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Comparison of desulfatohirudin (REVASC) and heparin as adjuncts to thrombolytic therapy with reteplase in a canine model of coronary thrombosis

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1 We compared the direct thrombin inhibitor, desulfatohirudin (REVASC) and the indirect thrombin inhibitor, heparin, as adjuncts to thrombolytic therapy with reteplase in a canine model of coronary artery thrombosis.

2 Reteplase (BM 06.022) is a recombinant unglycosylated variant of human tissue-type plasminogen activator. Thrombus formation in anaesthetized open chest dogs was induced by electrical injury. Left circumflex coronary artery blood flow was monitored for 210 min with an electromagnetic flow probe. Twenty eight dogs were randomized to receive i.v. heparin (120 iu kg^{-1} bolus plus 80 iu kg^{-1} per h) or i.v. hirudin (2.0 mg kg^{-1} bolus plus 2.0 mg kg^{-1} per h) 10 min before thrombolysis preceded by i.v. acetylsalicyclic acid (20 mg kg^{-1}) 5 min prior to anticoagulation. Every dog received an i.v. double bolus injection of $0.14+0.14$ u kg^{-1} ($=0.24+0.24$ mg kg^{-1}) reteplase, 30 min apart, 1 h after thrombus formation.

3 At comparable reperfusion rates (12 out of 12 vs. 15 out of 16 dogs), hirudin enhanced time to reperfusion (14.3 ± 1.4 vs. 23.2 ± 3.4 min; $P<0.05$) and completely prevented reocclusion after reperfusion in contrast to heparin (0 out of 11 vs. 7 out of 11 dogs; $P<0.05$). Coronary blood flow quality was improved by hirudin as shown by a higher maximum blood flow after reperfusion (130 ± 14.3 vs. $83\pm9.3\%$ of baseline; $P<0.05$), a higher blood flow level at 20, 30, 40, and 50 min after onset of thrombolysis ($P<0.05$) and a longer cumulative patency time (195 ± 1.7 vs. 166 ± 12 min; $P<0.05$). Activated partial thromboplastin time and buccal mucosa bleeding time were prolonged ($P<0.05$) by either anticoagulant, but did not differ significantly between groups.

4 The direct thrombin inhibitor, desulfatohirudin, enhanced thrombolysis, prevented reocclusion and increased blood flow as compared with the indirect thrombin inhibitor, heparin, when investigated at one dose level each and used in conjunction with reteplase.

Keywords: Thrombolysis; plasminogen activator; reteplase; hirudin

Introduction

Current clinical data suggest that conjunctive treatment with thrombolytic agents such as recombinant tissue-type plasminogen activator (rt-PA), aspirin and intravenous heparin (Popma & Topol, 1991) to reduce reocclusion, is associated with substantial morbidity and mortality rates (Ohman *et al.*, 1990). However, the antithrombotic efficacy of heparin may be limited primarily because it is an indirect inhibitor of thrombin requiring antithrombin III but also because heparin may be neutralized by substances released from platelets and may itself stimulate platelets (Hirsh, 1991). Thrombin plays a pivotal role in both the platelet activation and the fibrin generation inherent in this process leading to rethrombosis (Lefkovits & Topol, 1994). Therefore, more effective thrombin inhibition might offer a clinical benefit.

Hirudin has gained much interest because it is a direct thrombin inhibitor blocking both the active catalytic site and the anion binding exosite (Lefkovits & Topol, 1994). Hirudin was shown to inactivate clot-bound thrombin *in vitro* more effectively than heparin (Weitz *et al.*, 1990). Animal experiments indicated that hirudin compared with heparin facilitated rt-PA-induced thrombolysis and maintained patency of coronary arteries particularly effectively (Haskel *et al.*, 1991). Early clinical experience with hirudin and rt-PA showed that hirudin compared with heparin reduced late reocclusion (Neuhaus *et al.*, 1993; Cannon *et al.*, 1994).

The purpose of the present study was to compare hirudin with heparin as adjuncts to the novel recombinant plasminogen activator, reteplase which is a bolus injectable thrombolytic agent. Hirudin and heparin were investigated at one dose level each. Recent experimental work done with hirudin showed that a maintenance dose of at least 2.0 mg kg^{-1} h^{-1} was necessary to achieve an at least 2 fold prolongation of the activated partial thromboplastin time in the dog (Homeister *et al.*, 1991; Haskel *et al.*, 1991; Sitko *et al.*, 1992). Lower doses were suboptimal in their antithrombotic efficacy (Rigel *et al.*, 1993; Nicolini *et al.*, 1994). In dogs, a higher dose of hirudin than in human subjects is required due to the faster renal clearance (Nowak *et al.*, 1988; Marbet *et al.*, 1993). The heparin dose was chosen to achieve anticoagulation comparable to that of hirudin and to be in accordance with previously used doses of heparin in animal studies (Haskel *et al.*, 1991; Sitko *et al.*, 1992). Reteplase was used at the clinically evaluated and selected dosing regimen (INJECT trialists, 1995).

The study aimed to evaluate whether hirudin and heparin differed in their effects on coronary artery blood flow after thrombolysis by reteplase in the canine model of coronary artery thrombosis.

Methods

Animal model

Adult female or male beagle dogs (7.0 to 11.8 kg, $n=28$) were anaesthetized with i.v. sodium pentobarbitone (35 mg kg^{-1} body weight), intubated and artificially ventilated. The right

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femoral vein was catheterized for administration of reteplase and blood withdrawal. Cannulae were placed in both brachial veins for administration of the anticoagulant and acetylsalicylic acid or fluid substitution, respectively. Arterial blood pressure was measured continuously by way of the right femoral artery. A thoracotomy was performed at the left fifth intercostal space and the heart was suspended in a pericardial cradle. A 2 cm section of the left circumflex coronary artery was isolated and instrumented as recently described (Martin *et al.*, 1992b). An electromagnetic flow probe was used for continuous blood flow monitoring. Blood pressure, heart rate and coronary blood flow were recorded continuously on a Schwarzer polygraph.

The left circumflex coronary artery thrombus was produced as recently described (Martin *et al.*, 1992b), based upon a method originally described by Romson *et al.* (1980). Briefly, the adjustable screw occluder on the left circumflex coronary artery was tightened to produce a 90% inhibition of the hyperaemic blood flow response to a 20 s occlusion of the coronary artery. A 150 μA continuous anodal current was applied to the coronary artery electrode and maintained until left circumflex coronary artery flood flow decreased to and remained at 0 ml min^{-1} for 3 min. This procedure results in deep, thrombogenic, vascular lesion (Bates *et al.*, 1992) leading to formation of a platelet-rich intravascular thrombus. The thrombus consists of a platelet-fibrin mass adherent to the vessel wall at the site of intimal injury (Romson *et al.*, 1980). The thrombus was allowed to age for 60 min. This experimental study with dogs conformed to the German Animal Protection Law and was approved by the local authority.

Drugs

Reteplase was manufactured by Boehringer Mannheim GmbH, Penzberg, Germany, as recently described (Kohnert *et al.*, 1992). Reteplase had a specific activity of 0.575 u mg^{-1}; one new unit (u) corresponds to one previously used mega unit (MU). Recombinant desulfatohirudin (REVASVC; CGP 39393) was supplied by Ciba Geigy Pharmaceuticals, Horsham, England. Standard mucosal sodium heparin was obtained from Hoffmann-LaRoche, Grenzach-Wyhlen, Germany. Acetylsalicyclic acid in a form suitable for i.v. administration was purchased from Bayer AG, Leverkusen, Germany.

Experimental protocol

Immediately after thrombus formation, the animals were randomly assigned to one of two treatment groups differing only in the anticoagulation, i.e. heparin or hirudin. The technician was 'blind' to the anticoagulant treatment. Forty five minutes after vessel occlusion, each dog received a single i.v. bolus injection of 20 mg kg^{-1} acetylsalicylic acid. Fifty minutes after vessel occlusion, the dogs received either heparin or hirudin. Heparin was administered as an i.v. bolus injection of 120 iu kg^{-1}, immediately followed by infusion of 80 iu kg^{-1} per hour. Hirudin was administered as an i.v. bolus injection of 2.0 mg kg^{-1}, immediately followed by an infusion of 2.0 mg kg^{-1} per hour. Ten minutes later, i.e. 1 h after vessel occlusion, each dog received the first i.v. bolus injection of 0.14 u kg^{-1} reteplase over 2 min, followed 30 min later by the second i.v. bolus injection of 0.14 u kg^{-1} at $t=30-32$ min. Fluid substitution was performed with 10 ml of 9% hydroxyethylstarch after surgical preparation and with 2.0 ml kg^{-1} h^{-1} of saline solution after thrombolytic treatment.

Measurements

Mean and phasic left circumflex coronary artery blood flow was monitored for 210 min after onset of thrombolytic treatment. The time to reperfusion was defined as the time from onset of treatment to the time of return of coronary blood flow to 33% of the control blood flow level before occlusion. Reocclusion was defined as a return of coronary blood flow to zero flow level, regardless of how long this level lasted. Time to reocclusion was the time interval from the time to reperfusion to the time that blood flow reached the zero flow level. The cumulative patency time represents the sum of time intervals during which the coronary artery was patent as determined by measurement of blood flow. At termination of the experiments, the left circumflex coronary artery segment was excised and opened longitudinally. The residual thrombus was removed and its wet weight determined.

Blood analysis

Blood samples for measurement of platelet count, red blood cell count and haematocrit were drawn on ethylenediamine tetraacetic acid 30 min before and 3 h after onset of thrombolytic treatment. Venous blood samples for measurement of the activated partial thromboplastin time by a clotting assay (Larrieu & Weiland, 1957) were collected 30 min before and serially after thrombolysis in 0.01 M of citrate. Bleeding time was determined with a spring-loaded device (SIMPLATE I, Organon Teknika, Eppelheim, Germany) on the buccal mucosa as recently described (Sitko *et al.*, 1992). Citrated blood samples were taken 30 min before and 90 min after onset of thrombolysis for measurement of platelet aggregation induced by 0.45 iu ml^{-1} thrombin, 10 μM adenosine diphosphate (ADP), and 10 μg ml^{-1} collagen as recently described (Martin *et al.*, 1992b). Citrated blood samples for determination of plasma concentration of functionally active reteplase were taken before and repeatedly after administration of reteplase. They were analyzed with the chromogenic substrate S-2251 method as published (Verheijen *et al.*, 1982). The pharmacokinetic parameters were calculated as recently described (Martin *et al.*, 1992b).

Statistical analysis

Frequency data are presented in fractionated form. Data described by continuous variates are reported as the mean $\pm$ s.e.mean. Endpoints in the unbalanced, randomized parallel-group design were checked for variance homogeneity by Levene's test. In the case of homogeneous variances, the parametric Student's t test was used. In the case of heterogeneous variances, the Welch t test was used. Repeated time measurement variables were analyzed by analysis of variance in the generalized linear model. Since all the repeated measures variables showed a significant time effect, pairwise between group comparisons were performed independently for each time point. Moreover, individual differences from the baseline values were evaluated. Dichotomous variables were analyzed by Fisher's exact test. A two-sided P value of $P<0.05$ was considered indicative of a significant difference.

Results

Baseline conditions

The two groups did not differ significantly in age, body weight, residual reactive hyperaemia, time to occlusion or preocclusion coronary blood flow. Dogs which died after successful reperfusion due to fibrillation were excluded from further analysis. The two groups did not show significant differences in arterial blood pressure and heart rate at baseline and during the course of the experiment. However, blood pressure decreased and heart rate increased at a level of statistical significance in both groups.

Thrombolytic efficacy

Reperfusion was achieved in all hirudin-treated animals and in 94% of heparin-treated dogs (Table 1). Conjunctive use of

Table 1 Characteristics of thrombolysis and reocclusion after double bolus reteplase plus acetylsalicylic acid plus heparin or hirudin in the canine model

Anticoagulant	*Time to occlusion* (min)	*Incidence of reperfusion (reperfused/ total)*	*Time to reperfusion* (min)	*Incidence of reocclusion (reoccluded/ reperfused)*†	*Time to reocclusion* (min)
Hirudin	54.3±14.0	12/12	14.3±1.4*	0/11*	–
Heparin	83.0±9.6	15/16	23.2±3.4	7/11	21.4±8.0

†Four dogs in the heparin and one dog in the hirudin group died after treatment and could not be used for further evaluation. Data represent mean values±s.e.mean or fractional data. *$P<0.05$ versus heparin group.

Table 2 Characteristics of coronary blood flow and thrombus weight after double bolus reteplase plus acetylsalicyclic acid plus heparin or hirudin in the canine model

Anticoagulant	*Maximum blood flow after reperfusion* (% of preocclusion)	*Cumulative patency time* (min)	*Residual thrombus wet weight* (mg)
Hirudin	130.9±14.3*	195±1.7*	1.5±0.3*
Heparin	83±9.3	166±12	3.7±0.8

Cumulative patency time = sum of time intervals in which the coronary artery was patent. Data represent mean values±s.e.mean; n = 11 per group (only reperfused animals which survived). *$P<0.05$ versus heparin group.

hirudin enhanced ($P<0.05$) the time to reperfusion as compared to heparin treatment (Table 1). The use of hirudin also completely prevented ($P<0.05$) reocclusion in reperfused animals in contrast to the use of heparin (Table 1). The residual thrombus wet weight at termination of the experiments was lower ($P<0.05$) in hirudin-treated animals than in heparin-treated animals (Table 2).

Coronary blood flow quality

Maximal coronary blood flow achieved after successful reperfusion was higher ($P<0.05$) in the hirudin group than in the heparin group (Table 2). Coronary blood flow was higher ($P<0.05$) in hirudin animals than in heparin animals at 20, 30, 40 and 50 min after onset of treatment, but subsequently was comparable (Figure 1).

Pharmacokinetics

The plasma concentration-time profiles of reteplase in the hirudin and in the heparin groups did not differ significantly in terms of clearance rate (4.0±0.12 vs. 4.1±0.17 ml min^{-1} kg^{-1}) and half-life (12.1±0.4 vs. 12.4±0.7 min) (Figure 2).

Haemostasis and haematology

Anticoagulation with heparin and hirudin resulted in more than 5 fold prolongation ($P<0.05$) of the activated partial thromboplastin time over the observation period, which did not differ significantly between the two treatment groups except at termination of the experiment. Both anticoagulant treatments also induced prolongation ($P<0.05$) of the buccal mucosa bleeding time at 90 min and 3 h compared with the pretreatment value, but did not show significant differences between the groups (Table 3). There were no significant differences between the two groups in the residual platelet aggregation induced by thrombin, adenosine diphosphate or collagen. There were also no significant differences in platelet count, red blood cell count and haematocrit between the two groups (Table 4).

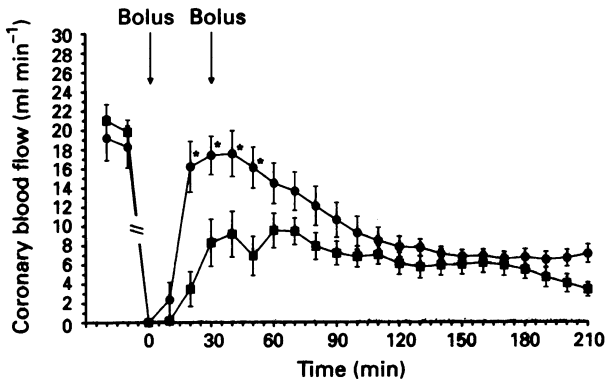

Figure 1 Time course of left circumflex coronary artery blood flow in dogs before and after coronary thrombosis. All animals received 20 mg kg^{-1} acetylsalicylic acid i.v. prior to double bolus i.v. injection of 0.14+0.14 u kg^{-1} reteplase, 30 min apart. Ten minutes before reteplase, animals received i.v. desulfatohirudin (●) as a bolus of 2.0 mg kg^{-1} followed by a 2.0 mg kg^{-1} per h infusion or i.v. heparin (■) as a bolus injection of 120 iu kg^{-1} followed by a 80 iu kg^{-1} per h infusion. Values represent the mean±s.e.mean; n=11 per group; *$P<0.05$.

Discussion

The findings of the present study suggest that direct thrombin inhibition by recombinant desulfatohirudin improved coronary artery blood flow after thrombolytic therapy with reteplase in comparison with indirect thrombin inhibition by heparin. The improvement was indicated by enhancement of thrombolysis, increase of coronary artery blood flow and complete abolition of reocclusion during the 210 min observation period. These findings are noteworthy because they were achieved by using reteplase as the thrombolytic agent.

Reteplase (BM 06.022) is a novel recombinant plasminogen activator and has a 4 fold longer half-life than alteplase, thus allowing intravenous bolus injection (Seifried *et al.*, 1992). The

German Recombinant Plasminogen Activator (GRECO) Study showed that a single intravenous bolus injection of 15 MU (=27 mg) reteplase achieved a 90 min patency rate of 76% despite the occurrence of very early reocclusions, i.e., before the 90 min angiographic time point (Neuhaus *et al.*, 1994b). Animal experiments suggested a double bolus regimen would be effective in preventing very early reocclusion after successful reperfusion (Martin *et al.*, 1992a). After demonstrating clinically the feasibility of the double bolus regimen (Tebbe *et al.*, 1993), the Recombinant Plasminogen Activator Angiographic Phase II International Dose Finding (RAPID) Study proved that the double bolus regimen of 10+10 MU reteplase, 30 min apart, achieves significantly higher patency rates than alteplase in the conventional 3 h infusion regimen (Smalling *et al.*, 1995). More recent angiographic investigations in the RAPID-2 study revealed that the 10+10 MU reteplase double bolus regimen also resulted in significantly higher patency rates than front-loaded alteplase in a direct comparison (Bode *et al.*, 1995). In a large-scale, randomized mortality trial, reteplase was shown to be at least as effective as streptokinase in reducing mortality after myocardial infarction (INJECT Investigators, 1995). Therefore, reteplase was used as the double bolus regimen in the present study.

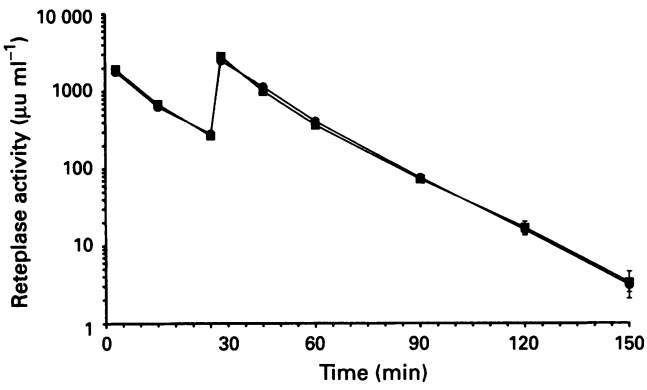

Figure 2 Plasma concentration time profiles for reteplase activity in dogs with coronary artery thrombosis. All animals received 20 mg kg^{-1} acetylsalicylic acid i.v. prior to double bolus i.v. injection of 0.14+0.14 u kg^{-1} reteplase, 30 min apart. Ten minutes before reteplase, animals received i.v. desulfatohirudin (●) as a bolus of 2.0 mg kg^{-1} followed by a 2.0 mg kg^{-1} per h infusion or i.v. heparin (■) as a bolus injection of 120 iu kg^{-1} followed by a 80 iu kg^{-1} per h infusion. Values represent the mean±s.e.mean; n=11 or 11 per group.

Table 3 Buccal mucosa bleeding time after double bolus reteplase plus acetylsalicyclic acid plus heparin or hirudin in the canine model

Anticoagulant	*Bleeding time (min) at*		
	-30 min	*90 min*	*180 min*
Hirudin	1.9±0.3	3.1±0.3	2.4±0.3
Heparin	1.6±0.1	2.2±0.3	1.9±0.2

Data represent mean values±s.e.mean; n = 10 per group.

Since acetylsalicylic acid is routinely used in the treatment of acute myocardial infarction (Popma & Topol, 1991), animals in both groups were pretreated with acetylsalicylic acid. Acetylsalicylic acid was given intravenously at the high dose of 20 mg kg^{-1} because recent investigations showed that high dose, but not low dose acetylsalicylic acid inhibited thrombus formation and stabilized blood flow in the dog (Mickelson *et al.*, 1993). This finding is supported by earlier canine experiments with administration of i.v. doses of 17 mg kg^{-1} (Yasuda *et al.*, 1990) or 35 mg kg^{-1} (Folts *et al.*, 1976). The requirement of high dose acetylsalicylic acid in the dog in contrast to low dose acetylsalicylic acid in the human subject may be explained by a different mechanism of thrombus formation in the dog model, anaesthesia or species-dependent variations in platelet responses to agonists (Mehta & Mehta, 1993). The role of prostacyclin which is inhibited by high dose acetylsalicylic acid may be negligible in the present experimental setting because the vessel occluder mechanically limits the reactivity of the injured coronary artery.

The canine model of coronary artery thrombosis induced by electrolytic injury is known to produce stable coronary artery occlusion for hours without thrombolytic therapy (Jackson *et al.*, 1990), even in the presence of heparin alone (Martin *et al.*, 1991) and of heparin or hirudin alone (Martin *et al.*, 1992c). Results of a recent experimental study comparing heparin and saline as adjuncts to reteplase suggested that heparin is required as an adjunct to reteplase (Martin *et al.*, 1993). Therefore, the results of the present study show further benefit by direct thrombin inhibition as compared with conventional anticoagulation by heparin.

A limitation of the present study is caused by the use of only one dose level of hirudin and heparin. However, both agents were used in the upper dose range of published experience with hirudin (Homeister *et al.*, 1991; Sitko *et al.*, 1992) and heparin (Haskel *et al.*, 1991; Sitko *et al.*, 1992) and achieved at least 5 fold prolongation of the activated partial thromboplastin time. Recent clinical experience with heparin and hirudin restricted the doses at a 2.5 fold prolongation of the activated partial thromboplastin time due to increased bleeding risk at higher prolongation of the activated partial thromboplastin time (Antman, 1994; The GUSTO IIa Investigators, 1994; Neuhaus *et al.*, 1994a).

The results of the present study provide a perspective for optimization of the clinical patency profile of reteplase, which was recently shown to be significantly higher than that of standard (Smalling *et al.*, 1995) and front-loaded (Bode *et al.*, 1995) alteplase. The introduction of the double bolus regimen reduced the occurrence of very early reocclusions seen during the 90 min angiographic observation period (Neuhaus *et al.*, 1994b), thereby increasing the patency rates. Nevertheless, patency rates after double bolus reteplase plus heparin plus aspirin are not yet maximal and capable of improvement, i.e., more rapid, more complete and more sustained patency (Lincoff & Topol, 1993).

The blood flow profile in the present experimental setting shows that the benefit provided by hirudin was mainly present during the first hour of thrombolysis, which may be regarded as the vulnerable phase. Subsequently, the blood flow profile in

Table 4 Haematological findings at 3 h after double bolus reteplase plus acetylsalicyclic acid plus heparin or hirudin in the canine model

Anticoagulant	*Platelet count* (% of baseline)	*Red blood cell count* (% of baseline)	*Haematocrit* (% of baseline)
Hirudin	134±10.3	105±3.7	106±4
Heparin	116±5.6	111±3.7	112±4.6

Data represent mean values±s.e.mean; n = 11 per group.

the hirudin group resembled that in the heparin group. If this observation can be extrapolated to human subjects one might conclude that the administration of hirudin could be restricted to the first hour of treatment with reteplase. Restricting hirudin administration in this manner might be beneficial in reducing the increased haemorrhagic risk seen to be associated with prolonged and high dose hirudin infusion in recent clinical studies (Antman, 1994; The GUSTO IIa Investigators, 1994; Neuhaus *et al.*, 1994a), while preserving the very early antithrombotic efficacy. The clinical patency profile of reteplase may justify this restricted administration of hirudin because reocclusion rates after the 90 min angiogram were low (Tebbe *et al.*, 1993; Neuhaus *et al.*, 1994b; Smalling *et al.*, 1995).

Clinical experience of hirudin with alteplase runs quite counter to the above perspective, as the effect of hirudin in combination with alteplase consisted mainly in reducing late reocclusion (Neuhaus *et al.*, 1993; Cannon *et al.*, 1994). Furthermore, in preclinical studies hirudin showed promising effects in combination with t-PA during the early phase of reperfusion. The failure of preclinical experiments to predict the clinical situation may be explained by the fact that subtherapeutic doses of t-PA were used, favouring the demonstration of synergistic effects of hirudin which might not have been as prominent at higher doses of t-PA (Haskel *et al.*, 1991). A direct comparison of reteplase with alteplase at therapeutic doses in a nonclinical study could help to explore differences in the susceptibility of the two fibrinolytics for interaction with hirudin. However, the short observation period in this model limits this exploration to the early phase during and after reperfusion. The high dose of hirudin used in canine experiments was required because hirudin is eliminated more rapidly in this species than in man. The dose of hirudin used in animal experiments thus cannot simply be extrapolated to man.

Another limitation associated with the anaesthetized preparation is the fall in blood pressure over time, which may at least partially explain the decrease in coronary blood flow during the late observation period. This experiment did not reveal significant differences in the safety profile of heparin and hirudin when combined with reteplase, probably because the parameters such as bleeding time or blood loss measured by haematocrit are not sensitive enough.

The direct thrombin inhibitor desulfatohirudin in combination with reteplase significantly enhanced thrombolysis, increased coronary blood flow and prevented reocclusion after successful reperfusion as compared with the indirect thrombin inhibitor, heparin in combination with reteplase, at least at the dose levels investigated. The relatively high number of animals needed to achieve a level of statistical significance in this study indicates that blood flow improvement was enhanced, but only to a small extent. Statistical aspects will therefore have to be given close consideration when planning clinical studies.

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