The low molecular weight heparin, enoxaparin, limits infarct size at reperfusion in the dog

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Abstract

Objective: Heparin (HEP) is used in the post-thrombolytic state to prevent vessel reocclusion, thereby aiding myocardial salvage. Side effects limit its benefits, but besides anticoagulant activity HEP has diffuse actions that may be potentially beneficial to jeopardized reperfused myocardium. This study compares the effect of therapeutic doses of HEP and enoxaparin (ENOX), a low molecular weight heparin, and to streptokinase (SK), on infarct size.

Methods: The left anterior descending coronary artery was occluded in dogs for 90 min, followed by 6 h of reperfusion with a residual critical stenosis in place. Five min before reperfusion, HEP 2800 IU was injected i.v., and perfused at 500 IU/h until sacrifice in group 2, while groups 3 and 4 received ENOX 2128 anti-Xa IU i.v. followed by 380 anti-Xa IU/h. Group 4 was also given 500000 IU SK over 30 min before reperfusion beginning at 55 min of occlusion (ENOX + SK), while group 5 received only SK. Controls (CON, group 1) received saline. P-selectin mediated platelet–neutrophil rosettes formation was also tested in vitro in the presence of HEP and ENOX.

Results: The area at risk delimited by dye perfusion was statistically similar among groups. Covariance analysis between infarct size (% of area at risk delimited with triphenyltetrazolium and collateral flow measured with radioactive microspheres confirmed that groups given ENOX (21.6 ± 5.5%) and ENOX + SK (24.9 ± 3.9%) developed smaller infarcts (P < 0.05) than CON (48.1 ± 4.5%), as opposed to HEP (32.2 ± 3.6%) and SK (46.8 ± 3.4%) groups. In-platelet counts in the infarct were reduced significantly by 64% in the ENOX group as compared to CON, and to a lesser extent 42%, n.s. in the ENOX + SK group, but were not reduced by HEP and SK treatments. Neutrophil accumulation in the infarcts was decreased significantly and by more than 75% in the ENOX and ENOX + SK groups versus CON, but not in the HEP and SK groups. Also, only ENOX (10–100 μg/ml) significantly inhibited platelet–neutrophil rosettes formation in a plasmatic milieu.

Conclusions: The ENOX treatment, as opposed to that of HEP, reduces myocardial platelet and neutrophil accumulations, and limits infarct size when given just before and during reperfusion. The benefits of ENOX on infarct size were not modified by SK, and may be related, at least in part, to an interaction with P-selectin-mediated cell adhesion. © 1998 Elsevier Science B.V.

Keywords: Low molecular weight heparin; Streptokinase; Stenosis; Reperfusion; Myocardial infarction; Platelet; Leukocyte; Dog

1. Introduction

Early coronary reperfusion salvages jeopardized myocardium, but overall benefits may be limited by reperfusion injury [1]. Reperfusion injury is a complex pathophysiologic process involving production and release of cytotoxic materials, such as free oxygen radicals, lysosomal enzymes, or lipid metabolites derived from neutrophils, platelets and ischemic tissue, and microcirculatory failure associated with leukocyte microcirculatory plugging, vasoactive agents or impaired vascular relaxation that compromise reflow and survival of reversibly injured myocytes [2].
The presence of a residual stenosis in the infarct-related artery during early reperfusion exacerbates tissue injury and increases infarct size by a platelet-dependent mechanism [3]. These observations are clinically relevant, because angiographically significant residual stenosis has been documented in > 75% of patients following thrombolysis [4], and that platelet activation occurring at severe coronary stenosis is responsible for cyclic flow variations [5], and could initiate platelet-related phenomena in the reperfused vasculature.

In addition to therapies aimed at preventing thrombotic reocclusion, drugs protecting reperfused myocardium would be beneficial in the set up of coronary thrombolysis in extending reduction of infarct size and associated morbidity and mortality. Heparin is used clinically with thrombolitics to prevent thrombotic reocclusion of the recanalized vessel [6], and pharmacologic doses of heparin at reperfusion reduce infarct size in the dog by a mechanism independent of its anticoagulant activity [7]. Heparins could be beneficial on postischemic jeopardized myocardium by modulating the activity of enzymes, such as elastase [8] and complement components [9], by interacting with leukocyte and platelet adhesion molecules [10,11], or by exerting antioxidant activity [12].

Low molecular weight heparins (LMWHs), such as enoxaparin, retain most of the properties of unfractionated heparin, and could represent a valuable alternative to unfractionated heparin in the context of coronary thrombolytic therapy. A lesser propensity for bleeding complications, such as reported in animal experiments [13], would be advantageous because intramyocardial hemorrhage is frequent in reperfused infarcts [14], and a worsening by anticoagulants could be potentially detrimental to salvaged myocardium. LMWHs have other potential advantages over unfractionated heparin that may be useful to limit reperfusion injury. LMWHs, as opposed to unfractionated heparin, do not increase vessel permeability [15], and therapeutic concentrations do not activate platelets [16] or neutrophil superoxide production [17]. In addition, LMWHs exhibit less non-specific binding to plasma proteins providing stable activity levels [18].

This study investigates the potential benefits of clinically relevant doses of heparins (unfractionated and LMWH) and streptokinase on postischemic myocardial preservation and infarct size in a non-thrombotic model of ischemia to avoid the situation of vessel reocclusion, but with reperfusion in the presence of a residual critical stenosis in the dog.

2. Methods

2.1. Ischemia and reperfusion

Mongrel dogs of either sex weighing 18–28 kg were anesthetized with sodium pentobarbital (30 mg/kg, i.v.), intubated and ventilated mechanically with room air. Blood gases and pH were measured and kept within normal limits. Lead II electrocardiogram was monitored and catheters were inserted in the femoral vein and artery for blood sampling. After injecting pancuronium bromide (0.1 mg/kg), a left thoracotomy was performed at the fifth intercostal space, and the heart suspended in a pericardial cradle. Catheters were introduced in the left ventricle through the left atrial appendage for pressure monitoring and into the left atrium for radioactive microsphere injection.

As described previously [3], the left anterior descending coronary artery (LADCA) was isolated distal to its first diagonal branch, and an electromagnetic flow probe was installed to measure coronary blood flow. Five minutes before occlusion, a micrometer occluder was adjusted on the LADCA to produce an eccentric critical coronary stenosis. The stenosis was sufficient to abolish the hyperemic response after the release of a 10-s occlusion, without affecting basal blood flow of the coronary. The LADCA was then occluded for 90 min and reperfused for 6 h with the critical stenosis left in place. Dogs presenting refractory ventricular fibrillation were excluded from the study. Also, animals that failed to develop persistent epicardial cyanosis and ST segment changes (< 0.1 mV) after 10 min of occlusion were discarded.

Studies adhered to the guidelines on the care and use of laboratory animals issued by the Canadian Council on Animal Care, and were approved by a local committee.
2.3. Infarct sizing

As described previously [20], the site of occlusion on the LADCA and the aorta above the coronary ostia were cannulated and perfused with saline (0.9%) for 5 min, and subsequently, the aorta was perfused with Evans blue (0.5% in saline; Sigma, Canada) and the LADCA with saline (0.9%), at a constant pressure of 100 mmHg for 5 min. The left heart was sliced in 7-mm-thick transverse sections, the slices weighed and immersed in triphenyltetrazolium chloride (Sigma, Canada) 1.5% in Tris buffer (2.4%, pH 7.8) for 10 min at 37°C. The normally perfused myocardium, the area at risk of ischemia, and the necrotic myocardium were delineated, and respective areas estimated on each slice by computerized planimetry. The area at risk was expressed in percentage of the left ventricle, and infarct size in percentage of the area at risk.

2.4. Regional and collateral myocardial blood flow

Regional and collateral blood flow were measured as described previously [20], using 15 μm diameter microspheres labeled with 99mTc (NEN Dupont Inc, Canada), 15 min after coronary occlusion. Briefly, 3–4 million microspheres were injected into the left atrium, and a reference arterial blood sample collected from the aortic catheter at a constant rate. For regional blood flow analysis, 3–4 central slices were dissected into non-ischemic and ischemic parts. Sections from the central ischemic and the non-ischemic wall opposite to the area at risk were divided into three equal subepicardial, mesomyocardial, and subendocardial portions. Each sample was weighed and counted in a gamma counter. After correcting for background and spillover between isotopes, blood flow was calculated and expressed in milliliters of blood per minute per gram of tissue.

2.5. Platelet isolation and 111In-labeling

The procedure for platelet isolation and labeling, preserving platelet aggregation, has been described elsewhere in details [3]. Venous blood from each dog was centrifuged to obtain platelet-rich plasma. After 4 successive washings with tyrode, the platelets were incubated with approximately 150 μCi 111In Indium-oxine (Amersham, Canada) for 1 min at 37°C. Radiolabeling efficiency ranged between 83.9% and 94.2%. The labeled platelets were finally suspended in 4.5 ml of citrated platelet-poor plasma and counted; 0.5 ml was saved to prepare standards for gamma counting, and the remaining (mean 1.96 ± 0.14 × 10^9) was injected intravenously a few minutes before occlusion. Venous blood was collected just before reperfusion to estimate the pool of 111In-platelets in the circulation, assuming a blood volume of 75 ml/kg b.w.

2.6. Isolation and 51Cr-labeling of red blood cells

Intramyocardial hemorrhages were estimated by tissue counting of 51Cr-labeled autologous red blood cells (RBC) [14]. The cells were isolated by centrifuging (10 min, 275 g) four 15-ml venous blood samples collected into 2.25 ml acid–citrate–dextrose. Hank’s balanced salt solution (HBSS) was added to the RBC pellet to a final volume of 15 ml in each tube and centrifuged (30 min, 1700 g) over 10 ml Percoll 71% (1.121 g/ml; Pharmacia, Canada). The RBC were washed twice in HBSS, and incubated with approximately 250 μCi of 51Cr (Na-chromate, Amersham, Canada). The suspension was incubated at room temperature and mixed gently every 5 min; unbound 51Cr was then eliminated by two successive washings in 15 ml HBSS. 51Cr-RBC labeling efficiency was 94.1%. 51Cr-RBC were finally suspended into 30 ml of platelet-poor plasma and injected intravenously before occlusion.

2.7. Platelet and red blood cell accumulations

Infarcted and non-infarcted myocardium samples from the area at risk and the normal non-ischemic myocardium from the opposite free wall were dissected, weighed and counted in a gamma counter for 5 min. The labeled-cell counts were determined by reporting the values obtained over the standards (1 × 10^6 platelets or 1 × 10^6 RBC), and accumulations were calculated per gram of tissue. As variable amounts of cells were injected, these values were normalized between dogs by using the mean labeled cell counts circulating just prior to reperfusion from all experiments [3].

2.8. Neutrophil accumulation in myocardial tissue

Neutrophil counts in myocardial tissue were estimated as previously [20] by measuring neutrophil-specific myeloperoxidase (MPO) activity using O-dianisidine (Sigma, Canada) and hydrogen peroxide as substrates. To establish a reference curve in each dog between MPO activity and neutrophil counts, blood neutrophils were isolated from the same blood sample as used for RBC isolation. The neutrophils obtained from the second cell layer were washed twice. Contaminating RBC were eliminated by hypotonic hemolysis. Suspensions containing 1 × 10^6 to 4 × 10^6 neutrophils were prepared, and the cell pellets frozen at −70°C for later use. Myocardial tissue was obtained from a central ventricular transversal slice rapidly frozen on a stainless-steel plate kept at −70°C. Samples of about 100 mg were taken systematically from the central ischemic area, two from the salvaged viable area (one at 2 mm above the infarct and one underneath the epicardium), and two from the infarct (one from the subendocardial layer, and the other from the outer edge of the infarct just below the salvaged live.
myocardium). One additional sample was taken from non-ischemic myocardium of the wall opposite to the area at risk.

Neutrophils and cardiac tissue samples were treated as reported by Bednar et al. [21]. MPO was assayed by adding to a 0.1 ml of sample, 2.9 ml of phosphate buffer containing $8.35 \times 10^{-3}$ % O-dianisidine dihydrochloride, and $\text{H}_2\text{O}_2$ 0.3%. Absorbency was read at 460 nm over 3 min. Values measured on the infarcted samples, or those of the viable area at risk were respectively averaged and results expressed in $10^6$ neutrophils/g wet tissue, by referring to the neutrophil reference curve constructed for each dog by using MPO measurements of blood neutrophil concentrations.

2.9. Serum creatine phosphokinase

Serum creatine phosphokinase levels were estimated directly on serum by use of an automatic analyzer (Hitachi 717, Boehringer Mannheim, Canada) according to the recommended procedure.

2.10. Blood coagulation assays

Venous blood (9 parts) was collected at 84 min occlusion, just before injecting heparin or enoxaparin, and after 2, 4 and 6 h of reperfusion, into 3.8% sodium citrate concentrates.

The aPTT was tested with phospholipid reagent Neothromtin (Behringwerke AG, Germany) on a Coag-a-Mate automate (General Diagnostics, USA). The thrombin time (TT) was measured by automated photodensitometry in an ACL-200 (Fisher Scientific, USA) system according to suggested methodology. A TT greater than 100 s was considered incoagulable. Anti-factor Xa plasma activity was determined with the Coatest heparin kit (Kabivitrum, Sweden), and a spectrophotometer equipped for kinetic studies.

The anti-Xa activity of HEP used in dog studies (group 2) was estimated over a reference anti-Xa curve of ENOX at concentrations ranging between 0.3 and 0.7 units/ml, so to obtain curve responses as closely parallel as possible [22]. Curve slopes were, respectively, 0.71 for HEP and 0.79 for ENOX. By referring the HEP curve values over the ENOX curve, we found that one international unit of HEP showed a mean activity of 0.76 anti-Xa unit.

2.11. Platelet–neutrophil adhesion assay

In a separate series of studies, the effects of HEP and ENOX on P-selectin mediated platelet–neutrophil adhesion were tested in vitro on rosette formation. Platelets and neutrophils were isolated from normal dog blood collected in ACD. After three successive centrifugations and washings, the platelets were suspended in tyrode pH 7.4 (500 × $10^6$/ml) activated with thrombin (0.1 U/ml) to elicit P-selectin expression, and fixed in 1% paraformaldehyde [23]. After neutralization of the fixative and three washings, the platelets were finally resuspended (400 × $10^7$/ml) in phosphate-buffered saline (PBS) containing Ca$^{2+}$ and Mg$^{2+}$ or in plasma anticoagulated with 133 U/ml hirudin (Sigma, Canada). Isolated neutrophil suspensions (10 × $10^7$/ml) were also prepared in a similar medium.

Platelet samples were incubated with HEP (Sigma, Canada) or ENOX at concentrations of 0, 10, 50 and 100 $\mu$g/ml for 30 min at room temperature under constant mixing before adding to neutrophils for rosette formation. The rosette formation and platelet quantification on neutrophils were performed according to Spagenberg et al. [24].

2.12. Statistical analysis

Results are expressed as mean ± s.e.m. for each group. A one-way analysis of variance (ANOVA) was used to investigate treatment differences and a Bartlett’s test carried out to verify the homogeneity of variances. A Bonferroni multiple comparison ($n = 5$) test was used for mean comparison between control (group 1) and each treated groups (2–5) and between groups 4 and 5. Intragroup hemodynamic data comparisons were analyzed by a one-way analysis of variance for repeated measures followed by a Bonferroni test between time values and values at baseline. A covariance analysis between infarct size (dependent variable) and collateral blood flow (covariate) was used in controlling the variability in infarct size due to collateral blood flow; the level of significance was set at $P < 0.01$. A two-tail Fisher’s exact test was applied to the incidence of ventricular fibrillation and sex distribution. A $t$-test or an alternate Welch test between control and each treated group, and also between groups 4 and 5 was used for comparisons of $111\text{In}$-platelet and neutrophil accumulations in the respective myocardial tissue areas. As 5 comparisons were made, the level of significance was set at $P < 0.01$. An ANOVA for repeated measures was followed by a paired Dunnet’s test for intragroup comparison of coagulation tests, and by an unpaired Bonferroni multiple ($n = 5$) comparison test as above for intergroup comparisons. Differences were considered statistically significant when $P < 0.05$ or when otherwise specified.

3. Results

3.1. Group characteristics and hematologic data

Seventy-one dogs were used in the study. Three dogs were excluded because of technical problems (2 CON, 1 ENOX + SK). Non-ischemic dogs (2 CON, 1 HEP, 1 ENOX, 1 ENOX + SK, 2 SK) with collateral subendocardial blood flow > 0.15 ml/g/min developing non-signifi-
cant infarcts were deleted [3]. Twenty-six dogs died of refractory ventricular fibrillation, 9 at occlusion, before initiating experimental treatments, and 17 at reperfusion (2 CON, 1 HEP, 3 ENOX, 4 ENOX + SK, 7 SK); differences in the incidence of refractory ventricular fibrillation between groups were not statistically significant. Thus, 35 dogs, 7 in each group, were used in the final analysis. There were no significant differences between groups for gender distribution, hematocrit (44.6 ± 1.8 to 48.1 ± 1.8%), hemoglobin (150.4 ± 6.2 to 168.1 ± 6 g/l), leucocyte (11.1 ± 0.8 to 15.1 ± 0.9 × 10⁹/l) and platelet (304 ± 18 to 376 ± 40 × 10⁹/l) counts. However, body weights differed slightly, but significantly, in groups 2 (24.3 ± 1 kg) and 5 (24.1 ± 1.5 kg) vs. group 1 (19.7 ± 0.7 kg).

3.2. Hemodynamic data

Differences in heart rate, peak left ventricular pressure, pressure-rate product and LADCA flow were not statistically significant between compared groups at any time during the study. However, during the course of the experiments, the pressure-rate product (heart rate × left ventricular pressure/100) decreased to a statistical level (P < 0.05) in all groups (CON, 208.7 ± 16.3 to 127.3 ± 10.4; HEP, 258.7 ± 26.9 to 174.0 ± 13.7; ENOX, 219.6 ± 16.9 to 117.1 ± 9.5; ENOX + SK, 239.7 ± 18 to 125.5 ± 17.5) at 6 h reperfusion, with the exception of the SK group (189.7 ± 22.8 to 176.8 ± 26.7). These changes were associated with significant reductions in heart rate in the CON (153.6 ± 7.8 to 122.1 ± 14.9), ENOX (164.6 ± 7.4 to 117.9 ± 8.2) and ENOX + SK (167.9 ± 8.6 to 119.6 ± 10.6) groups and with significant decreases in left-ventricular pressure in the HEP (153.1 ± 8.8 to 115.1 ± 5.9), ENOX (133.7 ± 9.7 to 99.7 ± 5.8), ENOX + SK (142.3 ± 5.9 to 103.4 ± 7.5), and SK (125.9 ± 7.9 to 110.3 ± 8.6) groups. LAD flow decreased in all groups after 6 h of reperfusion (CON, 20.1 ± 2.7 to 15.7 ± 2.1; HEP, 21.7 ± 3.9 to 15.7 ± 2.7; ENOX, 17.1 ± 2.5 to 13.0 ± 4.4; ENOX + SK, 16.4 ± 2.4 to 11.2 ± 1), but changes reached statistical significance only in the SK group (28.4 ± 4.44 to 15.1 ± 4.3).

3.3. Infarct size, and collateral blood flow (Table 1 and Fig. 1)

The area at risk was similar between groups and involved approximately 40% of the left ventricle (Table 1). However, infarct size expressed as a percentage of the area

Table 1

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>1 Control</th>
<th>2 Heparin</th>
<th>3 Enoxaparin</th>
<th>4 Enoxaparin + streptokinase</th>
<th>5 Streptokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of dogs</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Area at risk (AR) (% of LV)</td>
<td>38.4 ± 1.7</td>
<td>44.7 ± 5.4</td>
<td>44.0 ± 3.0</td>
<td>35.8 ± 3.1</td>
<td>44.8 ± 2.0</td>
</tr>
<tr>
<td>Infarct size (% of AR)</td>
<td>48.1 ± 4.5</td>
<td>32.2 ± 3.6</td>
<td>21.6 ± 5.0</td>
<td>24.9 ± 3.9</td>
<td>46.8 ± 3.4</td>
</tr>
<tr>
<td>Blood flow at 15 min of occlusion (ml/min/g)</td>
<td>117.1</td>
<td>26.9 to 174.0</td>
<td>8.2 to 122.1</td>
<td>14.9</td>
<td>164.6</td>
</tr>
<tr>
<td>Ischemic area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subendocardium</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Mesomyocardium</td>
<td>0.06 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.10 ± 0.02</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Subepicardium</td>
<td>0.11 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.15 ± 0.04</td>
<td>0.15 ± 0.02</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>Transmural</td>
<td>0.07 ± 0.02</td>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Non-ischemic area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subendocardium</td>
<td>1.42 ± 0.22</td>
<td>1.34 ± 0.15</td>
<td>0.83 ± 0.19</td>
<td>0.72 ± 0.10</td>
<td>1.41 ± 0.10</td>
</tr>
<tr>
<td>Mesomyocardium</td>
<td>1.34 ± 0.18</td>
<td>1.35 ± 0.16</td>
<td>1.01 ± 0.20</td>
<td>0.78 ± 0.06</td>
<td>1.30 ± 0.09</td>
</tr>
<tr>
<td>Subepicardium</td>
<td>1.19 ± 0.16</td>
<td>1.18 ± 0.08</td>
<td>1.00 ± 0.22</td>
<td>0.92 ± 0.08</td>
<td>1.31 ± 0.07</td>
</tr>
<tr>
<td>Transmural</td>
<td>1.32 ± 0.18</td>
<td>1.29 ± 0.13</td>
<td>0.94 ± 0.20</td>
<td>0.80 ± 0.07</td>
<td>1.34 ± 0.08</td>
</tr>
</tbody>
</table>

Results are means ± s.e.m. LV = left ventricle.

a P < 0.05 vs. control.
b P < 0.05 vs. streptokinase.
at risk was reduced ($P < 0.05$) by about 50% in the ENOX (21.6 ± 5.5) and ENOX + SK (24.9 ± 3.9) groups, but results in the HEP (32.2 ± 3.6) and SK (46.8 ± 3.4) groups were not statistically different from the CON group (48.1 ± 4.5). Infarct size in the ENOX + SK group was smaller ($P < 0.05$) than the SK group.

Transmural and regional collateral blood flows were not statistically different between groups (Table 1), and correlation between infarct size and transmural collateral flow in the ventricular wall is illustrated in Fig. 1. Covariance analysis confirmed that only the ENOX and ENOX + SK groups developed smaller ($P < 0.05$) infarcts than the CON group; the ENOX + SK group also differed statistically from the SK group.

Regional myocardial blood flow in the non-ischemic area was statistically lower in subendocardium in the ENOX + SK vs. CON and SK groups, and in the mesomyocardium as compared to the SK group. Differences between compared groups in subepicardial and transmural flow were not otherwise statistically significant.

3.4. Creatine phosphokinase (CK)

CK serum levels increased gradually during reperfusion from 725 ± 146 at 90 min occlusion to 10642 ± 2073 U/l after 6 h of reperfusion in the CON group. Similar changes were measured in the HEP group (657 ± 97 to 9376 ± 3478 U/l) and the SK group (443 ± 65 to 9546 ± 1594 U/l). However, increases in the ENOX (737 ± 277 to 4736 ± 1098 U/l), and the ENOX + SK group (527 ± 80 to 5776 ± 1662 U/l) were reduced by about 50% as compared to control, suggesting a milder myocardial damage in the ENOX-treated groups, but these differences were not statistically significant.

3.5. Platelet accumulations in myocardium (Fig. 2)

$^{111}$In-platelets circulating at onset reperfusion averaged 992 ± 88 X 10⁶, and differences between groups were not statistically significant. Fig. 2 illustrates the $^{111}$In-platelet accumulations in the non-ischemic, the viable area at risk and the infarcted myocardium. Values in 3 dogs (1 ENOX, 1 ENOX + SK, 1 SK) were excluded because of excessive (> 75%) $^{111}$In-platelets sequestration off the circulating pool.

Intragroup analysis of $^{111}$In-platelet accumulations indicated that differences in the platelet counts between the viable area at risk and the normal non-ischemic zone were not significant in the 5 study groups, with values ranging between 3274 and 86260 $^{111}$In-platelet/g myocardium. These results contrast with the accumulations seen in the infarcted myocardium that statistically exceeded ($P < 0.05$) those in the viable area at risk and the normal myocardium in all groups, with the exception of the ENOX group. $^{111}$In-platelet accumulations in both the viable area at risk and the non-ischemic myocardium were also statistically similar between groups. However, values per g of infarct were reduced by about 64% in the ENOX group (77 178 ± 16853; $P < 0.01$), and by 42% in the ENOX + SK group (122 648 ± 10 822, n.s.) as compared to CON (213 040 ± 37 230); values between the ENOX + SK (122 648 ± 10 822) and SK (367 951 ± 32 782) groups differed statistically ($P < 0.01$). Values in the HEP and SK groups were not statistically significant from the CON group.

Accumulation ratios (data not shown) between the outer epicardial and inner endocardial layers of the infarct were statistically similar among groups (CON: 0.50 ± 0.11; HEP: 0.37 ± 0.13; ENOX: 0.35 ± 0.10; ENOX + SK: 0.40 ± 0.09; SK: 0.31 ± 0.12) and indicate that about twice as many $^{111}$In-platelets accumulated deep in the infarct as compared to the outer top layer.

3.6. $^{51}$Cr-RBC counts in myocardial tissue

The mean count of circulating $^{51}$Cr-RBC of all dogs at onset of reperfusion was 215 ± 12 X 10⁹ cells. Normalized

![Image](https://academic.oup.com/cardiovascres/article-abstract/37/3/656/320270/661)
\[ {^{51}}\text{Cr-RBC} \text{ mean counts (} \times 10^6 /\text{g} \text{) were not statistically significant between groups in the different myocardial areas (infarct, } 5.51 \pm 1.01 \text{ to } 15.16 \pm 4.35; \text{ area at risk, } 4.18 \pm 0.58 \text{ to } 5.85 \pm 1.59; \text{ non-ischemic, } 4.31 \pm 0.58 \text{ to } 10.83 \pm 2.87), \text{ indicating that none of the treatments enhanced intramyocardial hemorrhage as compared to the CON group. However, upon intragroup comparisons, only the SK group showed increased (} P < 0.05 {^{51}}\text{Cr-RBC counts/g in the infarct versus non-ischemic myocardium and the viable area at risk, respectively (15.2} \pm 4.3 \times 10^6 \text{ vs. } 4.7 \pm 1.4 \times 10^6 \text{ and } 4.3 \pm 0.6 {^{51}}\text{Cr-RBC/g).}

3.7. Neutrophil accumulations in myocardial tissue (Fig. 3)

Neutrophil counts in the non-ischemic myocardium varied non-significantly between groups from 0.18 \pm 0.08 \text{ to } 1.52 \pm 0.55 \times 10^6 /\text{g}. \text{ For the viable area at risk, only the SK group showed a significant increase in neutrophils as compared to non-ischemic myocardium, indicating that the viable area at risk does not mobilize significant numbers of neutrophils in CON and in the other treated groups (HEP, ENOX, ENOX + SK).}

Fig. 4. Blood coagulation assays; values are mean \pm \text{s.e.m.} \text{ Only comparisons between treated and CON groups are illustrated; for intragroups comparisons, please refer to Section 3. Top panel: only the HEP group at 2 h of reperfusion showed significant prolongation (about 2-fold) of the aPTT (} P < 0.05 {). \text{ After 6 h of reperfusion, the SK group had shorter aPTT than the ENOX + SK group (} P < 0.05 {). Middle panel: thrombin time was slightly prolonged after SK administration versus CON (} P < 0.05 {) at onset of reperfusion, but only the HEP group differed statistically (} P < 0.05 {) from CON group during reperfusion. Bottom panel: anti-Xa levels remained unchanged in the CON and SK groups throughout reperfusion. Levels differed statistically (} P < 0.05 {) between the ENOX + SK and SK groups, throughout reperfusion.}
Accumulation of neutrophils in the infarct was, however, reduced (P < 0.05) vs. the CON group in dogs given ENOX ± SK (CON, 13.6 ± 2.0; ENOX, 2.6 ± 0.8; ENOX + SK, 3.2 ± 0.8 × 10⁶/g), but not in the HEP (15.4 ± 4.4 × 10⁶/g) and SK (16.4 ± 3.3 × 10⁶/g) groups. Neutrophils in the infarct were reduced in the ENOX + SK group as compared to the SK group (P < 0.05).

3.8. Blood coagulation assays (Fig. 4)

As compared to values measured before reperfusion, the aPTT was prolonged (P < 0.05) throughout reperfusion in the HEP, ENOX and ENOX + SK groups, and after 6 h of reperfusion in the SK group. However, only the HEP group differed statistically (P < 0.05) from the CON group at 2 h of reperfusion (P < 0.05). Also, the aPTT was significantly shorter in the SK group as compared to ENOX + SK treated dogs after 4 and 6 h of reperfusion.

Thrombin time was increased (P < 0.05) in the HEP group and, to a much lesser extent, in the ENOX group throughout reperfusion, and after 6 h of reperfusion in the ENOX + SK group. Changes in the SK group were not significant. However, thrombin time was slightly prolonged in the SK group (P < 0.05) as compared to control at onset of reperfusion, but only the HEP group differed statistically throughout reperfusion from the CON group.

Plasma anti-Xa activity was below detectable levels (< 0.01 U/ml) in every group just prior reperfusion and throughout reperfusion in the CON and SK groups. Levels initially increased in the HEP group to 0.56 ± 0.14 U/ml (P < 0.05) after 2 h of reperfusion, but declined below therapeutic levels to 0.34 ± 0.10 U/ml (P < 0.05) and to 0.28 ± 0.1 U/ml (n.s.) after 4 and 6 h of reperfusion, respectively. However, the anti-Xa activity remained stable (0.60–0.75 U/ml) and significantly increased (P < 0.05) throughout reperfusion in groups given ENOX and ENOX + SK.

Between-group differences in anti-Xa activity versus the CON group were significant (P < 0.05) at only 2 h in the HEP, and throughout reperfusion in the ENOX and ENOX + SK groups. Anti-Xa activity levels in the ENOX + SK group also differed significantly (P < 0.05) from the SK group.

3.9. Effect of HEP and ENOX on platelet–neutrophil adhesion (Fig. 5)

Platelet adhesion to neutrophils when tested with cell preparation in PBS was inhibited (P < 0.05) to the same degree by HEP and ENOX and in a dose-dependent manner, values ranging between 11 ± 3.2% at 10 µg/ml and 30 ± 5.2% at 100 µg/ml. However, while ENOX maintained a similar inhibitory (P < 0.05) activity in plasma, HEP was devoid of significant activity at a concentration of 100 µg/ml (4.0 ± 2.6%).

4. Discussion

The present study demonstrates that LMWH enoxaparin, started intravenously a few minutes before the end of a 90-min coronary occlusion and through reperfusion reduced infarct size by about 50%. The cardioprotection was associated with decreases in both platelet and neutrophil accumulations in the reperfused infarct, suggesting that enoxaparin has likely interacted with platelet–leukocyte interrelated mechanisms.

Platelets and neutrophils are found together in thrombosis and inflammatory sites, and cooperation between these cellular elements modulates each other function, and results in increased production of free radicals [25], and lipid mediators of the inflammatory response including leukotrienes, and new lipid derivatives [26]. Platelets thus play an active role in the inflammatory processes, either directly by producing or releasing active agents, or by activating neutrophils [27]. Activation induces expression on platelet membrane of P-selectin (GMP-140) stored in α-granules, allowing platelet binding to neutrophil ligand sialyl Lewis-X (SLex), a tetrasaccharide [28]. Activated endothelial cells also express P-selectin which is involved with L-selectin, a leukocyte selectin, in leukocyte rolling and initial adhesion to endothelial cells, a first step in neutrophil trafficking to inflammatory sites. P- and L-selectins appear to play an important role in reperfusion injury [29]. Inhibition of P-selectin by a SLex analog [30] or with a monoclonal antibody against P-selectin [31], reduce neutrophil accumulation in the reperfused myocardium and infarct size in dog and feline models of ischemia and reperfusion. In the present study, we found that enoxaparin can modulate P-selectin-mediated platelet–neutrophil adhesion in a plasmatic milieu, supporting the hypothesis that reduction of infarct size by enoxaparin could be related, at least in part, to an inhibition of P-selectin function or blockade of P-selectin receptor sites on leukocytes by yet
unidentified enoxaparin fragments, preventing both platelet and neutrophil accumulations in reperfused myocardium. Failure of tested dose of heparin to influence platelet and neutrophil accumulations is likely related to its neutralization by non-specific binding [18], or its inhibition by platelet factor 4 [32].

Over the past decade, several studies [33] have established the concept that neutrophils invading the myocardium attenuate the benefits of coronary reperfusion on infarct size limitation. An early and massive accumulation at reperfusion has been correlated to an increase of infarct size [34]. Plugging of the microvasculature and release of vasoactive substances by neutrophils are features of the no-reflow phenomenon [2] that may contribute to postischemic damage or reperfusion injury. The release of toxic material, such as free oxygen radicals and lysosomal enzymes may compromise reversibly injured myocardium [33], and limitation of infarct size by interfering with leukocyte counts, activation, adhesion and function has been generally successful [35]. On the other hand, presence of a residual critical stenosis during reperfusion in the dog is responsible for a further increment of infarct size through a platelet-dependent mechanism [3]. Therefore, observed benefits of enoxaparin on infarct size with such a model give further support to the hypothesis of an interaction of the LMWH with platelet-related mechanisms.

Despite our failure to reduce infarct size with unfractionated heparin, there was a favorable trend toward a reduction in the treated group, suggesting that additional mechanisms than P-selectin modulation by heparins may be involved, or that the dose of unfractionated heparin used in our study was too low to achieve cardioprotection. The dosage of heparin that we used was derived from clinical protocols assessing the benefits of unfractionated heparin with thrombolytics in acute myocardial infarction [6]. After adjustment for body weight, the treatment doubled the aPTT during the first 2 h of reperfusion and elicited anti-Xa plasma levels within the clinical range, and similar to those produced by enoxaparin. However, in contrast to enoxaparin, levels of anti-Xa activity declined in the last h of reperfusion in the unfractionated heparin-treated dogs, suggesting that blood anticoagulation was not then optimal. Whether or not the maintenance of the stable anti-Xa activity obtained with enoxaparin throughout reperfusion is connected to the limitation of infarct size by the LMWH cannot be ruled out. However, as only unfractionated heparin prolonged the thrombin time, it is unlikely that thrombin inhibition is related to the observed reduction of infarct size by enoxaparin. This conclusion is in agreement with the study of Friedrichs et al. [36] who observed reduction of CK release following global ischemia and reperfusion of isolated rabbit hearts, and that of Black et al. [7] who limited infarct size with N-acetyl heparin, which is devoid of anticoagulant activity.

High doses of unfractionated heparin were reported to reduce neutrophil accumulation and to limit infarct size in a similar dog model as the one we used in the present study [7], and the cardioprotection was associated with an inhibition of complement induced tissue damage. Complement activation is another mechanism implied in reperfusion injury [37], with the production of neutrophil chemoattractants C3a and C5a, the expression of P-selectin [38] and the cellular damage mediated by the membrane attack complex C5b-9. Because enoxaparin could be even more potent in this regard than unfractionated heparin [39], we cannot rule out an inhibition of the complement system as being also involved in the cardioprotection seen with enoxaparin. Additional studies are needed to clarify this issue.

Unfractionated heparin has ambivalent effects that could influence its benefits on reperfusion injury. Low and therapeutic concentrations are promoting platelet [40] and neutrophil functions [17], whereas high doses have the opposite effect [11]. Recently, heparin was reported to up-regulate neutrophil adhesion molecule CD11b and to release IL-8 [41], a neutrophil activator associated with reperfusion injury. In contrast with this, LMWHs do not activate platelets [16], and inhibit neutrophil function at low and high concentrations [42].

Infusion of a therapeutically relevant dose of SK before release of the coronary occlusion failed to alter platelet and neutrophil accumulations in the reperfused myocardium and to limit infarct size. These results are in agreement with other studies [43,44], but contrast with reports suggesting that SK may exhibit cardioprotective effects independent of clot lysis and coronary reperfusion [45,46]. Interestingly, the cardioprotection elicited by enoxaparin was not altered by concomitant administration of SK, although it has been reported in patients with acute myocardial infarction that SK activates complement [47], and primes oxidative metabolism of neutrophils [48].

Intramycocardial hemorrhages estimated by 51Cr-RBC were not exacerbated after 6 h of reperfusion by either heparin, enoxaparin, and SK, or by the association of enoxaparin and SK, although there was a trend for aggravation with SK. Therefore, the observed benefits of enoxaparin over heparin on infarct size are unrelated to development of intramyocardial hemorrhages which occurred, as reported [14], within the borders of the infarct.

4.1. Study limitations and conclusions

We conclude that limitation of infarct size by enoxaparin likely involves, at least in part, an interaction with P-selectin-mediated platelet and neutrophil adhesion reactions in the reperfused myocardium, an effect that could not be achieved by a corresponding therapeutic anti-Xa dose of unfractionated heparin. However, this failure of unfractionated heparin must be interpreted with caution as the selected doses of HEP and ENOX do not allow full comparison of the two compounds, and differences in dose or pharmacokinetics may account for the observed differ-
ferences. Future experiments are needed to determine if the observed cardioprotection with enoxaparin is sustained or transient, if the benefits are conditioned by the presence of a residual critical stenosis during reperfusion, and if unfractionated heparin in the upper therapeutic dose range can also limit infarct size.

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