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Enoxaparin Does Not Ameliorate Limb Ischemia-Reperfusion Injury

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Objective. **Since low molecular weight heparin has greater bioavailability and sustained serum levels *in vivo* than unfractionated heparin, it has been used to supplant unfractionated heparin to achieve therapeutic anticoagulation in humans. These studies were designed to determine whether treatment with enoxaparin could protect murine skeletal muscle from ischemia reperfusion injury.**

Methods. **C57BL6 mice were divided into four groups. Sham control animals underwent 90 min of anesthesia alone. All other groups underwent 90 min of unilateral hindlimb ischemia. At the onset of reperfusion, animals received either normal saline (control and saline) or 4 mg/kg of enoxaparin subcutaneously twice daily. Groups were followed for 24 or 48 h reperfusion. Hindlimb skeletal muscle blood flow was measured by laser Doppler, and muscle was removed for histological and protein analysis. Tissue thrombosis was evaluated by thrombin antithrombin III (TAT III), local inflammation by measurement of proinflammatory cytokines (macrophage inflammatory protein-2: MIP-2, monocyte chemoattractant protein-1: MCP-1), and neutrophil infiltration by myeloperoxidase (MPO) using enzyme-linked immunosorbent assay. Plasma levels of Factor Xa were measured during reperfusion to confirm therapeutic levels of anticoagulation. Comparisons were calculated using analysis of variance.**

Results. **At 24 h reperfusion, there was increased expression of MIP-2, MCP-1, MPO, and TAT III in saline and enoxaparin treated mice compared with control (*$P < 0.05$). By 48 h reperfusion, all parameters measured remained greater than control except for the enoxaparin treated mice whose TAT III levels were significantly less than untreated mice ($P < 0.05$). Despite documented therapeutic anticoagulation and decreased levels of markers of thrombosis in enoxaparin treated mice, there was no difference in tissue cytokines, inflammatory markers, degree of muscle fiber injury (31% ± 8% *versus* 30% ± 5%) or muscle flow between ischemia-reperfusion groups (2447 ± 141 *versus* 2475 ± 74 flux units) at 48 h reperfusion.**

Conclusions. Post hoc **administration of enoxaparin did not affect local tissue thrombosis, inflammatory markers, or muscle necrosis. This suggests that despite its potent *in vivo* activity, enoxaparin did not modulate skeletal muscle injury, thrombosis, or inflammatory following ischemia reperfusion. enoxaparin may not be useful in mediating skeletal muscle injury when administered in a clinically relevant scenario.**

Key Words: **heparin; ischemia; reperfusion; skeletal muscle; cytokines.**

INTRODUCTION

Low molecular weight heparins (LMWH) are now widely accepted to be viable, and perhaps even preferential, alternatives to unfractionated heparin in a variety of clinical situations including prophylactic and therapeutic treatment of deep venous thrombosis [1–3], treatment of pulmonary embolism [4], and as an adjunct to elective percutaneous coronary intervention [5–7]. In addition to their role as anticoagulant and antithrombotic agents, much attention has been directed to the anti-inflammatory effects of unfractionated heparin and the LMWH. LMWH have been shown to decrease inflammation in animal models of renal transplantation and wound healing [8–11]. LMWH were also associated with reduced concentrations of inflammatory markers and oxidative stress observed in patients undergoing hemodialysis [12], and are used as pharmacological adjuncts for treating active ulcerative colitis [13] and reducing postoperative inflammation following cataract surgery [14].

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The anti-inflammatory properties of LMWH make them potentially useful adjuncts in the initial treatment of acute limb ischemia. Acute ischemia of the lower extremities, which affects over 200,000 patients annually in the United States, is a devastating event that is associated with a mortality of 25% and a major amputation rate of 20% in survivors [15, 16]. Treatment options are largely limited to systemic anticoagulation with intravenous unfractionated heparin and restoration of blood flow, either through percutaneous endovascular intervention, thrombectomy, or surgical bypass. Previous experimental work in this area suggests that unfractionated heparin decreases skeletal muscle injury and vascular permeability [17–19]. However, in all of these experiments, heparin was administered prior to the onset of acute hindlimb ischemia in a protocol that is not useful for managing patients suffering from acute limb ischemia reperfusion. Therefore, these experiments were designed to determine whether *post hoc* administration of the LMWH enoxaparin sodium modulated the inflammatory response to ischemia-reperfusion injury in a murine model of unilateral hindlimb ischemia. While there is pharmacologic inequivalence of low molecular weight heparins [3, 20], enoxaparin has been widely used in clinical trials in humans [5, 6, 13, 14, 21–23].

METHODS

Animal Care Protocol

Adult male C57BL6 mice (weight 25 to 28 g) were used for this experiment. Clean surgical technique was used for all procedures. Animals were anesthetized by intraperitoneal injection of pentobarbital sodium (Abbott Laboratories, Chicago, IL, dosed at 50 mg/kg in a bolus of 0.5 mL normal saline). Additional doses of anesthetic were given in 10 mg/kg increments as needed to maintain general anesthesia for the duration of the ischemic interval (90 min). During the ischemic interval and immediate recovery period, animals were placed on a heating pad to maintain body temperature at 37°C. Animals recovered from the procedure in separate cages with free access to food and water. Once fully recovered from anesthesia, they were returned to their original cages and had free access to food and water. Mice were kept in the vivarium in a 12 h light/dark cycle, and the room temperature was kept constant between 24 to 26°C. All experimental procedures were approved by the Massachusetts General Hospital's Institutional Review Board and were in accordance with the Principles of Laboratory Animal Care (Guide for the Care and Use of Laboratory Animals, National Institutes of Health publication no. 86-23, revised 1996).

Experimental Groups

The mice were distributed into one of four experimental groups: control-saline, control-enoxaparin, saline-IR, and enoxaparin-IR. Within experimental groups, mice were allocated to laser Doppler imaging, histology, serum factor Xa inhibition assay, serum cytokine assays, biochemical assessment of skeletal muscle inflammation, and tissue thrombosis.

Induction of Hindlimb Ischemia

Hindlimb ischemia was induced by applying a 4.5-oz orthodontic rubber band around the upper thigh as previously described by our group [24]. After 90 min of ischemia, the bands were removed and the animals were allowed to recover from anesthesia. Animals were then followed for 24 or 48 h postprocedure (reperfusion interval). At the end of the reperfusion interval, animals undergoing laser Doppler imaging [24–26] underwent general anesthesia (50 mg/kg pentobarbital sodium bolus with additional 10 mg/kg doses as needed), hindlimb imaging, and were then euthanized (200 mg/kg pentobarbital sodium, intraperitoneal injection). All other animals were euthanized at the reperfusion end point. Blood and hindlimbs were harvested and used for subsequent biochemical and histological analysis.

Saline/enoxaparin Administration

At the end of the 90 min ischemic interval, and just prior to band removal in the ischemia-reperfusion groups, animals in the control-saline and saline-IR groups received 250 μL of normal saline subcutaneously (s.c.) with repeat doses administered twice daily (BID) for the duration of the reperfusion interval. Animals in the control-enoxaparin and enoxaparin-IR groups received enoxaparin sodium (Aventis Pharmaceuticals Inc., Bridgewater, NJ) in 250 μL normal saline s.c. with repeat doses administered BID for the duration of the reperfusion interval.

Confirmation of Therapeutic Anticoagulation

enoxaparin sodium (enoxaparin) was dosed to maintain therapeutic levels of systemic anticoagulation, which is defined as the concentration of enoxaparin required to inhibit Factor Xa, measured 4 h after administering the last dose, to the same degree as 0.4–1.1 International Units/mL (IU/mL) of unfractionated heparin [27, 28]. To ensure that therapeutic anticoagulation was maintained throughout the reperfusion intervals, treatment group animals (control-saline, control-enoxaparin, saline-IR, and enoxaparin-IR groups) underwent assessment of Factor Xa activity using a commercially available Factor Xa inhibition assay (American Diagnostica Inc., Stamford, CT). All blood samples were collected 4 h after the last dose of either enoxaparin or saline and were processed immediately according to the manufacturer's protocol. Enoxaparin concentration was determined using an unfractionated heparin standard curve and concentration was expressed in IU/mL. The calculated residual Factor Xa activity is inversely proportionate to the effective heparin or enoxaparin concentration [27, 28] and is expressed as percent residual activity.

Determination of Tissue Blood Flow

A laser Doppler imager (Moor Instruments, Wilmington, DE) was used to assess limb perfusion [24, 25, 29]. For experiments involving laser Doppler imaging (LDI), after induction of general anesthesia, fur was completely removed from both hindlimbs with an electric shaver. The LDI source was mounted on a movable track that was fixed exactly 10 cm above the mice limbs when the animals were restrained on a warming table. The laser beam (780 nm), reflected from moving RBCs in nutritional arterioles, capillaries, and venules, was detected and processed to provide a computerized, color-coded image. Image analysis software (Laser Doppler Perfusion Measure, V3.08; Moor Instruments, Wilmington, DE) was used to calculate the mean flux, representing tissue perfusion, from the relative flux units in scanned areas of the hindlimb. All animals that underwent LDI had two separate scans performed. The first scan was performed on the shaved limbs and the mean flux was calculated from the relative flux units corresponding to the areas of the medial thigh, leg, and dorsal aspect of the paw. Upon completion of the first scan, the skin was removed from the thighs of both hindlimbs and scanning of the