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ANTITHROMBOTIC AND BLEEDING EFFECTS OF A LOW MOLECULAR WEIGHT HEPARIN FRACTION

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Abstract—Low molecular weight (LMW) heparin prevents venous thrombosis by potentiating the inhibition of coagulation factor Xa. Heparin, however, has other biological properties whose role in the prevention of thrombosis is still unknown. The aim of our study was to compare the antithrombotic activity of a LMW heparin and its parent molecule in an attempt to understand better the mechanism and structural requirements for heparin's antithrombotic effect. We studied a preparation of an unfractionated pig mucosal heparin pure by any accepted criteria (electrophoresis in various systems, conductimetric titration and NMR spectra) and a LMW heparin fraction obtained from the former by fractional precipitation with ethanol. Both heparins completely prevented thrombus formation in an experimental model of stasis-induced venous thrombosis in rats.

When administered intravenously to rats, the unfractionated heparin had an *ex vivo* anti-Xa/APTT ratio of 1.67, versus 6.60 of the LMW heparin fraction.

Unexpectedly, both heparins induced a significant prolongation of tail bleeding time, performed by two different techniques, the "transection" (mostly exploring blood clotting) and the "template" (exploring the platelet/vessel wall interactions). This study suggests that, beside anticoagulation, other effects may play a role in both the antithrombotic and haemorrhagic effects of some heparins and LMW heparin fractions.

The main efforts in heparin fractionation studies have been recently directed toward the preparation of heparin species with lower molecular weight than the parent molecule; these fractions would still have a high antithrombotic effect with a reduced haemorrhagic potential [1]. In most heparin preparations described so far, this has been achieved by enhancing the anti-Xa activity of the molecule at the expense of total anticoagulant properties [2]. Relatively little emphasis has been placed in all these studies on the possible effect of the heparin fractions on primary haemostasis. The latter can be studied in experimental animals using bleeding time tests [3], which have been recently standardized in rats [4]. We report here that a low molecular weight (LMW) heparin obtained by ethanol precipitation of a mucosal heparin pure by any accepted criteria retained not only the antithrombotic effect of the parent molecule, but also its ability to impair haemostatic plug formation in rats. This observation offers fresh clues to the understanding of heparin's effects on the haemostatic system.

MATERIALS AND METHODS

Unfractionated heparin. For all the experimental studies reported a pig mucosal heparin preparation was used (Diosynth batch N. 57442111). This preparation was pure by any accepted criteria (electrophoresis in various systems, conductimetric titration and NMR spectra) [5]; it had an anticoagulant activity of 171.7 USP UI/mg (data from manufacturer) and molecular weight (by gel filtration) of 14,600 daltons [6].

LMW-heparin fraction. The heparin fraction tested was obtained from unfractionated heparin (17% yield) by fractional precipitation with ethanol. The mean molecular weight of the LMW fraction, as determined by gel-filtration (on Sephadex G-75), was 6500 daltons. Figure 1 shows the electropherogram on barium acetate [7] of the starting material and of the heparin fraction, the latter consisting only of fast moving species, as usually observed for LMW heparins [5].

Ex vivo *anticoagulant assays.* CD-COBS male rats (Charles River, Italy, Calco), weighing 250 g, were treated i.v. with heparin or saline. After 15 min blood was collected by intracardiac puncture in open-chested animals under ether anaesthesia. Blood was drawn in plastic syringes prefilled with 0.126 M sodium citrate (9 parts of blood and 1 part of citrate). Platelet-poor plasma (PPP) was prepared by centrifugation of blood at 3000 *g* for 15 min at room temperature. The activated partial thromboplastin time (APTT) [8] and the Anti-Xa clotting assay [9] were performed on collected blood. The anticoagulant activity of both heparins in these assays was evaluated against a reference curve obtained with the 3rd International Standard for Heparin. The ratio between both activities was calculated as a conventional Anti-Xa/APTT index.

Experimental venous stasis model. To assess the antithrombotic activity of heparins we used a venous

‡ Recipient of a fellowship in memory of avv. Federico Maironi.

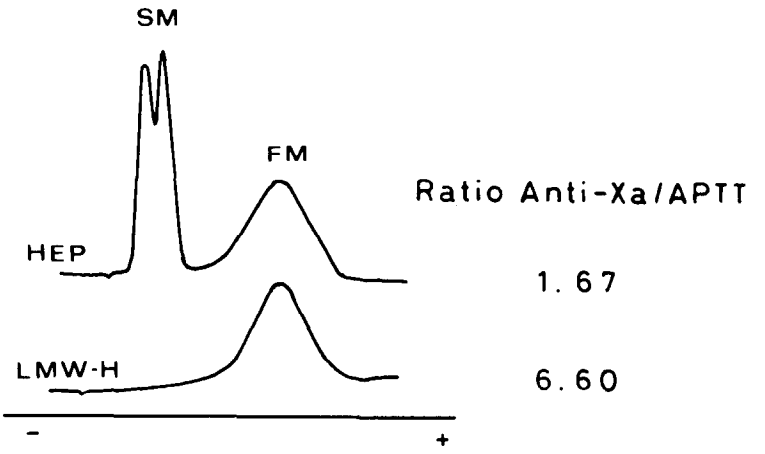

Fig. 1. Electropherogram (Ba acetate) of parent heparin (HEP) and its fraction (LMW-H) obtained by precipitation with ethanol of slow-moving (SM) species.

thrombosis model based on formation of a red thrombus under a ligature applied to the inferior vena cava of CD-COBS rats [10]. Venous stasis was applied 15 min after i.v. treatment of animals with either heparin or saline as control. The stasis-induced model of venous occlusion represents a system of localized fibrin deposition which is susceptible to clotting activation and appears to be selectively sensitive to the antithrombotic activity of heparin [10].

Experimental model of bleeding. In order to evaluate the bleeding tendency induced by heparin, we used the "tail transection" and the "template" bleeding time tests as described by Dejana *et al.* [4]. CD-COBS rats, 250 g body wt, were treated i.v. with heparins or saline at different doses (0.19, 0.38, 0.75 mg/kg) and bleeding time(s) were measured at intervals after treatment.

Platelet count. Platelet count was made by phase contrast microscopy on samples prepared using the Unopette diluting system (Becton Dickinson, Novate Mil., Italy).

RESULTS

Figure 2 shows the anticoagulant activity in plasma of rats treated with unfractionated heparin and LMW heparin fraction. In response to different doses of heparins, the APTT was markedly prolonged by the starting material and very mildly by the LMW heparin fraction, whereas the anti-Xa system was affected to a similar degree by both heparin preparations.

The ratio Anti-Xa/APTT was expectedly higher with the LMW heparin fraction than with the starting material. The ratio between the two activities was calculated as a conventional index of the balance between the antithrombotic and haemorrhagic properties of heparin.

Table 1 shows the results with an experimental model of venous stasis used to assess the antithrombotic properties of both heparin preparations. Both the incidence of thrombus formation and the weight of the thrombus were recorded. Tested at the dose of 0.75 mg/kg body wt, both unfractionated heparin and LMW heparin fraction, injected 15 min before venous stasis, protected the animals from thrombus formation. At the dosage of 0.38 mg/kg body wt, both preparations also had good antithrombotic activity.

To assess the bleeding tendency induced by heparin, both "template" and "tail transection" bleeding time(s) were measured. Figure 3 shows the results of both tests. It is worth mentioning that the LMW heparin fraction, although ineffective on APTT, significantly prolonged "transection" bleeding time, similarly to the starting material at the doses

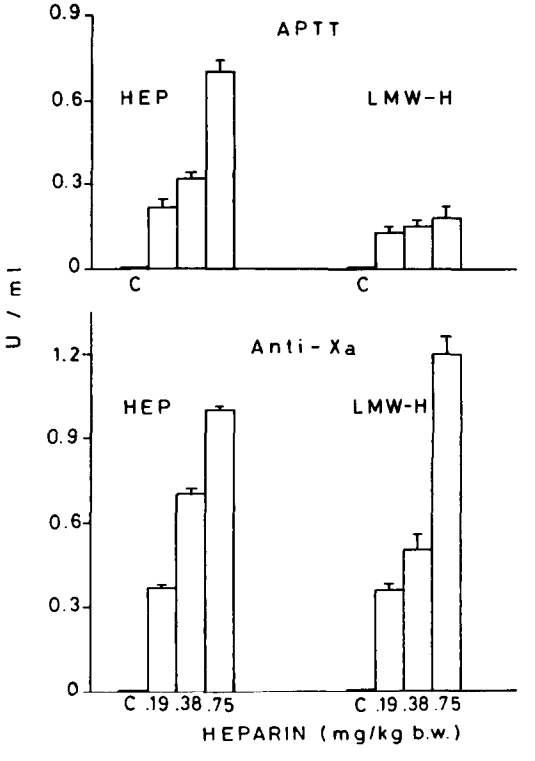

Fig. 2. APTT and anti-Xa activity in rat plasma after intravenous injection of parent heparin (HEP) and LMW heparin fraction (LMW-H) at different doses expressed as mg/kg (mean + S.E.; N = 5).

Table 1. Thrombus weight (mean + S.E.; N = 10) and percent of occlusion in rats treated with parent heparin (HEP) and LMW heparin fraction (LMW-H) at different doses

	Occlusion (%)	Thrombus weight (mg)
Control	70	2.05 + 0.38
HEP		
0.75 mg/kg	0	—
0.38 mg/kg	30	0.57 + 0.22
LMW-H		
0.75 mg/kg	10	0.20
0.38 mg/kg	20	4.2; 1.7*

* The individual weight of the two thrombi is reported.

of 0.75 and 0.38 mg/kg body wt. Unexpectedly, at the higher dose, both heparins significantly prolonged "template" bleeding time too. The dose of 0.38 mg/kg body wt had virtually no effect on "template" bleeding time.

In order to understand better the connections between the anticoagulant and "haemorrhagic" effects of the LMW heparin fraction, we followed them at intervals after i.v. administration. As reported in Fig. 4, the anticoagulant effect followed a similar time-course for anti-Xa and APTT. In both cases, the strongest effect was seen after 5 min, but anticoagulation was still detectable after 1 hr. The effect of the LMW heparin fraction on bleeding time was much more short-lived; it appeared after 5 min in the "tail transection" and "template" system, reached a maximum after 15 min, but was back to control values at 60 min (Fig. 5). When bleeding times were maximally prolonged (i.e. 15 min after administration of either heparin, 0.75 mg/kg body wt), no changes were observed in blood platelet count (data not shown).

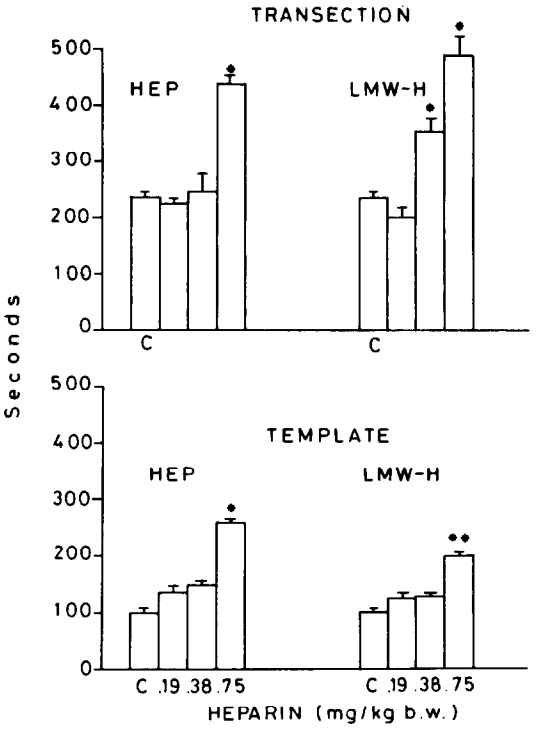

Fig. 3. Effect of parent heparin (HEP) and LMW heparin fraction (LMW-H) on "template" and "transection" bleeding time(s) after intravenous injection to rats at different doses expressed as mg/kg (mean + S.E.; N = 8). * $P < 0.01$ Duncan's test. ** $P < 0.05$ Duncan's test.

DISCUSSION

This study shows that a low-molecular weight heparin fraction of mucosal origin, with antithrombotic and anti-Xa activity, markedly impaired also primary haemostasis, as indicated by the prolongation of the "template" bleeding time. This effect was maximal 15 min after i.v. injection of the drug and declined thereafter. Concomitantly, "tail transection" bleeding time was markedly prolonged. The latter is believed to measure other phenomena besides early plug formation, since arrest of bleeding from the relatively large tail vessels depends upon both the platelet and the clotting-fibrinolysis systems. In other words, "tail transection" test can be regarded as an "*in vivo* clotting time".

The dose-dependency and time-course of the effect on "template" and "transection" bleeding times were still different from those of the Anti-Xa

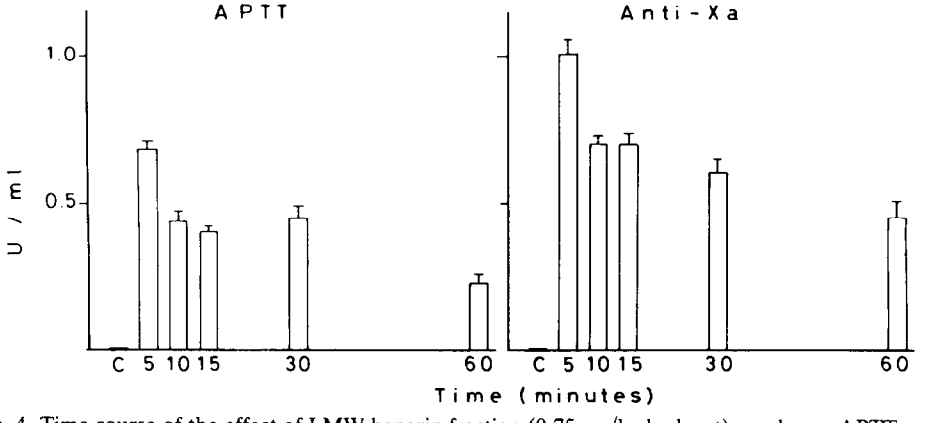

Fig. 4. Time course of the effect of LMW heparin fraction (0.75 mg/kg body wt) on plasma APTT and anti-Xa activity after intravenous injection to rats (mean + S.E.; N = 5).

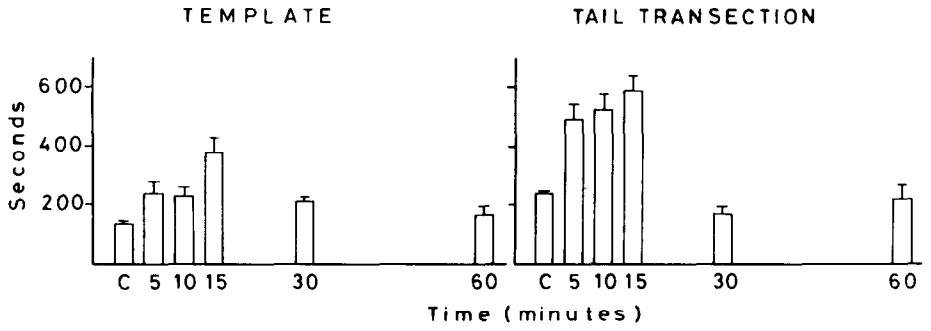

Fig. 5. Time course of the effect of LMW heparin fraction (0.75 mg/kg body wt) on "template" and "tail transection" bleeding time(s) after intravenous injection to rats (means + S.E.; N = 5).

activity and antithrombotic effects. Both Anti-Xa activity and prevention of stasis-induced venous thrombosis were already seen after 0.38 mg/kg of the drug, whereas the "template" bleeding time was only affected by the highest dose used, 0.75 mg/kg body wt.

Thus, at least at the higher doses, this LMW heparin may act as an antithrombotic agent not only by enhancing plasma anti-Xa activity, but also by affecting some still unknown mechanism involved in primary haemostasis. Platelet counts performed concomitantly with the bleeding time test ruled out the possibility that the prolongation of bleeding time was due to an assumed thrombocytopenic effect of heparin [11].

It is of interest to observe that the ability to markedly prolong the "template" bleeding time was already a property of the parent heparin and that this, together with the Anti-Xa activity (indeed unusually high as compared with that of the USP standard and literature values), was retained through the fractionation procedure. By contrast, the anticoagulant effect, as measured by APTT, was significantly lower for the LMW heparin fraction, in accordance with literature data [12]. Whereas several reports indicate that LMW heparin fractions and fragments cause less bleeding than unfractionated preparations [13, 14], occasional haemorrhagic effects were reported also for LMW heparins [15, 16].

When evaluating the possible connections between anticoagulant, haemorrhagic and antithrombotic effect of both heparins studied, it appears that the intermediate dosage used (0.38 mg/kg body wt) had a marked antithrombotic effect, in association with a significant anticoagulant effect, but no haemorrhagic effect. Similarly, considering the time course of both activities, for LMW heparin fraction there is a time interval, from 30 min on, when an anticoagulant effect is still present but the haemorrhagic effect has already vanished.

It is therefore conceivable that LMW heparin fraction works in some conditions mainly through its anticoagulant (Anti-Xa) effect. However, it cannot be excluded that platelet–vessel wall interactions contribute to the degree of its antithrombotic and haemorrhagic effects. Further work is required to establish which steps in the platelet–vessel wall interplay are particularly affected by this LMW heparin fraction.

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