing concentrations of dabigatran increased the lag time to initiation of clot formation, and decreased the overall clot lysis time, in a linear fashion. The profile of the overall hemostasis potential assay with the highest concentration of dabigatran, 450 ng/ml, was slightly different than the other curves, even though the lag time was further prolonged and the lysis time reduced. This reflects an artifact of the system, since the turbidity of the plasma is being measured and at this concentration of dabigatran clot formation is no longer normal, but the clots that form are very soft, do not retract and thus create a more turbid effect even though there is not more clot formation. Thus it represents a type of fibrin monomer/short chain “suspension” which has a higher absorbance than clear control.

**Lag Time to Initiation of Clot Formation**

At baseline, there was a significant difference in lag time between all pediatric groups and adults, with children having a shorter lag time to clot initiation ($P = 0.005$). However, there were no differences in lag time across the pediatric age groups ($P = 0.64$). Ratio data were used to correct for baseline differences. Across the pediatric groups, there was no difference in the ratios at any dabigatran concentration: 50 ng/ml, $P = 0.12$; 250 ng/ml, $P = 0.08$; 450 ng/ml, $P = 0.07$. Comparing all pediatric groups versus the adult group, there was a statistically significant decrease in response at lower dabigatran concentrations (50 ng/ml, $P < 0.001$; 250 ng/ml, $P = 0.001$), but no difference at 450 ng/ml ($P = 0.18$). The variations in response to clot initiation were minor, with 6% to 7% difference between the adult and pediatric plasma at lower dabigatran concentrations.

**Time to 50% of Clot Lysis**

At baseline, there was a significant difference between all pediatric groups and adults, with the pediatric groups having a slightly longer $t_{50}$ lysis time ($P < 0.001$). However, there was no difference in $t_{50}$ lysis time across pediatric age groups ($P = 0.23$). Correcting for baseline differences, there was no significant difference among all pediatric age groups when comparing the ratio data over all dabigatran concentrations. Also, no significant difference was seen in the $t_{50}$ lysis ratio comparing adult and children over all age groups and all dabigatran concentrations.

**Discussion**

Novel oral anticoagulants such as dabigatran etexilate are used in adults in fixed doses without routine anticoagulation monitoring. In certain situations, such as bleeding into critical organs, a suspected overdose, or emergency surgery, the anticoagulant status may need to be determined using a suitable assay. In addition, a reliable assay is useful for determining plasma drug concentrations in clinical trials, including pharmacokinetic studies. In children, the most critical determinant of anticoagulant dosing is age, because age has a profound effect on drug clearance with younger children requiring increased doses [27,28]. Therefore, monitoring of novel anticoagulants may be necessary in children, as the dosing may change in relation to the age of the child during treatment, as is the case with warfarin and heparin. The objective of the current study was to determine coagulation assay response over childhood at fixed concentrations of dabigatran using various laboratory assays as compared with adults.

The range of dabigatran concentrations tested represented the anticipated therapeutically relevant range and above. For example, population pharmacokinetic modelling based on adult patients with atrial fibrillation provides an estimated steady-state peak plasma dabigatran concentration of 184 ng/ml (5th and 95th percentiles 64–443 ng/ml) and a trough concentration of 90 ng/ml (31–255 ng/ml, respectively) after 150 mg twice-daily dosing with dabigatran etexilate. A 220 mg once-daily regimen results in a lower trough level of 37 ng/ml (10–96 ng/ml, respectively) [22].

The assays used in our study have been evaluated previously in adult plasma samples and results of the current study support the previous key findings [22]. The least sensitive measure of dabigatran was the INR. The aPTT showed moderate sensitivity and a consistent but nonlinear concentration–response curve. The dTT, TT, and ECT were linearly and sensitively correlated with plasma dabigatran concentrations. However, the TT and ECT were too sensitive, resulting in extremely prolonged clotting times – negatively impacting the reproducibility of the assay measurements.
the assays. From a practical perspective, the dTT is the most suitable assay for measuring dabigatran concentrations within and in excess of the therapeutic range in children, which is in agreement with findings in adults [22]. The dTT performed consistently across all age groups and the clotting times were similar to (or slightly shorter than) those in adult plasma. Furthermore, the dTT showed good reproducibility. The assay is commercially available with dabigatran standards for calibration [22].

Within each of the 5 assays, there were no remarkable differences among the coagulation assay responses to dabigatran from the different pediatric groups. In these assays, the pediatric plasma pools had slightly longer clotting times than the adult pool in control and dabigatran-spiked samples, possibly reflecting differences in the hemostatic system of healthy children [14]. Significant age-related differences in the effects on standard coagulation assays in vitro have been reported previously for several anticoagulants. For example, at each concentration of UFH tested, the aPTT was longer and anti-IIa activity was increased in plasma from children compared with adults. In addition, the difference in effect from that in adult plasma was more marked in the younger children (ages <1 year and 1 to <5 years) than in the older children [18]. A similar pattern of aPTT responses has been reported for danaparoid (a low-molecular-weight heparinoid) and lepirudin (a direct thrombin inhibitor) [19]. The ECT response to lepirudin was lower (shorter coagulation times) in children compared with adults [19]. Differing effects on thrombin generation assays between children and adults have also been reported for UFH, LMWH, and warfarin [17,20]. In contrast, no age-related effect was seen on the TT assay with LMWH [29]; on aPTT or PT with rivaroxaban (a direct factor Xa inhibitor) [30]; on anti-Xa assays with LMWH [29], fondaparinux (an antithrombin-dependent factor Xa inhibitor) [19], rivaroxaban [30], or danaparoid [19]; or on an anti-IIa assay with lepirudin [19].

The overall hemostasis potential assay quantifies the plasma capacity to both generate a fibrin clot and lyse the fibrin clot, allowing assessment of coagulant and fibrinolytic capacity in one system; this is more comprehensive than thrombin generation assays, which only assess the capacity to generate thrombin [26]. The overall hemostasis potential assay uses plasma, offering distinct advantages over the thromboelastography tests, which require fresh whole blood and do not allow for pooling of samples. A similar assay to the overall hemostasis potential assay, the clot formation and lysis assay, has been used in children with DVT [31]. In vitro studies in adults have assessed the anticoagulant effect of dabigatran using the overall hemostasis potential assay [32,33]. Results from both studies indicated that dabigatran decreased fibrin formation and enhanced fibrinolysis. Data from the current study using adult plasma demonstrated that the response in the overall hemostasis potential assay to increasing dabigatran concentrations for both the clot generation and clot lysis were in agreement with previously published studies [32,33]. In terms of clot generation, there was an increase in the lag time to clot initiation with increasing dabigatran concentrations, reflecting the thrombin inhibition by the anticoagulant. Regarding the capacity for fibrin clot lysis, there was a decrease in the t50 clot lysis time, reflecting dabigatran’s ability to enhance clot susceptibility to fibrinolysis. When corrected for baseline, the response to dabigatran was comparable in children and adults. Of note, the anticoagulant response was identical in children across all ages, which is an important observation for this drug in pediatrics: all other current anticoagulants show a differing response in children compared with adults [17,20]. The fact that the response to dabigatran in pediatric plasma is relatively consistent with adult plasma is promising, possibly indicating that the clinical response is comparable to that in adults. Also, the response is consistent across all pediatric ages, suggesting that age-related differences in the hemostatic system do not alter the response to dabigatran. While the in vitro response to dabigatran does not vary over age, the current study was not designed to assess dose requirements over age, which is being assessed in properly designed dose-finding Phase II pharmacokinetic/pharmacodynamic studies. Finally, the efficacy and safety of dabigatran in children of all ages must be assessed in adequately powered clinical trials.

Due to restrictions on acceptable volumes of blood from children for research purposes there was insufficient sample to do subjects individually. Therefore, one of the limitations of the study was that testing was done on spiked pooled plasma from the various age groups rather than individual plasmas spiked with dabigatran. However, this approach is reasonable and a relatively common approach to such studies in children.

Dabigatran etexilate is currently being assessed for safety and efficacy in children in carefully designed clinical trials. A total of six clinical trials, four Phase II and two Phase III have been undertaken and one Phase II trial has been completed (http://clinicaltrials.gov). Although it is hoped that dabigatran etexilate could ultimately be used in children without routine monitoring (as in adults), the availability of a simple assay that provides consistent results across age groups is invaluable in helping to identify suitable doses during the pediatric clinical development process.

In conclusion, the coagulation assay response to dabigatran for all tests was similar in pediatric plasma compared with adult plasma. The ECT and TT were more variable between adult and pediatric plasma, particularly as dabigatran concentrations increased, reflecting inaccuracies in reproducibility of these assays as the clotting times become prolonged. From a practical perspective, the dTT is an appropriate tool for measuring dabigatran concentrations in children, as also holds true in adults [22]. The dTT performed consistently across all age groups, and showed good reproducibility between repeat measurements and appropriate sensitivity and linearity of response within and above the therapeutic range. In addition, the anticoagulant response to dabigatran, as assessed by the capacity to generate a fibrin clot, is consistent over childhood and comparable to the adult response. Finally, the increased susceptibility to fibrin clot lysis associated with dabigatran is consistent over childhood and similar to that in adults. However, it is important to note that the results of the current study are preliminary pending confirmation in vivo in pediatric patients receiving dabigatran.

Author Statement

LGM conceived the idea, designed the project and drafted the manuscript. KD and LS conducted the experimental laboratory work and drafted the manuscript. JvR helped to draft the manuscript. All authors read and approved the final manuscript. No author received an honorarium, grant, or other form of payment to produce the manuscript.

Disclosure of Conflicts of Interest

LGM is paid for consulting as a member of the International Steering Committees for Pediatric studies for Boehringer Ingelheim, EISAI and Bristol-Myers Squibb. JvR is a full-time employee of Boehringer Ingelheim Pharma GmbH & Co. KG. KD and LS have no conflicts of interest.

Role of the Study Sponsor

This study was funded by a nonrestricted research grant awarded to LGM by Boehringer Ingelheim. The study sponsor had no role in the study design, or in generating or analyzing the data and the decision to submit the manuscript for publication. JvR reviewed provided editorial input as it related to the technical aspects and interpretation of coagulation assays and reviewed the manuscript.

Acknowledgments

The authors would like to thank Keith Day, PhD, of PAREXEL, for writing support, which was funded by Boehringer Ingelheim. Writing
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