Combinations of Low Doses of Unfractionated Heparin and of Low-Molecular-Weight Heparin Prevent Experimental Venous Thrombosis

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Key Words

Low-molecular-weight heparin · Experimental thrombosis · Rat vena cava

Abstract

Synergism between low-molecular-weight heparin and low doses of unfractionated heparin (UH) enhancing anti-factor Xa activity and the release of tissue factor pathway inhibitor was observed. The aim of this study was to verify whether this association is effective in preventing experimental venous thrombosis. Seventy rats were allocated into 7 groups: the control group treated with distilled water, the H₃₅₀ group treated with UH 350 IU/kg, the E₂ group treated with enoxaparin 2 mg/kg, the H₁₇₅ group treated with UH 175 IU/kg, the E₁ group treated with enoxaparin 1 mg/kg, the H₁₇₅ + E₁ group treated with UH 175 IU/kg plus enoxaparin 1 mg/kg, and the H₁₀₀ + E_{0.5} group treated with UH 100 IU/kg plus enoxaparin 0.5 mg/kg. Forty minutes after subcutaneous injection, thrombosis was induced in vena cava. Three hours later, if present, thrombi were withdrawn and weighed. Bleeding time, activated partial thromboplastin time, thrombin time (TT), and anti-factor Xa were measured at the beginning and end of the experiment. Forty-eight other animals were treated, but without inducing thrombus, and tests were performed 40 min after injection.

Thrombus developed in 90.9% of control animals, 20% of the H₃₅₀ group, 22.2% of the E₂ group, 10% of the H₁₇₅ + E₁ group, and 30% of the H₁₀₀ + E_{0.5} group; there was a difference between group C and the other groups. Only in the H₃₅₀ and H₁₇₅ + E₁ groups were TT and activated partial thromboplastin time prolonged in relation to control at the end of the experiment. Forty minutes after injection, TT was prolonged in the H₃₅₀ and H₁₇₅ + E₁ groups. In conclusion, combinations of low doses of low-molecular-weight heparin and low doses of UH were as effective as high doses of each one used alone in preventing thrombus development in rat vena cava.

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Introduction

The standard drug for prevention and treatment of thromboembolism is unfractionated heparin (UH); it has a heterogeneous structure, molecular weight, anticoagulant activity and chromatographic affinity. Moreover, it presents pharmacokinetic limitations such as binding to plasmatic proteins, endothelial cells, and leucocytes, resulting in a complex clearance mechanism, variability of anticoagulant response, and resistance to heparin [1, 2].

Low-molecular-weight heparins (LMWHs) produce an antithrombotic response with a better dose/effect re-

lationship, reflecting greater bioavailability, a longer half-life, and dose-dependent clearance, and is considered at least as efficient and safe as UH in preventing and treating thromboembolic events. Their pharmacokinetic difference to UH may be explained by the lower affinity for plasma proteins, endothelial cells, and leucocytes. The possible lower incidence and magnitude of bleeding may be a consequence of low platelet function inhibition, no increase in microvascular permeability, and low affinity for endothelial cells and von Willebrand factor [2, 3]. Like UH, LMWH also induces liberation of tissue factor pathway inhibitor (TFPI) by endothelial cells, thus increasing inhibition of FXa and FVIIa/TF [4–8]. At least, LMWH is as efficient and safe as UH for the prevention and treatment of thromboembolic events. Moreover, LMWH is easily administered and, in most cases, does not need laboratory monitoring [1, 2, 4, 5, 7, 9–11]. The limiting factor to its large-scale use in developing countries is its high price.

In 1997, Perez-Requejo et al. [12] demonstrated, *ex vivo*, the synergic effect of UH plus LMWH, observing that the anti-factor Xa (A-FXa) activity generated after subcutaneous injection of 1,000 IU of UH plus 20 mg of enoxaparin was greater than that expected by administering each drug separately.

In 1999, Altman et al. [13] demonstrated that, when LMWH is administered with a low dose of UH, there is synergy in the liberation of free TFPI by endothelial cells, and the consequent increase in free TFPI in plasma remains longer than with UH or LMWH alone.

The aim of this study was to investigate the effect of associating UH and LMWH in lower doses than those efficient when administered separately to prevent the development of experimentally induced thrombus in rat posterior vena cava.

Material and Methods

Animals

One hundred and eighteen male Wistar rats, weighing 230–530 g, from the University animal house were used according to the Brazilian Law on Animal Experiments. The experimental protocol was approved by the University Committee on Animal Research. The animals were kept at ambient temperature; access to food was restricted 12 h before the experiment.

Drugs

UH: Lique mine[®], Produtos Roche Químicos e Farmacêuticos SA, São Paulo, Brasil.

LMWH: Clexane[®], Rhodia Pharma, São Paulo, Brasil.

Reagents

Reagent for TT: Dade[®] Thrombin Reagent, Dade Behring, Marburg, Germany.

Reagent for APTT: Platelin[®] LS, Organon Teknika Corporation, Durham, N.C., USA.

Spectrolyse[®] Heparin (Xa): chromogenic assay Kit (Biopool International).

Experimental Groups

The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg) and were randomly allocated into the following groups:

- group C (control group): 11 animals treated with 0.1 ml/kg distilled water;
- group H₃₅₀: 10 animals treated with 350 IU/kg UH;
- group E₂: 9 animals treated with 2.0 mg/kg enoxaparin;
- group H₁₇₅: 10 animals treated with 175 IU/kg UH;
- group E₁: 10 animals treated with 1.0 mg/kg enoxaparin;
- group H₁₇₅ + E₁: 10 animals treated with 175 IU/kg UH plus 1.0 mg/kg enoxaparin;
- group H₁₀₀ + E_{0.5}: 10 animals treated with 100 IU/kg UH plus 0.5 mg/kg enoxaparin.

The drugs were diluted in distilled, sterilized water so that all animals received 0.1 ml/kg, by subcutaneous injection.

In a previous experiment, doses in groups H₃₅₀ and E₂ were determined as the minimum to efficiently prevent thrombus formation in this model.

Thrombosis induction, evaluation of presence and weight of thrombi, as well as all the laboratory tests were performed in a blind manner.

Venous Thrombosis Induction

Forty minutes after drug administration, the abdomens of the animals were surgically opened and thrombosis induced according to a modified method of Reyers et al. [14] and Carvalho et al. [15]. Briefly, the caudal vena cava was isolated and its branches ligated up to 2 cm distal to the left renal vein confluence. Ligation of the vena cava was done by cotton thread at a point immediately caudal to the left renal vein.

Three hours after thrombosis induction, animals were reanesthetized and the abdomen reopened to verify the presence of thrombus. If present, the thrombus was withdrawn and weighed immediately and again after drying for 24 h at 37°C.

Bleeding Time

Bleeding time (BT) was measured before drug administration (initial BT) and at the time of thrombus evaluation (final BT). A 3-mm-long and 2-mm-deep incision was made in the ventral side of the tail, 8–9 cm from the extremity, and BT was measured from the time of incision until bleeding stopped [16–19].

Determination of TT, APTT and A-FXa Activity

After thrombus removal, 3 ml blood was collected by cardiac puncture, anticoagulated at 9:1 with 3.8% sodium citrate and centrifuged to separate plasma and measure TT, APTT and A-FXa activity.

Immediately after blood collection, the animals were euthanized by sodium pentobarbital overdose.

A-FXa activity was measured by chromogenic assay. For this measurement, a different calibration curve was constructed for each kind of heparin and heparin association, using rat plasma.

Table 1. Frequency of animals with thrombus and median of the weight of fresh thrombi

Group	n	Animals with thrombus		Median weight of fresh thrombi, g
		n	%	
C	11	10	90.9 ^a	0.03010 ^a
UH ₃₅₀	10	2	20.0 ^b	0.00000 ^b
E ₂	9	2	22.2 ^b	0.00000 ^b
UH ₁₇₅	10	7	70.0 ^{a, b}	0.00285 ^{a, b}
E ₁	10	7	70.0 ^{a, b}	0.00100 ^{a, b}
UH ₁₇₅ + E ₁	10	1	10.0 ^b	0.00000 ^b
UH ₁₀₀ + E _{0.5}	10	3	30.0 ^b	0.00000 ^b

In each column, results with the same letter did not differ statistically.

Table 2. Medians of initial and final BT

Group	Initial BT, s	Final BT, s
C	132	135
H ₃₅₀	138	160*
E ₂	123	128
H ₁₇₅	124	150*
E ₁	133	153
H ₁₇₅ + E ₁	131	156
H ₁₀₀ + E _{0.5}	142	140

* $p < 0.05$.

Another 48 rats were anesthetized and randomly allocated into the groups C', H'₃₅₀, E'₂, H'₁₇₅, E'₁, H'₁₇₅ + E₁, and H'₁₀₀ + E_{0.5} using the same medication scheme as the above groups.

Forty minutes after drug administration, blood was collected, by cardiac puncture, to measure TT, APTT and A-FXa. Immediately after blood collection, the animals were euthanized by overdose of sodium pentobarbital.

Analysis

The variance test was used to compare variables with normal distribution and homogeneous variance. Thrombi occurrence was compared between groups using the Goodman test. The Kruskal-Wallis test was used for variables without normal distribution and/or without homogeneous variance. The Wilcoxon test was used to compare initial and final BT. The Mann-Whitney test was used to compare A-FXa levels at different moments within each group [20, 21]. A p value ≤ 0.05 was considered significant.

Table 3. Medians of TT and APTT

Group	TT, s		APTT, s	
	40 min after injection	3 h after injection	40 min after injection	3 h after injection
C	15 ^c	14.0 ^c	23.0 ^c	25.0 ^c
UH ₃₅₀	360 ^a	350.0 ^a	122.0 ^a	54.5 ^a
E ₂	38 ^{a-c}	17.0 ^{b, c}	41.0 ^{b, c}	26.0 ^{b, c}
UH ₁₇₅	190 ^{a, b}	22.5 ^{a-c}	55.0 ^{a-c}	29.0 ^{a-c}
E ₁	21 ^{b, c}	15.0 ^c	29.0 ^{b, c}	25.5 ^c
UH ₁₇₅ + E ₁	360 ^a	282.0 ^{a, b}	63.0 ^{a, b}	39.0 ^{a, b}
UH ₁₀₀ + E _{0.5}	360 ^{a, b}	22.5 ^{a-c}	55.0 ^{a-c}	28.0 ^{b, c}

In each column, results with the same letter were not significantly different.

Results

Presence and Weight of Thrombi

In group C, 10 animals presented thrombus (90.9%). Animals from the H₃₅₀, E₂, H₁₇₅ + E₁, and H₁₀₀ + E_{0.5} groups had statistically fewer thrombi than the control group. There was no statistical difference in number of animals with thrombus in groups H₁₇₅ or E₁ and the other groups (table 1).

There was a significant difference between the medians of fresh thrombus weights of the control group and groups H₃₅₀, E₂, H₁₇₅ + E₁, and H₁₀₀ + E_{0.5} ($p < 0.05$). Groups H₁₇₅ and E₁ were not significantly different from the others, although there was an important reduction in the weight of the formed thrombi. Similar differences were observed in the results of dried thrombus weight.

BT Values

Statistical significance between initial BT and final BT was only seen in groups H₃₅₀ and H₁₇₅ (table 2). Comparing all groups, BT values measured 40 min after drug administrations were not statistically different ($p = 0.076$).

TT and APTT

At the end of the experiment, TT and APTT in groups H₃₅₀ and H₁₇₅ + E₁ were longer than in the control group ($p < 0.001$).

At 40 min, TT was longer in groups H₃₅₀, H₁₇₅, H₁₇₅ + E₁, and H₁₀₀ + E_{0.5} than in controls ($p < 0.001$); in groups E₁ and E₂ (enoxaparin only), TT was not significantly different from controls (table 3). APTT in groups H₃₅₀ and H₁₇₅ + E₁ was significantly longer than in the

Table 4. Median and 1st and 3rd quartiles (in parentheses) of A-FXa activity in groups E₁ and E₂ at two different times

Group	Time	
	40 min after injection	3 h after injection
C	0.039 (0.037; 0.045) ^{a, B}	0.032 (0.029; 0.045) ^{a, B}
E ₁	0.560 (0.430; 0.665) ^{a, A}	0.155 (0.086; 0.285) ^{b, A, B}
E ₂	0.560 (0.463; 0.690) ^{a, A}	0.300 (0.172; 0.397) ^{b, A}

In each column, results with the same letter were not statistically different. Lower case letters: to compare times in each group. Capital letters: to compare groups at each time point.

Table 5. Median and 1st and 3rd quartiles (in parentheses) of A-FXa activity in groups H₁₀₀ + E_{0.5} and H₁₇₅ + E₁ at each time point

Group	Time	
	40 min after injection	3 h after injection
C	0.030 (0.030; 0.034) ^{a, B}	0.022 (0.020; 0.029) ^{a, B}
UH ₁₀₀ + E _{0.5}	0.640 (0.625; 0.700) ^{a, A}	0.190 (0.086; 0.270) ^{b, A, B}
UH ₁₇₅ + E ₁	0.980 (0.600; 1.100) ^{a, A}	0.360 (0.330; 0.580) ^{b, A}

In each column, results with the same letter did not differ statistically. Lower case letters: to compare times in each group. Capital letters: to compare groups at each time point.

control group ($p < 0.001$), whereas APTT in group H₃₅₀ was longer than in the two enoxaparin only groups.

A-FXa Activity

In group E₁, at 40 min, A-FXa levels were not different from those of group E₂, but in both groups, the levels were significantly higher than in the control group. After 3 h, this increase was still different from the control only in the group E₂. Comparing A-FXa activity at two different times, there was no difference in the control group, but in groups E₁ and E₂, levels at 3 h were significantly less than at 40 min (table 4).

At 40 min, A-FXa activity levels were not different in groups H₁₀₀ + E_{0.5} and H₁₇₅ + E₁, but these were significantly higher than in controls. After 3 h, this increase was still different from the control only in the group H₁₇₅ + E₁. Comparing A-FXa activity at two different times, there was no difference in the control group. In groups H₁₀₀ + E_{0.5} and H₁₇₅ + E₁, activity at 3 h was significantly less than at 40 min (table 5).

It was not possible to determine the activity of A-FXa in the rats treated with unfractionated heparin at the moment of thrombus induction, because the levels in both groups were too high above the sensibility of the method.

Discussion

Our results indicate that the combinations of UH with LMWH, in low doses, that do not prevent thrombus formation when used alone, in this model, have an antithrombotic effect at least as efficient as higher doses of each drug administered separately. Increased A-FXa activity levels were seen at thrombus induction time in all groups treated with enoxaparin and heparin combinations when compared with controls, but there was no correspondence with the development or prevention of thrombus. Perez-Requejo et al. [12] demonstrated potentiation of the A-FXa effect after low-dose injections of UH plus LMWH. In our study, it was not possible to determine whether thrombus prevention was due to addition or potentiation of the effect of drugs. We have no explanation for the high levels of activity of A-FXa in the groups treated with UH. As the plasmas of all the groups were tested simultaneously, i.e. in a blind manner, a technical problem seems to be unlikely.

Young et al. [22] observed a synergic increase in A-FXa activity when low antithrombin (AT) affinity fragments were added in vitro with heparins. These authors administered UH or LMWH to healthy volunteers and to patients submitted to post-surgical prophylaxis for deep venous thrombosis; to the plasma of these individuals they added, in vitro, low AT affinity fragments. Plasma of UH-treated individuals showed an increase of 350% in A-FXa activity after addition of the fragments that theoretically dislocate heparin with anticoagulant activity from plasma proteins. The same addition to plasma of LMWH-treated individuals provoked a 25–35% increase in A-FXa activity. In our experiment, one of the possible explanations could be that the addition of low AT affinity molecules contained in UH provoked an increase in antithrombotic activity.

Additionally, Perez-Requejo et al. [12] suggest the importance of anti-FIIa for the antithrombotic activity. Ofo-su et al. [23] showed that UH equally inhibited both thrombin and FXa and the low-molecular-weight derivatives were weaker inhibitors of thrombin generation. These authors have suggested that thrombin inactivity may be critical to the efficiency of heparins, heparin-like compounds, and other antithrombotic agents, and that

some degree of thrombin inhibition seems to be necessary for an excellent antithrombotic effect.

Other mechanisms, besides those associated with AT, may be related to the antithrombotic action observed with the combination of UH and LMWH. Some recent studies suggest that TFPI may have a significant role in the antithrombotic action induced by heparin [6, 24]. Many studies have demonstrated that UH and LMWH promote TFPI liberation by endothelial cells [24]. Altman et al. [13] demonstrated that enoxaparin associated with low doses of UH exerts a synergic effect on endothelial cell TFPI liberation *ex vivo*. Hiebert et al. [25] studying oral reviparin action in rats did not find any indication of TFPI in plasma with the administered doses until the 6th hour, with low levels appearing in samples taken 8 h after oral administration. In the present study, we tried to dose TFPI in our rats, using the same method employed by these authors, but no difference between treated and control animals was found. As our animals were euthanized 3h and 40 min after injection, we do not know whether there would have been a later increase in the inhibitor level. On the other hand, it is also possible that the antibodies directed against human TFPI, presented in the ELISA test employed, does not have any or only low cross-reactivity with rat TFPI.

There was significant alteration in BT between final and initial times in the UH-treated groups. This agrees with other experimental studies in rats, other animals,

and humans [16, 18, 26–28]. In this experiment, there was no significant alteration of BT in the enoxaparin groups compared with controls. The combinations used in our experiment did not alter BT either.

There was no correlation between TT and inhibition of thrombus formation, since no thrombi were formed in animals treated with 2 mg/kg enoxaparin, with no prolongation of TT; in animals treated with UH 175 IU/kg, there was thrombus in spite of prolonged TT at 40 min. Further, there was no correlation between APTT and thrombus formation. In accordance with previous studies, there was no evidence of TT or APTT alterations in LMWH-treated animals [3, 4, 29].

In conclusion, the combinations of low-dose unfractionated heparin and LMWH were as effective as high doses of each substance individually in preventing thrombus development in rat vena cava. Further experimental and clinical studies must be performed to confirm the efficacy and safety of this combination treatment, allowing its use in clinical practice and possibly lowering the costs of treatment.

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