Decreased Concentrations of Heparinoids Are Required to Inhibit Thrombin Generation in Plasma from Newborns and Children Compared to Plasma from Adults due to Reduced Thrombin Potential

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Keywords

Heparin, thrombin, anticoagulant, newborn, plasma

Summary

Thrombin generation is decreased and delayed in plasma from newborns and children compared to adults. We hypothesized that lower doses of heparinoid anticoagulants are required to give similar thrombin generation in newborn (umbilical cord) and child plasmas compared to that of adults. Thrombin generation was performed in either the absence or presence of unfractionated heparin (UFH), low molecular weight heparin (LMWH) or a covalent antithrombin-heparin complex (ATH). After contact activation and recalcification of each plasma, thrombin activity was measured by periodic sub-sampling into chromogenic substrate. UFH inhibited thrombin generation to a greater extent compared to LMWH in all plasmas. Cord plasma was more sensitive to inhibition and displayed a greater difference in the effectiveness of UFH compared to LMWH than other plasmas. Lower concentrations of UFH and LMWH were required to inhibit thrombin generation in cord and child plasmas compared to adult plasma. In comparison, ATH strongly inhibited thrombin generation in all 3 plasmas. Similar peak thrombin concentrations were observed at lower ATH concentrations (0.1 U/mL) compared to either UFH (0.25 U/mL) or LMWH (0.25 U/mL). As with UFH and LMWH, cord plasma was more sensitive to inhibition by ATH than the other plasmas and lower ATH concentrations inhibited thrombin generation in cord and child plasmas compared to adult plasma. Decreased thrombin generation with heparinoids in cord and child plasmas compared to adult plasma coincided with decreased rates of prothrombin consumption and increased proportion of thrombin-α2-macroglobulin inhibitor complexes. In summary, lower doses of UFH, LMWH or ATH result in similar peak thrombin generation in newborn and child plasmas compared to adult plasma. Cord plasma was the most sensitive to inhibition, with ATH being more effective than UFH or LMWH.

Introduction

Thromboembolic disease (TEs) in children has been described as a new epidemic in tertiary paediatric care (1, 2). The increased recognition of TEs as a significant cause of morbidity and mortality in children already suffering from major primary illnesses, has led to more aggressive attempts to provide prophylactic and therapeutic anticoagulation therapy for children (3-5). However, current anticoagulation therapy in children is based on recommendations developed for adult patients. In particular, target therapeutic ranges for all anticoagulation therapy in children are directly extrapolated from adult data, with no clinical trials to determine the optimal therapeutic or prophylactic target ranges in children.

Target ranges for antithrombotic therapy in adults are unlikely optimal for children for two major reasons. First, there are vast differences in the epidemiology of TEs in children compared to adults, in particular with respect to frequency of co-morbid conditions which may alter the risk of bleeding (6-10). Second, developmental haemostatic differences between children and adults are well documented in numerous studies (1, 6, 11-14). Thrombin generation is decreased and delayed in plasma from newborns and children compared to adults (15, 16). Prothrombin is decreased and α2-macroglobulin increased in plasma from the young, compared to that of adults (11, 17, 18), further reducing plasma thrombin potential (19-21).

Given the reduced thrombin potential of plasma from newborns and children compared to adults, we hypothesised that reduced doses of anticoagulants may inhibit thrombin generation in newborns and children to the same extent as in adults. Effective thrombin inhibition using reduced doses of anticoagulant drugs, would have clear clinical benefits, as many children requiring anticoagulation, such as premature infants and post-cardiac surgical patients, have significant bleeding risks. Prior to performing clinical trials of reduced dose anticoagulant therapy in children, the aim of this study was to compare the effects of a variety of heparinoid anticoagulants, including unfractionated heparin (UFH), low molecular weight heparin (LMWH) and a covalent antithrombin-heparin complex (ATH) on thrombin generation in plasma from newborns, children and adults in vitro.

Subjects, Materials and Methods

Subject Population

The study received approval from the ethics board of the Hamilton Health Sciences Corporation and informed consent was obtained for venous blood sampling from adult subjects and either the children or their parents. Venous
plasma was obtained from umbilical cords taken after full-term, uneventful deliveries at St Joseph’s Hospital, Hamilton, Ontario, Canada. Plasmas from healthy children and adults were also collected. The healthy children were having routine blood work performed prior to elective day surgery, in the paediatric outpatient clinic at the Children’s Hospital at Chedoke-McMaster (CHCM), Hamilton, Canada. The healthy adults were volunteers.

### Blood Sampling and Plasma Preparation

Three mL blood samples were obtained from all 3 study groups at the time of blood sampling for clinical purposes. Blood samples were collected into tubes or syringes which contained 3.2% buffered sodium citrate (volume of blood:volume of citrate = 9:1), immediately centrifuged (3000 × g for 20 min) and platelet-poor plasma removed and stored at −80°C for future coagulation studies. For the measurement of thrombin generation, prothrombin consumption, and thrombin inhibitor complex formation, plasma pools were prepared from the 3 study groups. Each plasma pool was comprised of plasmas from a minimum of 5 individuals. The APPT and PT were measured using an ACL machine (Instrumentation Laboratory, Milan, Italy). PT values were expressed as INRs. Plasma concentrations of coagulation factors were measured using standard microtechniques (18, 22).

### Thrombin Generation Experiments

Thrombin generation was quantitated using a previously described technique. In brief, plasma (500 μL) was defibrinated by incubation with Arvin (15 μL of 6 U Ancrod/mL of 0.036 M Na acetate, 0.036 M Na diethylbarbiturate, 0.145 M NaCl, pH 7.4 buffer) for 10 min at 37°C, followed by a further 10 min incubation on ice (with any clot formed being wound out using a wire loop after each incubation). Reaction mixtures (at 37°C) consisted of a solution (pre-incubated for 3 min) of 100 μL of defibrinated plasma + 50 μL of APTT reagent, to which 50 μL of 0.04 M CaCl₂ in buffer was added. At various times after CaCl₂ addition, a subsample (25 μL) of reaction mixture was mixed with 0.005 M Na₂ EDTA (475 μL) on ice and 25 μL of the resultant time sample-EDTA solution was then mixed with 775 μL of 0.00016 M S-2238 in buffer. After incubation of the S-2238 containing mixture for 10 min at 37°C, 200 μL of 50% (v/v) acetic acid was added. The amount of thrombin generated was determined by comparing the absorbance at 405 nm (amidolysis of S-2238 substrate) to that produced by the reaction of S-2238 with known amounts of purified thrombin. The final plasma dilution after reaction with substrate was 1/1280. Total amidolytic thrombin activity was measured for a duration of up to 5 min and at least every 15 s when peak amidolytic activity appeared.

Since thrombin bound to α-M retains activity against small substrates (24), formation of thrombin-α-M complexes was quantitated by a previously described method [thrombin generations carried out as above, except that thrombin not bound to α-M was neutralized with antithrombin (AT) + heparin prior to mixing with EDTA] and subtracted from the total thrombin activity to give the free thrombin activity (23). The amidolytic activities were plotted against time to produce thrombin generation curves for all plasma pools. Prothrombin levels were measured in all plasma pools and in each sample used for thrombin generation to study prothrombin consumption in conjunction with thrombin generation. Prothrombin, thrombin-antithrombin complex and thrombin-heparin cofactor II complex concentrations during the thrombin generations were determined by analysis of the time sample-EDTA mixtures using ELISA kits (Affinity Biologicals, Hamilton, ON, Canada).

In order to study the effect of anticoagulants on thrombin generation in the different plasma systems, prior to thrombin generation, plasma pools were spiked to various concentrations (range 0.1 to 0.5 anti-factor Xa U/mL) with either UFH (Leo Laboratories, Ajax, Ontario, Canada), LMWH (Clivar® 1.750, Nordmark, Germany) or covalent antithrombin-heparin complex (ATH). ATH was prepared by incubating AT (Bayer, Mississauga, Ontario, Canada) with UFH (Sigma, Mississauga, Ontario, Canada) in pH 7.3 buffer at 40°C for 10 days, followed by purification of the product on butyl agarose and DEAE Sepharose columns. The different heparinoid anticoagulants were mixed into the various plasmas at different anti-factor Xa concentrations according to the activities quoted by the manufacturer (UFH and LMWH) and as assayed (UFH, LMWH and ATH) by an anti-factor Xa heparin kit (Stachrom Heparin, Diagnostica Stago, Asnières, France) using an ACL300 machine (Instrumentation Laboratories, Lexington, MA, USA). Anti-IIa activities (catalytic inhibition of thrombin by antithrombin) of UFH, LMWH and ATH stock reagents were assayed using an anti-IIa heparin kit (ACTICHROME® Heparin, American Diagnostica Inc., Greenwich, CT, USA) and the ACL300. Anti-factor Xa activity, anti-IIa activity and mg heparin/mL for UFH, LMWH and ATH are listed in Table 1. Thus, the relative anti-factor Xa/anti-IIa ratios for UFH, LMWH and ATH in plasma were 1.24, 2.44 and 1.03, respectively (Table 1). During the thrombin generations, defibrinated plasma samples containing 0.25 U (anti-factor Xa) UFH/mL, 0.25 U LMWH/mL or 0.1 U ATH/mL were tested, corresponding to 1.72 μg/mL, 2.02 μg/mL or 0.14 μg/mL (in terms of heparin), respectively.

### Statistical Analyses

An analysis of variance (ANOVA) or general linear model ANOVA were performed to compare test results between groups. In some cases a Student’s t-test was performed to analyze differences between groups. A p-value of < 0.05 was considered significant. All values are expressed as means ± SEM.
Results

Plasma Pool Groups

Pools were collected from 3 different age groups. Pools contained a minimum of 10 donors for each age group. Plasma from newborn umbilical cord veins had slightly prolonged APPT and PT values compared to that of children and adults. The ages of the children ranged from 2 to 13 years with an average age of 6.7 years and a median age of 5 years. Coagulation parameters (APPT, PT, INR) and concentrations of most coagulation factors (fibrinogen, contact factors) in the child group were similar to those of the adults. However, plasma prothrombin concentrations were decreased (80%) in children compared to adults. Values for APPT, PT/INR in the adults were well within the normal range (11).

Effect of Age on Thrombin Generation

Free thrombin generation in plasmas from newborns (cord), children and adults is shown in Fig. 1. Peak thrombin concentrations in children were slightly decreased compared to adults. Generation of free thrombin was significantly decreased and delayed (t-test, p < 0.01) in newborns compared to adults. This result corresponded to decreased starting prothrombin concentrations and a reduced rate of prothrombin consumption in newborns compared to adults (Fig. 2).

Effect of Anticoagulants on Thrombin Generation in Different Age Groups

Table 2 shows the peak thrombin concentrations achieved for representative heparinoid concentrations in newborn, child and adult plasma (results from other heparinoid concentrations not shown). At similar anti-factor Xa concentrations, UFH inhibited free thrombin generation to a greater degree than LMWH in all age groups. ATH inhibited thrombin generation to a similar extent compared to UFH and LMWH in all three plasma groups but at decreased anti-factor Xa concentrations. Thrombin generation experiments with UFH, LMWH and ATH at other concentrations further verified the above findings.

The effects of UFH (0.25 U/mL), LMWH (0.25 U/mL) and ATH (0.1 U/mL) on newborns, children and adults are compared in Figs. 3a, 3b and 3c, respectively. For UFH, thrombin generation is delayed and reduced in children compared to adults, and virtually absent in newborns. Thrombin generation is similar in adults and children at the same concentration of LMWH, however thrombin generation in newborns is significantly reduced. A similar pattern is observed for ATH, although thrombin generation in the newborns is virtually absent.

Table 2  Peak thrombin concentrations during thrombin generation in plasmas containing different heparinoids

<table>
<thead>
<tr>
<th>Heparinoid</th>
<th>Newborn Plasma</th>
<th>Child Plasma</th>
<th>Adult Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFH (0.25 U/mL)</td>
<td>15.6 ± 0.5</td>
<td>293 ± 28</td>
<td>354 ± 100</td>
</tr>
<tr>
<td>LMWH (0.25 U/mL)</td>
<td>426 ± 6</td>
<td>813 ± 10</td>
<td>820 ± 44</td>
</tr>
<tr>
<td>ATH (0.1 U/mL)</td>
<td>11.7 ± 1.1</td>
<td>616 ± 5</td>
<td>715 ± 16</td>
</tr>
</tbody>
</table>

The effects of the anticoagulants on thrombin generation were mirrored by corresponding effects on prothrombin consumption in newborns, children and adults (Figs. 4a, 4b and 4c, respectively). Prothrombin consumption was more delayed in all age groups by UFH.
Fig. 3 Effect of plasma type on inhibition of plasma thrombin generation by heparinoids. The effect of age on plasma thrombin generation with either: a) 0.25 U/mL unfractionated heparin (UFH), b) 0.25 U/mL low molecular weight heparin (LMWH) or c) 0.1 U/mL covalent antithrombin-heparin complex (ATH) was investigated by taking time samples into EDTA and measuring total thrombin activity in the time sample-EDTA mixtures. Subtraction of activity due to thrombin-α2-macroglobulin complexes from the total thrombin activity gave free thrombin activity, which was converted to nM concentrations using a purified thrombin standard curve. Results are mean ± SEM (n ≥ 5).

Fig. 4 Effect of plasma type on inhibition of plasma prothrombin consumption by heparinoids. The effect of age on plasma prothrombin consumption with either: a) 0.25 U/mL unfractionated heparin (UFH), b) 0.25 U/mL low molecular weight heparin (LMWH) or c) 0.1 U/mL covalent antithrombin-heparin complex (ATH) was investigated by taking time samples into EDTA and assaying the time sample-EDTA mixtures for prothrombin by ELISA. Results are mean ± SEM (n ≥ 5).
than by LMWH, at similar anti-factor Xa concentrations. In addition, prothrombin was consumed at a similar rate in the three groups when ATH was present compared to when UFH or LMWH was present, but at reduced anti-factor Xa concentrations. Newborns were more sensitive than either children or adults to inhibition of prothrombin consumption by ATH.

### Inhibitor Complex Formation

Concentrations of the thrombin-inhibitor complexes formed at the end of the thrombin generation time courses (at final equilibrium) are shown in Table 3. In the absence of heparinoid, significant amounts of inhibitor complexes formed in all three groups, with a higher proportion of thrombin being inhibited by α2M in children and newborns compared to adults. When heparinoids were present, as expected, there was an increase in thrombin-antithrombin complexes as a percentage of the total inhibitor complexes in each plasma type. Nevertheless, similar to experiments without heparinoids, proportionately more thrombin-α2M complexes were formed in plasmas from children and newborns compared to that of adults for UFH, LMWH and ATH (Table 3). Total inhibitor complexes were similar for UFH and LMWH. Furthermore, similar inhibitor-complex concentrations were observed with ATH compared to UFH and LMWH, but at reduced ATH anti-factor Xa activities. Similar to the measurements of free thrombin generated (Fig. 3) and prothrombin consumed (Fig. 4), newborns were the most sensitive to inhibition of inhibitor-complex generation by the heparinoids.

### Discussion

Developmental haemostasis, which refers to age-dependent changes in the coagulation system, is likely an important reason for age-related differences in the epidemiology of TEs. Despite these age-related differences, antithrombotic therapy in children is based on adult therapeutic guidelines. In particular, target therapeutic ranges for anticoagulant drugs are extrapolated directly from adult data. This is likely suboptimal, and given the physiological differences in thrombin regulation, we hypothesised that reduced target therapeutic ranges may be appropriate in newborns and children. The results of this study show that reduced concentrations of heparinoid compounds are required to inhibit thrombin to the same extent in newborns and children compared to adults. This data supports our hypothesis, and provides a rational basis on which clinical trials of reduced target ranges for newborns and children could be considered.

Generation and inhibition of thrombin are the key processes occurring during clot formation. Thrombin is generated from the zymogen prothrombin by the prothrombinase complex, which consists of factors Xa, Va, calcium and phospholipid surfaces (26). Once generated, thrombin exerts several procoagulant activities including: activation of factors V and VIII (thereby augmenting its own formation) (27-29); proteolysis of fibrinogen to release fibrinopeptides A and B (resulting in fibrin formation) (30); and initiation of platelet aggregation (31-34). Physiological clot formation depends on the initial activation of the prothrombinase complex by the factor VII/tissue factor pathway (35,
followed by rapid potentiation of thrombin generation due to a positive feedback loop involving predominantly factors XI and IX (37).

The overall effect of the unique age-dependent features of the coagulation system on the formation of the intrinsic and extrinsic tenase complexes has not been measured directly. However, the rate of thrombin generation by the prothrombinase complex has been examined and is both delayed and decreased in newborns compared to adults (19, 23, 38, 39). Thrombin generation remains reduced by 25% throughout childhood (11). The amount of thrombin generated is directly proportional to plasma concentrations of prothrombin (19), while the rate at which thrombin is generated is dependent upon plasma concentrations of all procoagulants.

In terms of the heparinoid compounds used in this study, our results confirm that at similar anti-factor Xa concentrations, UFH inhibited thrombin generation to a greater degree than LMWH, in plasma from newborns, children or adults. ATH inhibited thrombin generation to a similar extent compared to UFH in all 3 plasma types, but at significantly lower anti-factor Xa concentrations. ATH inhibits thrombin directly at a rate [3.1 \times 10^8 \text{M}^{-1} \text{s}^{-1}] (25)] which is an order of magnitude faster than the catalytic rate of UFH [1 \times 10^8 \text{M}^{-1} \text{s}^{-1} with antithrombin 25]) and 5-6 orders of magnitude faster than antithrombin alone [7.0 \times 10^8 \text{M}^{-1} \text{s}^{-1} (40)]. The rapid inhibition of thrombin by ATH likely prevents the positive feedback loop from significantly potentiating initial thrombin generation, hence reducing the total thrombin generated.

Our results highlight the vastly different responses to all heparinoids in newborns compared to adults. In newborns, thrombin generation was absent at 0.25 U/mL UFH and 0.1 U/mL ATH, compared to significant thrombin generation in the adult. Further, at 0.25 U/mL LMWH, thrombin generation was delayed and reduced by approximately half in newborns compared to adults. These differences were matched by reductions in rates of prothrombin consumption. Thus, increased inhibition of initial amounts of thrombin generated (lag in appearance of thrombin activity, Fig.3) would delay the thrombin feedback on prothrombin activation (Fig. 4).

The differences between adults and children were less marked, with children showing delayed thrombin generation with UFH compared to adults. However responses to LMWH and ATH were not dissimilar. Our results are consistent with previous studies which demonstrated reduced thrombin generation in children compared to adults when treated with either low dose UFH or oral anticoagulation (16, 41). Although responses to LMWH and ATH have not previously been assessed, the relative complement of activities in these heparin derivatives suggests that they would express different anticoagulant potencies, especially in plasmas with varied concentrations of pro- and anticoagulants. While equivalent amounts of anti-factor Xa activity for UFH and LMWH were mixed into the plasmas, the relative amount of LMWH anti-IIa activity added would be significantly reduced (Table 1). Thus, peak thrombin generation activity was increased for LMWH, especially in newborn plasma where the high anti-IIa potency of UFH may take greater advantage of the elevated antithrombin/prothrombin plasma molar ratio compared to that of the child or adult (38). In comparison, ATH gave significant inhibition of thrombin generation, but at much reduced anti-factor Xa activities. Similar to UFH, ATH has essentially equal anti-factor Xa and anti-IIa activities, but much higher specific activity [145 IU/mg versus 731 IU/mg for UFH and ATH, respectively (Table 1)]. The additional rapid non-catalytic (direct) inhibitory capability of ATH (25, 42), not found with UFH or LMWH, would significantly add to its strong anti-factor Xa and anti-IIa potency.

Decreased free thrombin generation due to the reduced rate of prothrombin activation in plasmas from newborns and children containing heparinoids is consistent with previous work on thrombin generation in the presence of UFH in children and adults (16). Earlier investigations have shown that decreased plasma prothrombin concentrations in children (16) and, particularly, newborns (19) was a major factor in the reduced free thrombin generated compared to adults. Furthermore, increased levels of $\alpha_M$ in newborn (17, 18) and child (11) plasmas compared to adults is also a significant factor involved in the decreased risk of thrombotic complications in the young (21, 43, 44). Less free thrombin generation in newborns (20, 45, 46) and children (16, 41) compared to adults has been strongly linked to increased thrombin-$\alpha_M$ complex formation. Similarly, we found that for all heparinoids studied, the proportion of thrombin complexed to $\alpha_M$ was greater in plasmas from newborns and children compared to plasma from adults (Table 3). Thus, a combination of reduced prothrombin and increased $\alpha_M$ in plasmas from the young likely contributed to decreased free thrombin potential requiring less heparinoid anticoagulation.

The potential clinical significance of our results is considerable. UFH is one of the most frequently prescribed drugs in pediatric tertiary care centres. LMWHs are used with increasing frequency for paediatric patients with TEs as they have a number of potential advantages including consistent reproducible pharmacokinetics, subcutaneous administration, minimal need for monitoring, decreased risk of heparin-induced thrombocytopenia, and decreased risk of osteoporosis, making long-term use a feasible option instead of oral anticoagulation (47, 48). ATH is a recently described covalent complex with favourable kinetics, compared to heparin and antithrombin, however ATH has not yet progressed to clinical trials (25). There is a clear need to optimise the therapeutic target range of heparinoids, as this will reduce the risk of bleeding complications while maintaining effective antithrombotic therapy. Our results provide evidence to suggest that the optimal target range is likely reduced in newborns and, possibly, also in children compared to adults.

In conclusion, our study provides the first in vitro data demonstrating that heparinoid thrombin inhibition is age-dependent. In particular, decreased concentrations of heparinoids are required to inhibit thrombin in vitro in newborns compared to adults, with less marked differences observed between children and adults. Whether these differences are significant in vivo remains to be determined, however clinical trials of reduced target ranges for heparinoids in newborns and children are justified.

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