The Relationship Between the Hemorrhagic and Antithrombotic Properties of Low Molecular Weight Heparin in Rabbits

By C. J. Carter, J. G. Kelton, J. Hirsh, A. Cerskus, A. V. Santos, and M. Gent

We have compared the hemorrhagic and antithrombotic effects of a low molecular weight (LMW) heparin fraction and standard heparin in rabbits. Similar LMW heparin fractions have antithrombotic effects when tested in animals, but their hemorrhagic effects relative to standard heparin have not been established. Standard porcine mucosal heparin (mol wt 15,000 daltons) was depolymerized by nitrous acid to a low molecular weight fraction (mol wt 4600 daltons). Using equal USP units, the standard and Dep LMW heparin were compared in vitro, ex vivo, and in vivo. In vitro, when diluted in rabbit plasma, the Dep LMW heparin at equivalent anti-Xa activity showed less prolongation of thrombin clotting times or activated partial thromboplastin times. Ex vivo, platelets from rabbits treated with the Dep LMW heparin showed less inhibition of collagen-induced aggregation. The relative hemorrhagic properties of the two heparins were compared in vivo in rabbits using a sensitive blood loss assay, and the antithrombotic properties were compared in a thrombin-induced venous stasis model. By using an optimal threshold heparin dose in each test system, it was possible to demonstrate that equal USP units of Dep LMW heparin caused less blood loss but showed greater antithrombotic activity than standard heparin.

EPIARIN is an effective antithrombotic agent, but its clinical use is limited by its major side effect, bleeding.1,3 It has generally been assumed that bleeding associated with heparin therapy, like its antithrombotic properties, is directly related to its effect on blood coagulation, and therefore, this side effect is unavoidable. Most clinical studies, however, have failed to demonstrate a relationship between bleeding and the anticoagulant effect of heparin in individual patients.2,3

The anticoagulant effect of heparin has been well characterized and occurs by accelerating the inhibitory effect of antithrombin III on factors XIIa, XIa, Xa, IXa, and thrombin.4 A number of investigators have separated heparin into fractions of varying molecular weights. A consistent observation has been that when compared to standard heparin, the low molecular weight heparin fractions have a relatively greater inhibitory effect on the activated Xa clotting time than on the activated partial thromboplastin time.5,6 We have recently demonstrated that low molecular weight heparin has pronounced antithrombotic properties in experimental animals even though it only minimally prolongs the activated partial thromboplastin time.7 This finding would be of potential clinical value if the low molecular heparin produced less hemorrhage for equivalent antithrombotic effect than the standard heparin from which it was derived.

Heparin has other effects on hemostasis, and while it is likely that the hemorrhagic properties of heparin are related to its anticoagulant effect, other factors may also be important. Heparin interacts with platelets and has been reported to both induce and inhibit platelet aggregation.8,11 Heparin also prolongs the bleeding time in patients and volunteers, so it is possible that the hemorrhagic properties are related in part to an effect on platelet function.12 Recently, Salzman and associates reported that the interaction between heparin and platelets was less for a low molecular weight fraction, particularly if it had a high affinity to antithrombin III.13 We now report that at equivalent doses expressed either as USP units or as anti-Xa units, depolymerized low molecular weight heparin (Dep LMW heparin) has a greater antithrombotic effect in rabbits compared to standard heparin, but produces less bleeding.

MATERIALS AND METHODS

Heparins

Both heparins were prepared by Riker Laboratories Inc. (Northridge, Calif.). The standard heparin was of porcine mucosal origin, manufactured to standard clinical specifications. It had a mean weight molecular weight of 16,000 daltons and a specific activity by both USP and anti-Xa assay of 145 U/mg when compared with a standard USP heparin (Hepalean, Harris Laboratories, Brantford, Ontario, Canada). The low molecular weight heparin was prepared as follows: 20 g of USP grade sodium heparin (the standard heparin described above) was dissolved in 1 liter of 15% aqueous acetic acid. After stirring at 20° C for 3.5 hr, the solution was frozen and then lyophilized. A yield of 19.8 g of a granular white solid was recovered. The following analytic data were obtained on this depolymerized heparin: weight, average molecular weight, 4600 daltons; polydispersity, 1.9; USP and anti-Xa of potency 41 U/mg; percent

*Referred to in the text as Dep LMW heparin.
sulphur, 10.4; and percent nitrogen, 1.5. Chemical analysis showed a substantial percentage of anhydromannose end groups. The USP activity was determined by the standard procedure and the anti-Xa activity was described below.

Coagulation Reagents and Assays

The in vitro effects of the heparin preparations on the thrombin clotting time and the activated partial thromboplastin time were assessed using heparin diluted in rabbit platelet-poor plasma. The concentrations were in USP units on the basis of the previously determined specific activities of the two heparins (standard, 145 USP U/mg; Dep LMW, 41 USP U/mg). The in vitro effects on the above clotting times of the addition of both heparins to rabbit plasma were determined in quadruplicate on two different rabbit plasma pools. The following reagents were used: Bovine thrombin (Parke Davis and Co., Brockville, Ontario) was diluted in saline to a concentration of 1000 U/ml and then further diluted to a concentration of 100 U/ml with 50% glycerol in 0.85% saline. This was then divided into 0.5-ml aliquots and kept frozen at −70°C until immediately before use. Human brain cephalin was prepared according to the methods of Bell and Alton. Kaolin was obtained from Baker Company, Phillipsburg, N. J.

Heparin levels were determined using three methods. (1) A protamine sulphate neutralization using a modification of the method of Refn and Vestgaard. (2) A Xa coagulation time as described by Chen, Hirschgold and Wilson, modified by adding pooled human plasma in the initial incubation phase of the assay. (3) An anti-Xa assay using the chromogenic method of Teinen et al. with the substrate S2222. The Xa used in all assays was prepared from crude bovine thrombin (Parke-Davis, Brockville, Ontario) by the method of Yin and Wessler. In order to obtain a comparative value for the coagulant anti-Xa activities for the standard and Dep LMW heparins, a second standard heparin (Hepalean, Harris Laboratories, Brantford, Ontario, Canada) was serially diluted in rabbit plasma and the dose–response curve drawn so that 1 USP unit was equivalent to 1 anti-Xa unit. Similar serial dilutions in USP units were then made with the standard and the Dep LMW heparin and their relative anti-Xa heparin units read off the original standard curve. 125I-Fibrinogen was prepared from rabbit plasma using the method of Regoezzi and iodinated by the chloramine-T method. The final preparation had a specific activity of 100 μCi/ml and a clottability of >95%. Plasma fibrinogen was assayed by the Clauss method.

In Vivo Experiments

The rabbits used in the hemorrhage and antithrombotic experiments were inbred New Zealand white rabbits of similar weight (mean 2.5 kg, range 2.2–2.8 kg).

Blood Loss Assay

The method for measuring blood loss has previously been described in detail. In brief, rabbits were anesthetized with sodium pentobarbital, their ears shaved, and the animals infused with 1 ml of 51Cr-labeled red cells from a common donor animal. The first ear was immersed into a bath containing 950 ml of 1.2% saline that was continuously stirred and kept at 37°C. Immediately prior to immersion, a heparin or a saline placebo was administered and blood samples taken for baseline clotting tests and blood radioactivity. The ear was then raised from the saline bath, and under transillumination, 5 full thickness stabs were made with a Bard Parker scalpel blade, no. 11 (Becton, Dickinson and Co., Rutherford, N. J.). The ear was then reimmersed in the saline bath for a further 10 min. Blood was again taken for coagulation studies and radioactivity measurements. Aliquots of the saline bath, which now contained the shed erythrocytes, were then made with the standard and the Dep LMW heparin. These procedures were balanced to ensure that the first treatment ear (left or right) for each rabbit had an equal opportunity of being tested after the injection of Dep LMW or standard heparin. These procedures were done to ensure balance of any unexpected hemodynamic or pharmacologic effects.

Measurement of the Antithrombotic Effect of Heparin

Rabbits were anesthetized by infusing sodium pentobarbital into the marginal ear vein. The neck was then opened ventrally and the jugular veins and left carotid artery exposed. The left carotid artery was cannulated with a polyethylene cannula, through which samples were taken. 125I-labeled rabbit fibrinogen was infused into the rabbits. After allowing 10 min for equilibration of the labeled fibrinogen, samples were taken through the carotid cannula for coagulation studies, for plasma fibrinogen assay, and for measuring radioactive fibrinogen. Saline placebo or equal USP units of test heparin were injected via the marginal ear vein. After 4 min, a blood sample was taken for heparin assays and 1 min later 50 U/kg of highly purified thrombin were injected via the marginal ear vein. This thrombin was purified using a modification of the method of Lundblad et al. and was assayed by plasma clotting methods using an international thrombin standard. One minute after injection of the purified thrombin, the caudal ligature was elevated and the vein allowed to reach maximum filling. The cephalad ligature was then raised, trapping the blood in a vein segment of approximately 1 cm in length. Assessment of the size of the vein segment was subjective, but bias was avoided because the experiments were blinded and randomized and any errors in judgment would operate to decrease potential differences between various medications. Thirty seconds later the left jugular vein was tied off in a similar fashion. Fifteen minutes were allowed to elapse and then the rabbit was killed, and the jugular veins exposed, slit open, and examined for thrombi. Thrombi, if present, were removed, washed in saline and then placed in tubes for gamma counting. Samples of whole blood for fibrinogen radioactivity were counted in a similar manner. Plasma fibrinogen levels were measured as described above, and by relating the specific activity of the plasma fibrinogen to the radioactivity of the thrombi, the amount of fibrinogen converted to fibrin clot in each rabbit was calculated.

Platelet Aggregation Studies

Rabbits were anesthetized with sodium pentobarbital. A carotid cannula was inserted into the jugular vein for blood sampling and for giving medication. A prepararin blood sample was collected in 3.12% sodium citrate (1 part citrate:9 parts blood). The rabbits were then injected with 100 USP units of either Dep LMW heparin or standard heparin. Five minutes postheparin, a second blood sample was taken into 3.12% sodium citrate. Both pre- and postheparin blood samples were centrifuged at 260 g for 15 min at room temperature. Platelet-rich plasma (PRP) was prepared and platelet counts adjusted to 300,000/μl mm using autologous platelet-poor plasma. Platelet aggregation studies were performed using purified acid soluble collagen and an aggregometer (Payton Instruments Ltd., Scarborough, Ontario, Canada). Aggregations were per-
either Dep LMW heparin or standard heparin. After the injection of blood loss caused either by placebo or the heparins. In analysis of the same rabbit, a paired

neutralization of thrombin clotting times.6

Porath et al.27 Human antithrombin-III (100 mg) was coupled to Al-Ill to activated sepharose were carried out using the method of collagen. This same concentration of collagen was then used on

M 0.01

M 0.1 5

M 0.01

M 0.01

with 2

expressed

0. 1 5

starting buffer,

Iris, pH 7.4 (starting buffer). All fractionations were

thrombin clotting time assay was a commercially available porcine

the column was eluted, but the quantity was insufficient to assess the

third peaks were, individually, extensively dialyzed

against 0.1 5

the second

peak was determined by a thrombin clotting time assay and

the column effluent was monitored by a turbidometric assay (using

Heparin Clearance Studies

Mucosal heparin (Hepalean, Harris Laboratories, lorraine, Canada, Uppsala, Sweden). The column was equilibrated with 0.01

buffer

were

performed

to exclude the possibility that the nitrous acid depolymer-

Antithrombin-lll (A T-III) Binding Experiments

tions were then expressed as a percent of the aggregation obtained

each rabbit's postheparin infusion platelet-rich plasma prepared

the preheparin sample without any heparin addition.

with the preheparin sample without any heparin addition.

Iwo USP units

of either heparin were then added to another

arm PRP was recorded as 100% aggregation for the particular rabbit

blood samples. The maximum deflection obtained with the prehep-

the

the

apparent overload peak that predominantly rechromatographed

in

formed

on the preheparin infusion blood sample with and without

Effect on Coagulation Tests In Vitro

comparison by analysis of variance a Newman-Keuls multiple

compared. The results showed a log-normal distribution, and there-

antiocoagulant activity of each

and 1MW heparin (open circles) expressed as USP concentrations

compared with standard heparin, Dep LMW heparin

assays following their addition to rabbit plasma. When

however, the Dep LMW heparin caused less prolonga-

tion of the thrombin clotting time and the APTT than

However, the Dep LMW hepanin caused less prolonga-

had a comparable inhibiting effect on the inhibition of

hepanins on the APTT, thrombin clotting time, and Xa

were tested. Iwo USP units

us

Fig. 1

Continued
Table 1
Summary of Ear Blood Loss Experiments in Rabbits Treated With Saline Placebo

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Blood Loss (ml)</th>
<th>Heparin Level (Anti-Xa U/ml)</th>
<th>Value ± SD</th>
<th>p (Anti-Xa U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Heparin</td>
<td>0.053</td>
<td>0.053</td>
<td>&lt;0.025</td>
<td>ND</td>
</tr>
<tr>
<td>Saline</td>
<td>0.006</td>
<td>0.006</td>
<td>&gt;0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Depolymerized Low Molecular Weight (Dep) Heparin</td>
<td>0.023</td>
<td>0.023</td>
<td>&lt;0.005</td>
<td>ND</td>
</tr>
<tr>
<td>Saline</td>
<td>0.003</td>
<td>0.003</td>
<td>&gt;0.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

Both heparins were given as a 500 USP U bolus. ND, not determined. The heparin levels are in coagulation anti-Xa units (see text).

Hemorrhagic Effects
At a USP dose of 500 U per rabbit, both heparins caused significantly increased bleeding compared with saline placebo (Table I). The amount of blood lost from ear to ear after saline injection was very similar. When compared directly in the same animal after the administration of 500 USP U of each heparin, the standard heparin produced a significantly greater amount of blood loss than the Depolymerized Low Molecular Weight (Dep) heparin (p < 0.05).

Antithrombotic Effects
The antithrombotic effects of the heparins were compared with each other and with a saline infusion at heparin concentrations of 20 and 10 USP U/kg (Table 2). At the higher dose, a trend to reduced thrombus size was observed with both heparins, but only Depolymerized Low Molecular Weight (Dep) heparin significantly inhibited thrombus formation compared to saline placebo (p < 0.05).

In the majority of these experiments, no thrombi were detected with either heparin so the results obtained did not allow a sensitive comparison to be made between the two heparins. At the lower dose (10 USP U/kg), detectable thrombi were present in a majority of both heparin-treated groups of animals. Compared to saline placebo, a significant reduction in thrombus size was not observed in the animals treated with standard heparin, whereas a significant reduction was seen in animals treated with Depolymerized Low Molecular Weight (Dep) heparin (p < 0.05).

The Depolymerized Low Molecular Weight (Dep) heparin produced a greater reduction in thrombus size than standard heparin (p < 0.05).

The mean anti-Xa heparin levels (± SD) with Depolymerized Low Molecular Weight (Dep) and standard heparin in the 10 U/kg experiments were 0.39 ±0.06 U/ml for the Depolymerized Low Molecular Weight (Dep) heparin-treated animals and 0.28 ±0.08 U/ml for the standard heparin (p < 0.001).

Table 2. The Effects of Standard and Depolymerized Low Molecular Weight Heparin on Thrombin-Induced In Vivo Thrombus Formation Compared With Saline Placebo Treated Rabbits

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Geometric Mean Accretion (mm)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 20 USP U/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>71.2</td>
<td>NS</td>
</tr>
<tr>
<td>Standard heparin</td>
<td>14.6</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Depolymerized Low Molecular Weight (Dep) heparin</td>
<td>5.2</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>B. 10 USP U/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>81.6</td>
<td>NS</td>
</tr>
<tr>
<td>Standard heparin</td>
<td>80.5</td>
<td>p = 0.05</td>
</tr>
<tr>
<td>Depolymerized Low Molecular Weight (Dep) heparin</td>
<td>24.6</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>
platelet aggregation when compared with Dep LMW heparin ion ($p < 0.05$). There was also a greater inhibition of collagen-induced aggregation caused by standard heparin when added in vitro to preheparin infusion platelet-rich plasma, but this difference was not statistically significant ($p > 0.05$).

**Heparin Clearance Studies**

When injected in a dose of 100 USP U/kg, both Dep LMW and standard heparin showed similar clearance characteristics over the first 30 min of observation, and thereafter, the Dep LMW heparin appeared to be cleared more slowly (Fig. 3).

**Antithrombin-III Binding**

Using a thrombin clotting time assay in human plasma, unfractionated, standard, and Dep LMW heparins had anticoagulant activities of 145 U/mg and 45 U/mg, respectively. This was in good agreement with their USP specific activities. Both heparins were separated into predominantly three peaks of no, low, and high affinity to AT-III by affinity chromatography on AT-III-Sepharose 4B (Fig. 4).

Fifty-five percent of the standard heparin and 66% of the LMW heparin eluted in the first two peaks. The first peak, peak I, consisted almost entirely of low specific activity material, which on rechromatography eluted in the same position as peak II. The anticoagulant activities of the low AT-III affinity fractions, peak II, for standard and LMW heparins were 13 antithrombin U/mg and 4 antithrombin U/mg, respectively. The high affinity fractions, peak III, corresponding to 43% of the standard heparin and 33% of the Dep LMW heparin, had anticoagulant activities of 180 antithrombin U/mg and 77 antithrombin U/mg, respectively. A further 1%–2% of each heparin was eluted with 2 M NaCl (not shown), but the amount was too small to accurately establish anticoagulant activity. When pooled fractions from peaks II or III were dialyzed against starting buffer and rechromatographed on AT-III-Sepharose 4B, greater than 90% of the heparin from each pool eluted in the same respective fraction.

**DISCUSSION**

A number of investigators have reported that low molecular weight heparin has different anticoagulant properties and different effects on platelets than standard heparin. A possible explanation for the molecular basis of the relatively greater anti-Xa activity of LMW heparin compared with its effect on the thrombin clotting time was recently provided by Oosta and colleagues. We have previously reported that a depolymerized low molecular weight heparin fraction

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**Fig. 2.** The inhibition of collagen-induced platelet aggregation after the in vitro addition of standard and LMW heparin to platelet-rich plasma and upon ex vivo platelet-rich plasma after heparin infusion into the rabbit, expressed as a percentage of the aggregation obtained in the absence of in vitro or infused heparin ($\pm$ 1 SE).

**Fig. 3.** Heparin clearance determined after a bolus dose of 100 USP U/kg of (A) standard heparin and (B) LMW heparin.
heparin used in the antithrombotic and hemorrhagic experimental models were selected to maximize the difference in the effects of these two heparins on these measurements. The heparin levels obtained in the antithrombotic experiments were approximately 0.3 anti-Xa U/ml and, therefore, similar to levels achieved clinically. On the other hand, in the hemorrhage experiments, the mean observed heparin levels were approximately 3.5 anti-Xa U/ml, which would be equivalent to those obtained clinically immediately after an intravenous bolus dose of heparin of 10,000 U. These high heparin levels were selected on the basis of dose finding studies that indicated that they were required to produce a consistent and statistically significant difference in bleeding between the groups of animals studied. It should not be assumed that these differences would also occur at lower heparin concentrations and, therefore, their relevance will require validation by formal clinical trial.

The mechanism responsible for the differences in hemorrhagic antithrombotic properties between the standard and depolymerized low molecular weight heparin is uncertain. The depolymerized LMW heparin was effective as an antithrombotic agent in vivo in the thrombin-initiated thrombosis model despite having a relatively poor antithrombin effect in vitro. It is probable that the thrombogenic action of thrombin in this model is largely mediated through the acceleration of earlier steps in the coagulation pathway (e.g., via factor VIII and factor V activation) rather than through a direct effect of thrombin on fibrinogen. LMW heparin, particularly those with high AT-III affinities, interact less with platelets, and this could be an explanation of the difference found in the hemorrhage experiments, although affinity chromatography shows that Dep LMW did not contain an increased proportion of heparin with high affinity for AT-III. Both heparins tested showed similar clearance kinetics for a 100 USP U/kg i.v. bolus over the first 30 min, making it unlikely that more rapid heparin clearance could explain the lesser degree of hemorrhage observed with Dep LMW heparin.

Depolymerized low molecular weight heparin did not inhibit the collagen-induced platelet aggregation when this was tested ex vivo, whereas a significant inhibitory effect on collagen-induced platelet aggregation was observed with the standard heparin. It is possible, therefore, that the differences in the hemorrhagic effect between standard and depolymerized low molecular weight heparin was caused by differential effects on platelet function, which in turn do not influence the antithrombotic activities of the heparins in a stasis-hypercoagulability venous thrombosis model.

Although a number of investigators have suggested
that low molecular weight heparin might have a therapeutic advantage over standard heparin on the basis of its different effects on various blood coagulation tests, this study represents the first demonstration of a dissociation between the antithrombotic and hemorrhagic effects of heparin fractions tested in vivo. These findings have the limitations imposed by animal models, but they are sufficiently promising to warrant extensive investigation of Dep low molecular weight heparin by clinical trial.

ACKNOWLEDGMENT

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The relationship between the hemorrhagic and antithrombotic properties of low molecular weight heparin in rabbits

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