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A NOVEL NON-ANTICOAGULANT HEPARIN MAINTAINS VASCULAR ENDOTHELIAL CELL FUNCTION AND PREVENTS DISSEMINATED INTRAVASCULAR COAGULATION DURING SEPSIS

presented by

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By

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ABSTRACT

A NOVEL NON-ANTICOAGULANT HEPARIN MAINTAINS VASCULAR ENDOTHELIAL CELL FUNCTION AND PREVENTS DISSEMINATED INTRAVASCULAR COAGULATION DURING SEPSIS

By

Albert M. Morrison, M.D.

Although a novel non-anticoagulant heparin (i.e., GM1892) produces various beneficial effects after hemolytic shock, it remains unknown whether this agent has any salutary effects on the depressed vascular endothelial cell function and disseminated intravascular coagulation (DIC) during sepsis. To this end, Male Sprague-Dawley rats (275-325 gms) underwent cecal ligation and puncture (CLP) or sham operation. To study endothelial cell function, GM1892 (7mg/kg and 14mg/kg, provided by Glycomed), heparin (7mg/kg and 14mg/kg, Upjohn), or an equivalent volume of saline was administered via tail vein cannula at 1 h after CLP or sham operation. At 5 h postoperation, the thoracic aorta was isolated, cut into 2.5 mm rings, and placed in organ chambers. After obtaining near maximal contraction with norepinephrine ($2x10^{-7}$ M), dose-response relaxation curves were determined to Acetylcholine (Ach) and nitroglycerin (NTG), which stimulates endothelial derived relaxing factor (EDRF) and directly provides NO, respectively. The results indicate that sepsis induced depression of Ach-induced relaxation of vascular smooth muscle was significantly improved with GM1892 as well as conventional heparin treatment. In contrast, there was no significant

difference in endothelium-independent NTG-induced relaxation. To study GM1892's effect on DIC, a chronic model of sepsis was employed: CLP or sham operated rats were treated with continuous infusion of normal saline and GM1892 (5mg/kg/day), normal saline and conventional heparin (5mg/kg/day) or normal saline beginning 1 hour after operation continuously for 5 days, then sacrificed and DIC parameters drawn. The results indicate that thrombocytopenia was prevented, and the protime and activated partial thromboplastin time were reduced by both GM1892 and heparin treatment. Thus, GM1892 (which does not possess any significant anticoagulant properties) 1) appears to be a useful adjunct for maintaining vascular endothelial cell function during early polymicrobial sepsis and 2) appears to attenuate DIC associated with a polymicrobial sepsis.

This thesis is dedicated to my Wife, Kristin, for her love, kindness and self-sacrifice and to my son Thomas, may you continue growing to become strong and wise.

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ABBREVIATIONS

ADP = Adenosine Diphosphide
ATP = Adensine Triphosphide
DNA = Deoxyribonucleic Acid
IL-6 = Interleukin-6
IL-8 = Interleukin-8
IL-10 = Interleukin-10
TNF = Tumor Necrosis Factor

INTRODUCTION

In spite of improvements in its management, sepsis will result in more than 100,000 deaths this year in critically ill patients (Parnello et al., 1990). Improved survival will depend upon a more complete understanding of the mechanisms of sepsis to aid in the search for new pharmacologic interventions.

Recently, there have been significant advances in our understanding of the importance of the vascular endothelium in the pathophysiology of sepsis and other diseases (Rubanyi, 1991). Rather than being an inactive one cell layer barrier, the endothelium regulates vascular tone by actively releasing mediators which dilate or constrict smooth muscle (Furchgott et al., 1989).

One of these agents, endothelium-dependent relaxing factor (EDRF), has been shown to be nitric oxide (NO) or a closely related molecule (Palmer et al., 1987) which relaxes vascular smooth muscle (Moncada et al., 1990), inhibits aggregation of platelets (Radomski et al., 1987a; Radomski et al., 1987b), attenuates margination and activation of neutrophils (McCall et al., 1988), and protects against reactive oxygen species (Wink et al., 1993).

It is formed by NO synthase from its precursor L-arginine and induces the second messenger, 3',5'-guanosine monophosphate (cGMP), by increasing guanylate cyclase activity (Ignarro, et al., 1985; Holzman, 1982; Palmer et al., 1988). cGMP then acts to promote smooth muscle relaxation (Davies et al., 1993). After hemorrhage (Szabo et al., 1992; Csaki et al., 1991), anoxia (DeMey et al., 1983), and ischemia-reperfusion (Lefer et al., 1991), endothelium-derived NO is diminished resulting in decreased vascular smooth muscle relaxation. However, in rats after cecal ligation and puncture (CLP) to induce sepsis, (i.e., a polymicrobial sepsis model) (Wang et al., 1994a), a biphasal response occurs: NO is increased after 2 hours, diminished after 5 hours or longer with an equivalent zone in between at 3.5 hours.

Contrary to theories that increased non-constitutive (i.e., inducible) NO production found after shock is harmful (Petros et al., 1991; Gonzalez et al., 1992), many studies have shown that eliminating or restricting NO production is harmful. For example, blockage of NO production increases cytotoxicity of oxygen radicals to hamster lung fibroblasts (Wink et al., 1993), and it results in liver damage in Corynebacterium parvum treated mice given lipopolysaccharide (Harbrecht et al., 1992). Further studies have shown that NO blockers decrease survival in endotoxic dogs (Cobb et al., 1992), depresses renal function in mice (Spain et al., 1994), and increase pulmonary injury (Minnard et al., 1994). Thus, based

on this evidence, evaluation of agents that appear to preserve endothelial cell (EC) function and constitutive nitric oxide synthase (cNOS) activity might prove to be beneficial in ameliorating the deleterious effects of sepsis.

In regard to this, unfractionated heparin has been shown to have important anti-inflammatory properties (separate from its anti-coagulant properties) that appear to be beneficial in the overall outcome after sepsis, including modulating neutrophil activity (Labrouche et al., 1992), decreasing platelet activation (Barrett et al., 1984), and antioxidant activity (Hiebert and Liu, 1990). Furthermore, studies have shown that heparin decreases susceptibility to sepsis after trauma-hemorrhage (Wang et al., 1994b), decreases disseminated intravascular coagulation (DIC) (Hau et al., 1978), improves renal function (O'leary et al., 1979), diminishes sepsis induced leukopenia (Filkins et al., 1968) and improves survival in septic rats (Schirmer et al., 1987).

Unfortunately, concern over iatrogenic hemorrhage after heparin treatment of sepsis has severely limited its clinical usefulness in human patients. Recently, however, a novel non-anticoagulant heparin, GM1892, has been developed which has only about 2-10% of the antithrombin III activity of regular heparin. If this agent were to have retained heparin's other anti-inflammatory properties, it may prove useful in the treatment of sepsis without significant risk of hemorrhage at therapeutic dosages. Therefore, we sought to study this

agent's effects upon the vascular endothelium after sepsis in two manners: preservation of cNOS activity and modulation of the severity of DIC after sepsis.

LITERATURE REVIEW

SEPSIS

Sepsis has been defined as a generalized systemic host response to an infection originating from a focus without positive blood cultures; septicemia has been defined as a generalized systemic host response to an infection with positive blood cultures (Schwartz, 1994). In this dissertation the definition of sepsis encompasses both of these terms.

PATHOPHYSIOLOGY OF SEPSIS

Sepsis appears to be mediated by a complex array of humoral factors including lipopolysaccharide (LPS), cytokines, complement, vasoactive substances, chemoattractants, and inflammatory cells resulting in uncontrolled inflammation and tissue destruction that is often associated with disseminated intravascular coagulation (DIC) and with multiple organ failure.

Although various pathogens can promote sepsis, gram-negative bacteria contain an outer coat of lipoglycoproteins known as lipopolysaccarhide (LPS) or endotoxin that is an especially potent and capable initiator of a diffuse systemic response similar to sepsis. LPS contains core oligosaccharides and the Lipid A moiety that is primarily

responsible for its inflammatory properties (Stutz et al., 1991).

LPS stimulates macrophages and endothelial cells to produce TNF and IL-1 (Ertel et al., 1991). Similarly, LPS activates the coagulation and complement systems leading to hypercoagulability, microemboli, and DIC (Hardaway et al., 1961; Hardaway et al., 1993). LPS activates the intrinsic and extrinsic complement system directly which leads to further activation of inflammatory cells (Mason et al., 1970; Muller-Berghaus et al., 1971). C3a and C5a annaphalaxins cause vasodilation, aggregation and degranulation of platelets, and aggregation and activation of PMNs leading to more interaction between Ecs and these activated cells.

LPS stimulates activation of Factor XII (Hageman factor) that initiates the intrinsic coagulation cascade beginning with its direct activation of factor XI (Muller-Berghaus et al., 1971). Furthermore, Hageman factor converts prekallikrein to kallikrein, which converts high-molecular-weight kininogen to bradykinin, promoting vasodilation and vascular leakage (Mason et al., 1970).

Platelet activating factor (PAF) is released by platelets and lymphocytes by LPS stimulation which results in activation of platelets and inflammatory cells such as macrophages, and PMNs (Lefer, 1988). LPS effects are enhanced by LPS-binding protein (LBP) which when bound to LPS, binds to the CD14 receptor on macrophages and monocytes facilitating the

presentation of LPS to these cells. A soluble form of CD14 in serum also promotes the binding of LPS to endothelial cells, stimulating the release of cytokines and adhesion molecules. Furthermore, septin, similar to LPS may promote early presentation of small amounts of LPS to macrophages in early sepsis. To counter balance this, bactericidal/permeability-increasing protein (BPI), similar in structure to LBP appears to antagonize LBP presentation of LPS and may have therapeutic potential.

IL-10 and IL-8 also appear to be important mediators in shock. IL-8 is a chemoattractant and proinflammatory mediator while IL-10 appears to decrease the effects of IL-1 and TNF.

VASCULAR ENDOTHELIUM

The vascular endothelium in recent years has been recognized as an essential participant in the pathophysiology of sepsis. In health, it lines the entire cardiovascular system and serves as a nonthrombogenic and selectively permeable membrane between the circulatory system, interstitium and parenchymal tissue. The normal endothelium opposes coagulation; however, in disease it often promotes coagulation.

Healthy endothelium secretes agents inhibiting coagulation: NO, prostacyclin, thrombomodulin, tissue plasminogen activator, von Willebrand's factor, protein C, protein S, and glycosaminoglycans. These agents in the presence of non-activated platelets and PMNs facilitate the unencumbered

passage of blood cells through diminutive vessels and capillaries without emboli or clot formation. However, when sepsis occurs, this pattern is altered.

SEPTIC ALTERATIONS OF VASCULAR ENDOTHELIUM

Inflammatory mediators, such as cytokines, complement and bradykinin, increase the EC production of procoagulant factors, such as thromboxane A1 and PAF, while decreasing production of the aforementioned anticoagulatory factors (Rodriguez et al., 1990). As noted previously, sepsis results in increased TNF production. TNF has many important effects upon endothelial cells resulting in significantly altered or disrupted physiological function, which appears to promote sepsis. It increases EC IL-1 production (Nawroth et al., 1986). It appears to inhibit EC barrier function by increasing cyclic nucleotide phosphodiesterase (CNPDE) production, thereby decreasing cAMP levels (Koga et al., 1995). It has also been shown to promote coagulation by decreasing thrombomodulin (Koga et al., 1995). Furthermore, TNF increases EC permeability as demonstrated by EC culture studies in which extracellular levels and diffusion of substances such as sorbitol, inulin, and albumin are increased (Koga et al., 1995). There is a significant reduction in the production of the anti-inflammatory and anti-coagulatory mediators NO, protein C, prostacyclin, antithrombin, etc (Brandtzaeg et al., 1989; Grffith et al., 1988).

LPS not only disrupts normal EC function with production of TNF, but directly stimulates EC production of tissue factor (Schorer et al., 1985). Furthermore, it directly decreases the EC anion charges resulting in an increased permeability of the pulmonary vasculature (Gotloib et al., 1988).

SEPSIS PROMOTES INFLAMMATORY CELL ADHESION TO ENDOTHELIUM

Sepsis promotes PMN and EC adhesion by two pathophysiological mechanisms: 1) reduced sheer rate (Firrel et al,. 1989) and 2) increased expression and activation of adhesion molecules (Arnaout et al., 1990). Sheer rate is proportional to the rate and volume of red blood cells (RBCs) moving through vessels (Firrel et al, 1989). When sheering forces are high (high RBC rate and volume moving through the vessel) the ability of PMNs to marginate, undergo rolling adhesion or stationary adhesion is decreased (Firrel et al., 1989). Furthermore, studies have shown that a greater cell to cell surface area of contact must be present for adhesion to occur at high sheer rates than at low sheer rates (Bienvenu et al., 1993). Sepsis produces decreased sheering forces by releasing agents, such as leukotriene B_4 (LB_4) and platelet activating factor (PAF), which results in decreased microvascular blood flow via microemboli and leukocyte mediated endothelial damage (Kubes et al., 1990; Zimmerman et al., 1990). Moreover, this endothelial damage results in decreased NO production, which causes further vasoconstriction and platelet activation leading to fibrin microemboli and,

thus, greater decreases in sheering force in venules (Korthuis et al., 1994).

Sepsis also has been shown to increase the expression of adhesion molecules (Gimbrone et al., 1990). PMNs express L-selectin and integrins, including CD11a/CD18, CD11b/CD18 and CD11c/CD18. Studies have shown these to be increased upon exposure to TNF-alpha, Il-1 and Il-2, which are increased during sepsis (Arnaout et al., 1990).

ECs express P- and E-selectins, and the immunoglobulin superfamily molecules--ICAM-1 and ICAM-2 (Bevilqcqua et al., 1991). Lipopolysaccharide (LPS) has been shown to produce a conformational change in the selectins resulting in immediate increases in their activity. However, LPS increases ICAM activation via transcription related events, which results in a late increase in expression at 6 hours after exposure (Spinger et al., 1990). Thus LPS, produced during sepsis, increases PMN and EC adhesion. Activated PMNs adhere, marginate and migrate into tissue to release proteases, free radicals and other toxic compounds. Moreover, PMN adhesion to ECs further promotes endothelial damage, coagulation, and vascular permeability (Grant, 1973).

SEPSIS AND DIC

Recent studies have revealed that disseminated intravascular coagulation is a rather ubiquitous and uniform component of the septic process (Housholder, 1991; Bloom, 1990; Hesselvik, 1989). DIC occurs when microemboli form

diffusely throughout the vascular system impairing the microvascular blood flow, the delivery of substrates to tissue, and the removal of waste products. It appears to be incited by the mediators of the septic process, including TNF, IL-1, and Il-6, etc. There is new evidence to support the conclusion that DIC has a higher incidence in sepsis than previously recognized and may play a central role in the promotion of the entire process. For instance, one study found that 50% of 126 septic human patients developed significant signs and symptoms of DIC (Hernandez, 1989). Others who have evaluated new coagulation tests in septic patients, such as the D-dimer test, believe that a subclinical DIC can be diagnosed in the majority of septic patients and that initiating anti-embolic treatment in these patients improves survival (Wada et al., 1993). Further evidence supporting the ubiquity of DIC in sepsis is that routine rat and rabbit septic models have been shown to induce DIC so uniformly so as to be utilized as DIC models (Yang and Hauptman, 1994; Emerson et al., 1987). Finally, the previous description of LPS effects on the coagulation cascade, platelets and PMN adhesion to Ecs is compelling evidence that sepsis is a process closely related and partially dependant upon the generalized coagulopathy known as DIC.

NO BACKGROUND

NO, as previously mentioned, is an agent produced by healthy vascular endothelial cells that plays an essential role in maintaining the normal anti-coagulatory state, thus, inhibiting the septic process.

Ever since 1977 when Furchgott and Zawadzki (Furchgott et al., 1977) discovered the existence of endothelium-derived relaxing factor (EDRF) by demonstrating that only aortic blood vessels with an intact endothelium relax in response to acetylcholine, it has been recognized as an essential modulator of blood vessel tone. Moreover, since EDRF was determined to be nitric oxide (NO) or a closely related molecule, it has recently become recognized as one of the most important physiologic messengers known to exist today (Lowenstein et al., 1994).

NO EFFECTS

NO is an uncharged molecule with an unpaired electron that results in it being a highly unstable, lipid-permeable gas having diverse effects including vasodilatation (Lowenstein et al., 1994; Morris et al., 1994), neurotransmission, platelet inactivation (Radomski et al., 1987a; Radomski et al., 1987b), microbial destruction (Hotchkiss et al., 1992), diminished neutrophil adhesion (McCall et al., 1988), and immunomodulation (Van Dervort et al., 1993). Understanding its synthesis, regulation, and role in pathological conditions is essential in developing possible interventions that alter NO appropriately.

NO SITES AND MECHANISMS OF ACTION

NO has many different mechanisms of action depending upon its site and level of production.

Heme binding: Because of its non-polarity, NO diffuses freely into cells without using a cellular membrane receptor and binds to the iron in various complexes, such as the heme complexes in guanylate cyclase, cis-aconatase, and ubiquinone reductase (Lowenstein et al., 1994). After binding to guanylate cyclase, various kinases are activated resulting in relaxation of smooth muscle as well as neurotransmission.

ADP-ribosylation: Another important and recently recognized NO mechanism of action is ADP-ribosylation (Brune et al., 1992). NO facilitates the transfer of an ADP-ribose molecule to the ribonucleotide reductase enzyme and to the glycolysis enzyme, glyceraldehyde-3-phosphate dehydrogenase (Zhang et al., 1992), which results in their inactivation.

ATP Depletion: Thus, these mechanisms combine to produce cellular toxicity by reducing the availability of ATP through three specific pathways: 1) decreasing glycolysis by inactivating glyceraldehyde-3-phosphate dehydrogenase, 2) decreasing oxidative phosphorylation by blocking ubiquinone reductase, and 3) decreasing the Kreb's cycle by inactivating cis-aconatase.

DNA: Moreover, it also exhibits cellular toxicity at the level of DNA by inactivating ribonucleotide reductase (Lepoivre et al., 1991). Additionally, it appears to be directly toxic to

DNA through an unknown mechanism, adding to its significant pathogen and cellular toxicity (Wink et al., 1991).

Oxygen: Finally, its high reactivity with oxygen leads to free radical production; however, it also scavenges free radicals under appropriate conditions, which appears to serve a cytoprotective role (Stamler et al., 1992).

Platelets: Another target is the platelet into which NO diffuses or is produced in vivo. NO results in decreased platelet adhesiveness and aggregation via increased cGMP by either guanylate cyclase, adenosine cyclase or phospholipase C (Radomski et al., 1987a; Radomski et al, 1987b). This appears to decrease platelet thromboemboli and improve the microcirculation during pathologic states such as disseminated intravascular coagulation, sepsis, and ARDS. Similarly, it affects leukocytes by the following mechanisms. First, as previously mentioned, PMNs and monocytes have iNOS that produces large quantities of NO when the appropriate inflammatory signals are present, such as cytokines or even malarial schizonts (Morris et al., 1994). NO has been demonstrated to be highly lethal to these pathogens and an important entity in the elimination of intracellular pathogens, such as Mycobacterium, by the various mechanisms of cellular toxicity mentioned previously (Morris et al., 1994).

Adhesion molecules: Recently, NO has been shown to decrease EC and PMN adhesion (Thom et al., 1994; Niu et al., 1994; Kubes et al., 1994). The following discussion of three articles provides evidence for this recently recognized process.

Recently, Niu et al. revealed that NO inhibition increases PMN adhesion to ECs (Niu et al., 1994). In this in-vitro study, human umbilical vein endothelial cells (HUVEC) were collected, cultured and exposed to G-nitro-L-arginine methyl ester (L-NAME)--a competitive inhibitor of L-arginine. Radioactive ^{51}Cr-labeled PMNs were placed on the EC cell monolayer. Significant increases in PMN adhesion were found in the L-NAME treated (NO inhibited) cultures. The increased adhesion was found to be inhibited by monoclonal antibodies against B_2 integrin (CD18) and EC ICAM-1, intracellular free-radical scavengers, and platelet activating factor (PAF) receptor antagonist WEB 2086. Thus, this data suggests that NO inhibition causes a PAF- and oxidant-associated rise in B_2 integrin and ICAM function.

Further evidence in support of B_2 integrin modification by NO is found in another study performed by Thom et al. in which acute carbon monoxide poisoning, which increases extracellular levels of NO produced by platelets, was found to inhibit PMN B_2 integrin function in rats. The mechanism of carbon monoxide's effects appears to be its high affinity binding to intracellular heme-containing proteins, which blocks NO binding. This allows NO to diffuse out of the platelets,

rather than being inactivated after binding to heme-containing proteins.

In this study, blood obtained after rats were exposed to carbon monoxide was assayed for integrin function using Dupont scrubbed nylon fiber columns, in which white blood cell retention is highly correlated with expression and proper function of B_2 integrin. A significant decrease in B_2 integrin PMN function was found. Interestingly, when superoxide dismutase was incubated in the carbon monoxide poisoned blood, the increased B_2 integrin mediated adhesion was reversed, which further suggests that NO effects may be manifested by scavenging free radicals.

Thus, both studies collaborate that NO appears to inhibit PMN B_2 integrin function. The mechanism through which NO acts is not clear, as there was no purely quantitative determination of adhesion molecules; however, both studies point to a mechanism in which NO acts as a free radical scavenger or increases scavenger production.

In another article by Kubes et al. an in-vivo approach was utilized to study NO effects on PMNs. Using an ischemia-reperfusion (I/R) model in which cat small intestine underwent ischemia though 80% occlusion of the SMA for 1 hour and then reopened to 100%, PMN rolling adhesion and firm adhesion were quantitated by intravital microscopy (x1,400 magnification) and a video recorder. The microscope was positioned over the mesentery (blood vessel rich connective tissue to the bowel)

to view jejunal small venules. PMN rolling was defined as observation of cells moving slower than RBCs, which could be seen tumbling through the vessels; firm adhesion was defined by no movement of cells for greater than 30 seconds.

Cats undergoing I/R were assigned into various treatment groups, including NO antagonists (L-NAME), NO donors (nitroprusside treatment) and monoclonal antibodies against B_2 integrin, I-CAM-1 and P-selectin. NO donor groups exhibited severe decreases in PMN firm adhesion, while B_2 integrin antibodies produced similar effects. I-CAM-1 blockade caused a smaller decrease in PMN firm adhesion. Furthermore, NO donors, B_2 integrin, and ICAM-1 had no effect on rolling adhesion. As expected, L-NAME increased firm adhesion, while P-selectin had no effect on firm adhesion, but decreased rolling adhesion. Thus this study shows that NO acts primarily though a B_2 integrin related mechanism, some possible ICAM effects, but does not modify P-selectin function.

These results uniformly show that NO decreases PMN and EC adhesion by producing a significant reduction in the activity of B_2 integrin and moderate decreases in ICAM function. These studies do not confer the specific mechanism, but it appears to be at least partly mediated by free radical scavenging and possibly anti-PAF effects. NO, however, does not appear to have any effects upon the selectins.

NO PRODUCTION

NO is formed when the guanidino nitrogen from L-arginine and an oxygen molecule are combined by nitric oxide synthase (NOS) in the presence of flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH), heme and reduced thiol (Lowenstein et al., 1994). During this process L-arginine is converted to L-citrulline, which has been used as an assay to determine levels of NO synthesis.

NO SYNTHASES

There are at least three different genes encoding nitric oxide synthase (NOS): two constitutive isoforms (cNOS) and one inducible isoform (iNOS) (Morris et al., 1994). One of the constitutive enzymes is present in the human vascular smooth muscle and endothelium; the other is present in the central and peripheral nervous system, kidney, skeletal muscle, and pancreas (Lownestein et al., 1994). Since both of these isoforms are always present, they are appropriately termed "constitutive" enzymes, even though they produce NO intermittently in small, physiologic amounts. The endothelial cNOS enzyme isoform produces basal levels of NO to maintain vascular tone; the other cNOS isoform produces NO to function as a neurotransmitter (Lowenstein et al., 1994).

The iNOS enzyme, unlike the cNOS enzymes, is produced only upon induction by a physiologic stimulus of inflammatory mediators, such as endotoxin, IL-1, interferon, and TNF;

however, after induction it continuously produces large quantities of NO until its degradation (Morris et al., 1994). It can be induced in almost all cell types including macrophages, neutrophils (PMNs), liver cells, kidney cells, pancreatic cells, and myocardial cells (Morris et al., 1994).

CONSTITUTIVE NO SYNTHASE

The regulation of NO production differs markedly between iNOS and cNOS. cNOS is regulated by cellular calcium levels and phosphorylation, independently (Lowenstein et al., 1994). For instance, after cellular calcium levels increase, calcium and calmodulin bind together to form a complex, which then binds to the cNOS enzyme, activating it. More recently, it has been discovered that phosphorylation of both isoforms of cNOS also results in their inactivation (Morris et al., 1994). Finally, as NO levels build up, it reduces its own production by acting as its own negative feedback inhibitor (Lowenstein et al., 1994).

INDUCIBLE NO SYNTHASE

In contrast, iNOS activity appears to be primarily regulated at the mRNA level by transcription modulation and/or post-transcriptional stability (Morris et al., 1994). However, three separate studies appear to show conflicting results concerning which mechanism is predominant (Lorsbach et al., 1993; Vodovotz et al., 1993; Xie et al., 1992). Alternatively, once the enzyme is activated, concentrations of substrate and cofactors respectively, such as L-arginine and

BH_4, can limit the rate of NO synthesis (Baek et al., 1993). Similar to cNOS, iNOS is affected by direct NO negative feedback inhibition (Assreuy et al., 1993). Finally, in spite of iNOS being independent of calcium regulation, calmodulin is bound to iNOS permanently during synthesis of the enzyme, which appears to be the cause of its perpetually activated state (Cho et al., 1992).

Animal and human isoforms of iNOS are not identical, either. Murine macrophage iNOS is easily induced with LPS and other inflammatory signaling agents; human macrophage iNOS activity is much more difficult to induce (Denis et al., 1991). In contrast, human liver cell iNOS is easily induced, possibly due to greater homology with murine iNOS or due to its teleological importance in protecting hepatocytes from first-pass mesenteric pathogens (Nussler, et al., 1992).

NO ROLE IN DISEASE

Recently, much research has focused on the role of NO in sepsis, diabetes, hypertension, atherosclerosis and ARDS. Initially, NO was believed to be responsible for much of the pathogenesis of sepsis; however, recent research leads one to draw the opposite conclusion. Although iNOS mediated NO production is probably responsible in part for hypotension during sepsis (Kilbourn et al., 1990), using an inhibitor of NOS during sepsis has been shown to decrease survival (Cobb et al., 1992), decrease kidney function (Spain et al., 1994), and aggravates adult respiratory distress syndrome (ARDS) (Minnard

et al., 1994). Furthermore, NO inhibitors appear to increase susceptibility to pathogens that accompany a generalized loss of immune function (Minnard et al., 1994). In contrast, NO in a liquid form administered directly into the airway has been shown to significantly decrease pulmonary vascular pressure and decrease shunting in ARDS, which improves oxygenation (Rossaint et al., 1993). However, NO does appear to be destructive in cases of localized chronic activation. For instance, islet destruction in Type I diabetes mellitus appears to be mediated by NO through inappropriate IL-1 activation of iNOS (Corbett et al., 1991). Aortic aneurysms and atherosclerotic plaques also appear to be a result of inappropriate localized, chronic NO production (Watkins).

HEPARIN STRUCTURE

Heparin is a glycosaminoglycan, which is negatively charged. It has a molecular weight ranging from 3,000 to 30,000 in conventional preparations. Specifically, it is a glucosaminoglycan, while chondroitin sulfate, for instance, is a galactosaminoglycan.

HEPARIN AND ANTICOAGULATION

Heparin, while having well-known and important anticoagulant properties, also has many other important anti-inflammatory properties. Heparin's anticoagulant properties are due to its ability to activate anti-thrombin III and its interactions with factor Xa; both are necessary for optimal anti-coagulant effects.

HEPARIN'S ANTI-INFLAMMATORY PROPERTIES

Concerning anti-inflammatory properties, heparin has recently been extensively studied. For instance, it has recently been shown that in cultured endothelial cells heparin administration decreases cell mortality after exposure to oxygen free-radicals (Heibert & Liu, 1992). Another study has shown that heparin binds to superoxide dismutase and acts as a free-radical scavenger (Meyer, 1991). Others have shown that heparin appears to decrease vascular permeability induced by bradykinin, angiotensin, bacterial endotoxin, and histamine (Engelberg et al., 1991). Other important heparin effects during sepsis include diminished release of hepatic damage markers, aspartate aminotransferase (AST) and ornithine carbamoyltransferase (OCT), in endotoxic mice (Harbrecht et al., 1992); and enhanced reticuloendothelial phagocytosis and reduced reticuloendothelial dysfunction have been demonstrated after heparin treatment (Kaplan et al., 1981). In our laboratory, we have shown that preheparinization in the hemorrhage-resuscitation shock model in rats significantly improves cardiac output, renal function and hepatocellular blood flow compared to rats not receiving heparin (Wang et al., 1990).

Furthermore, more recent studies in our lab have shown that heparin pretreatment in a trauma-hemorrhage model restores NO mediated vascular smooth muscle relaxation and preserves

microvascular blood flow in the kidney and liver (Rana et al., 1992; Wang et al., 1993).

The mechanism of heparin preservation of endothelium-dependent relaxation and other positive effects on vascular endothelial function is currently under investigation. Yokokawa et al. have shown that heparin increases cGMP, NO production and synergistically increases thrombin mediated NO production in cultured human endothelial cells (EC) (Yokokawa et al., 1993a). This was associated with reduced endothelin-1 (ET-1), a vasoconstrictor, production theorized to have been caused directly by heparin mediated increases in NO. In another publication, Yokokawa et al. demonstrated that heparin blocks basal and agonist-induced ET-1 production at the transcriptional level (Yokokawa et al., 1993b). To determine whether heparin caused this by increasing NO or another mechanism, L-NMMA in the presence of heparin was used to inhibit NO production and 10^{-5} M L-NMMA was found to restore EL-1 mRNA production to levels found without heparin exposure. However, Imai et al. found that L-NMMA did not restore ET-1 mRNA production in the presence of heparin (Imai et al., 1993). Thus, it still remains controversial whether heparin decreases EL-1 production by increasing NO or another by another mechanism. Heparin has many other effects including being a thrombin inhibitor via antithrombin III, inhibiting vascular smooth muscle cell proliferation (Castellot et al., 1982), inhibiting proto-oncogene (c-fos, c-myc) induction

(Castellot et al., 1989), inhibiting neutrophil activity (Labrouche et al., 1992) and lowering the blood pressure in spontaneously hypertensive rats (Susic et al., 1982).

HEPARIN AND DIC

Concerning heparin's effects on DIC, there is much evidence supporting its ability to decrease the severity of DIC. Studies using heparin in experimentally induced peritonitis in rats shows that DIC parameters were significantly decreased, and there was a significant reduction in mortality (O'leary, 1979). Another study found significant improvement in survival and hepatic blood flow in heparin-treated vs. saline-treated controls (Schirmer, 1987). A criticism of these studies was that rats are not closely genetically related to humans as are other mammals and primates. But in a study evaluating DIC in baboons, heparin was found to significantly improve survival and reduce DIC parameters: protime, activated partial thromboplastin time, and platelet count (Du Toit et al., 1989). One study evaluated DIC in septic infant swine because of the similarity of swine to human infants (Griffin et al., 1990). Escherichia coli peritonitis was induced in two groups: swine treated with heparin 25 u/kg/hr verses swine treated only with an equivalent volume Lactated Ringer's solution. There was a statistically significant improvement in survival time (18.8 +/-2.2 hr. vs. 11.9 +/- hr., respectively). Two other studies have evaluated heparin use in septic animals and found that it alleviated hypotension

associated with sepsis in both a clinical and experimental study (Corrigan et al., 1970; Hardaway et al., 1963).

HEPARIN'S SIDE EFFECTS

The use of heparin in human patients clinically for treatment of deep venous thrombosis (DVT), DVT prophylaxis, and certain arterial occlusive conditions, such as TIA's has been widely utilized and is well accepted. However, heparin use for sepsis and its sequelae such as DIC and MOF has been limited primarily by the risk of iatrogenic-induced hemorrhage and because of controversy concerning its effectiveness--not all studies have shown consistent improvements in outcome. Another untoward side-effect that can limit its clinical usefulness is immune-induced platelet injury, which is an idiosyncratic reaction causing platelet destruction in a small percentage of patients receiving heparin due to production of antibodies, which destroy platelets. This can lead to a severe embolic disease, sometimes referred to as "White Clot Syndrome" (Chong, 1988).

NON-ANTICOAGULANT HEPARIN

Recently a new, chemically modified heparin (CMH) without significant in-vitro anticoagulant properties has been developed. Wang et al. have shown that it reveals promise as a possible therapeutic intervention (Wang et al., 1994b). For instance, GM1892 administration has been shown to improve hepatocellular function, cardiac output, microcirculation and

decrease susceptibility to sepsis in a trauma-hemorrhage resuscitation shock model.

Rationale for specific aims:

As previously mentioned in the introduction, sepsis has been shown to decrease vascular endothelium-derived relaxing factor (EDRF) within a few hours after the onset of sepsis. Decreased NO production appears to have deleterious effects as many studies have demonstrated that septic animals treated concomitantly with competitive NO synthase inhibitors have that rates of organ damage and mortality are increased by blocking NO production. Furthermore, heparin has been shown to have many important beneficial effects in the treatment of sepsis, including increasing survival, decreasing DIC and diminishing organ dysfunction. Because of the risk of iatrogenic hemorrhage from its administration for sepsis, its therapeutic usefulness in this setting is limited. However, recently a novel anticoagulant heparin, GM1892, has been synthesized which appears to have retained many anti-inflammatory properties, while essentially causing no significant coagulopathy. We, therefore, sought to show that this agent was able to maintain NO production without the significant risk of iatrogenic induced hemorrhage.

SPECIFIC AIMS:

1) To determine if administration of GM1892 after onset of sepsis will maintain endothelium-dependent vascular relaxation at 5 and/or 20 hours after induction of sepsis.

2) To determine if GM1892 is equivalent to unfractionated heparin in protecting endothelial function during sepsis.

3) To determine if GM1892 attenuates DIC in septic rats with peritonitis induced by CLP.

MATERIALS AND METHODS

SEPSIS MODEL

All experiments were performed with the approval of the All-University Committee on Animal Use and Care, Michigan State University, and in accordance with the National Institutes of Heath Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) weighing 275-325 gms were acclimated to their new environment for 1 week, given water and standard rat chow ad libitum, and then were anesthetized with methoxyflurane (Metofane, Lot B3D674, Pitman-Moore Inc, Mundelein, IL).

Rats underwent cecal ligation and puncture (CLP) (Wicterman et al., 1980). Briefly, the abdomen was shaved, and a 2-cm midline abdominal incision was made using dipolar cautery. After grasping and removing the cecum from the abdominal cavity, it was tied off distal to the ileo-cecal valve with a 3-0 silk suture in order to isolate the cecum without obstructing the gastrointestinal tract. The antimesenteric boarder of the cecum was punctured, both proximally and distally with an 18 gauge needle. After expressing small quantities of sucus entericus by applying light pressure upon the cecum, it was replaced within the abdominal cavity and a two-layer abdominal closure with 3-0 dexon was performed.

In rats undergoing sham operation, the abdomen was opened in a manner consistent with the CLP technique, after which the cecum was grasped and brought out through the incision. After handling the cecum, it was replaced and the abdomen closed, as described for CLP rats.

Immediately after abdominal closure, CLP and sham operated rats received 3ml/100g BW normal saline via subcutaneous injection. The tail vein was cannulated with a 24 gauge angiocath needle, and the rats were randomly assigned into treatment groups (n=6-8): CLP+heparin (7 & 14 mg/kg), CLP+GM1892 (7 & 14mg/kg), CLP+normal saline (NS) or sham+NS, in which the medication or NS was administered one hour after operation. The rats were then re-anesthetized with Metofane and sacrificed at 5 hours after surgery, for evaluating response to early sepsis.

Blood Vessel Ring Study

Immediately following sacrifice, a thoracotomy was performed to remove the aorta, which was immediately placed in Kreb's-Ringer HCO_3 solution (composition in mM): NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; Ca-EDTA, 0.026; glucose, 11.1), which was aerated with 95% O_2:5% CO_2 (pH 7.4; pO_2 = 580 mm Hg) at 37^0C. After carefully trimming all excess tissue and cutting the aorta into 2.5 mm rings weighing 1.7-2.0 mgs, the rings were mounted between an isometric pressure transducer (FTO3, Grass Instruments, Quincy, MA), which was coupled to a polygraph (Model 7D, Grass

Instruments), and a hook in the 28 ml glass organ chamber, using two 6-0 silk sutures tied in separate loops around the specimen. The glass organ chamber was filled with 20 ml of aerated Krebs-Ringer bicarbonate solution and the temperature was maintained at 37^0C using a circulating waterbath.

After equilibrating the aortic rings at 1000 mg of tension for one hour and rinsing the chamber every 15 minutes with buffer, norepinephrine (NE, Sigma, St. Louis, MO) 10^{-9} to 10^{-5} M was given to determine a dose-response curve. It was determined that 2 x 10^{-7} M resulted in about 75% of maximal contraction.

After the dose-response curve was performed, the chambers were rinsed with fresh, aerated Kreb's solution every 10 minutes. After 30 minutes, 2 x 10^{-7} M of norepinephrine was given to produce 75% of maximal ring contraction, so a relaxation curve could be obtained by the titrated addition of acetylcholine (Sigma; 10^{-8} to 10^{-5} M) and Nitroglycerin (American Regent Laboratories, Shirley, NY; 10^{-9} to 10^{-6} M). After obtaining the ACh relaxation curve and prior to obtaining the NTG curve, the rings were be washed with Krebs-Ringer bicarbonate solution and allowed to equilibrate for 30 minutes. Immediately following the NTG relaxation curve, the aortic rings were removed, blotted on tissue paper, and weighed.

Statistical Analysis:

The amount of contraction and relaxation is expressed as milligrams of tension starting from a baseline of 0 gms (not including the 1 gm resting tension). The multiple treatment group responses were statistically evaluated with One-Way ANOVA followed by the Student-Newman-Keuls Post-hoc test. The Arcsin squareroot transformation was used to normalize the percentage data before applying the parametric tests. A $p<0.05$ was considered significant.

DIC STUDY PROTOCOL

Sepsis Model:

The sepsis experimental protocol was followed for the induction of sepsis in the DIC protocol and is described on page 27.

In rats undergoing cecal ligation and puncture (CLP) or sham operation it was performed as described on pages 27-28, except that the neck was also shaved.

After the CLP or sham operative procedures were performed, in order to cannulate the right jugular vein, a vertical incision near the midline was made in the neck and the subcutaneous tissue spread to expose the right jugular vein. After skeletonizing the jugular vein, silastic tubing (.03 x .065; Baxter Healthcare Corporation, McGaw Park, IL) was inserted and fastened with 6-0 silk suture. It was tunneled through the subcutaneous tissue to the back of the neck. After the incision was closed, the tubing was threaded through

a 12 inch long metal-coiled spring, which was sutured to the neck epidermis.

The rats were assigned to one of four experimental groups: CLP + normal saline (NS); CLP + NS + Heparin given at 5mg/kg/day (heparin sodium, 1000 USP units/mL, Lot 930 WT, Upjohn Company, Kalamazoo, MI); CLP+ NS + GM1895 given at 5mg/kg/day (GM1892, Lot 230, Glyco/Med, CA); and Sham operation + NS. All groups received their respective intravenous (IV) fluids at a rate of 3 ml/hr through continuous intravenous infusion (harvard syringe infusion pump 22; Harvard Apparatus, South Natick, MA). Heparin and GM1892 were administered continuously beginning one hour post CLP. Daily urine outputs and weights were obtained and mortality was monitored.

On the fifth postoperative day, the rats were anesthetized, and the following clinical assays were obtained: complete blood count (Technicon H1; Dr. Roth's Laboratory, MSU Dept. of Pharmacology and Toxicology); thrombin time, activated partial thromboplastin time and prothrombin time (Dade Diagnostics, Baxter Healthcare Corp, Miami, FL); fibrin degradation products using Staphylococcal clumping method (Sigma Diagnostics, St. Louis, MO); blood urea nitrogen, serum creatinine, urine creatinine, antithrombin III (Sparrow Hospital Chemistry Dept. Laboratory, Lansing MI).

RESULTS

Vascular Endothelial Studies

NE-Induced Vascular Contraction:

The cumulative NE-induced vascular contraction dose-response curves are represented in Figures 1-4. The vascular ring NE-induced peak contractions in the dose-response curves were not found to differ significantly between any of the groups. This included the Sham + NS, CLP + NS, CLP + GM1892 (7 & 14 mg/kg BW), and CLP + heparin (7 & 14 mg/kg BW) at both 5 and 20 hours after operation. There were no obvious non-significant trends noted as the sham + NS group was found to vary in relation to the other groups in what appears to be a random fashion. However, it was determined through the cumulative dose-response curves that a dose of $2x10^{-7}$ M of NE resulted in about 75% of maximal vascular ring contraction. We subsequently utilized this dose to precontract the rings in preparation for the ACh and NTG-induced relaxation.

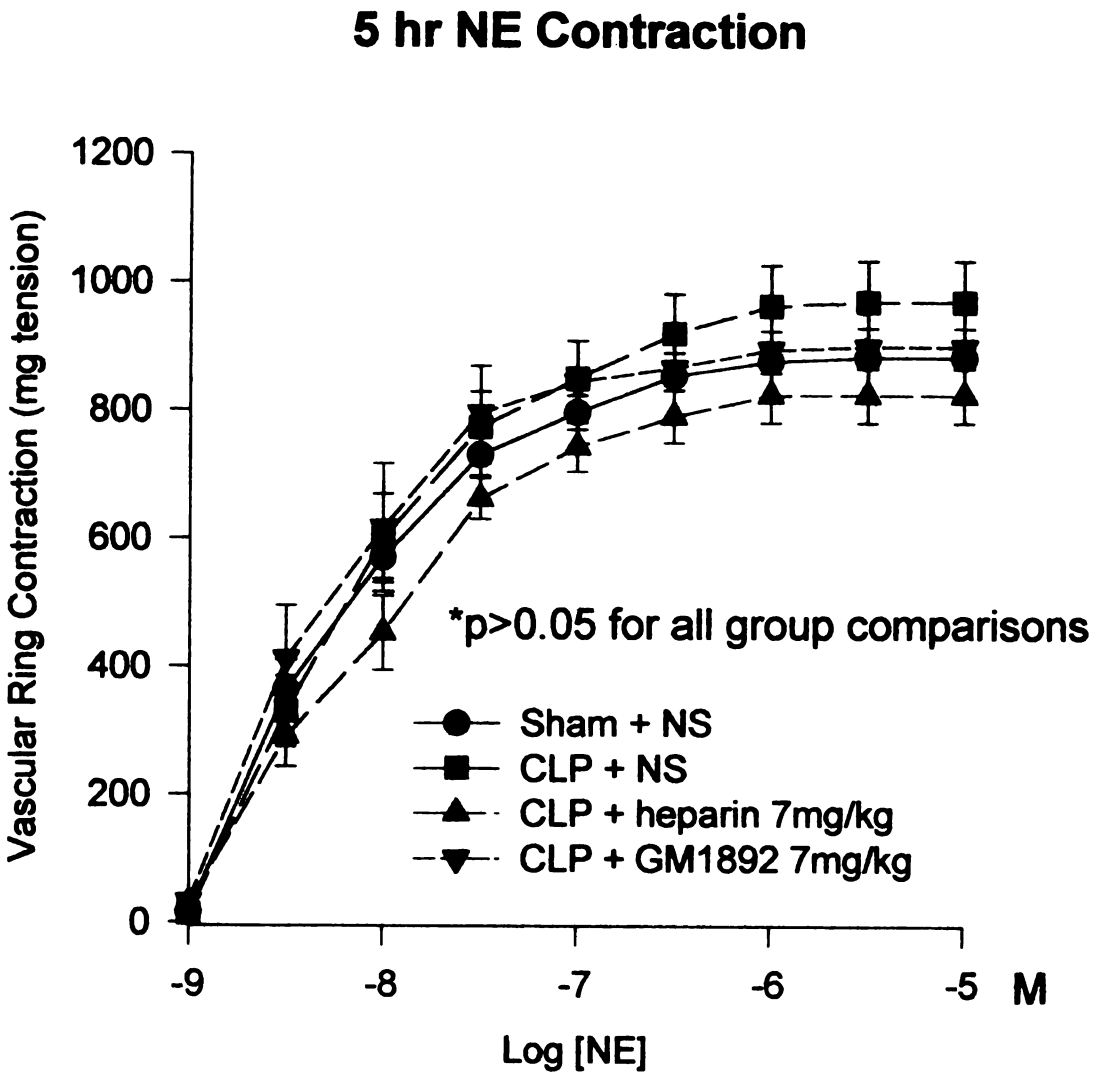

Figure 1. Cumulative dose-response relationship to norepinephrine in aortic rings isolated five hours after onset of sepsis from animals that underwent sham operation or CLP with either normal saline, unfractionated heparin or GM1892 at 7 mg/kg. There were seven to nine rats in each group with one ring from each animal. Values are means +/- SEMs and compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

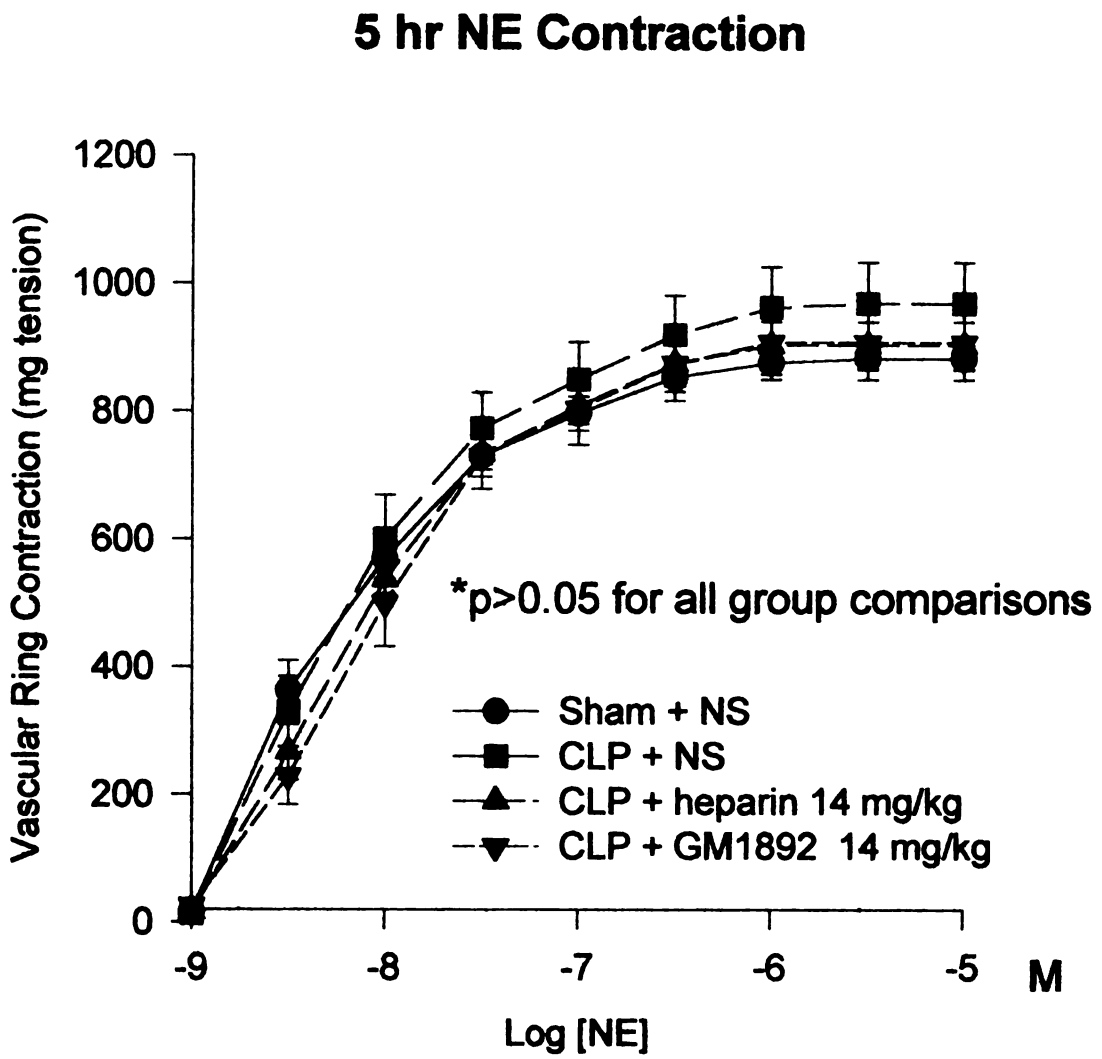

Figure 2. Cumulative dose-response relationship to norepinephrine in aortic rings isolated five hours after onset of sepsis from animals that underwent sham operation or CLP with either normal saline, unfractionated heparin or GM1892 at 14 mg/kg. There were seven to nine rats in each group with one ring from each animal. Values are means +/- SEMs and compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

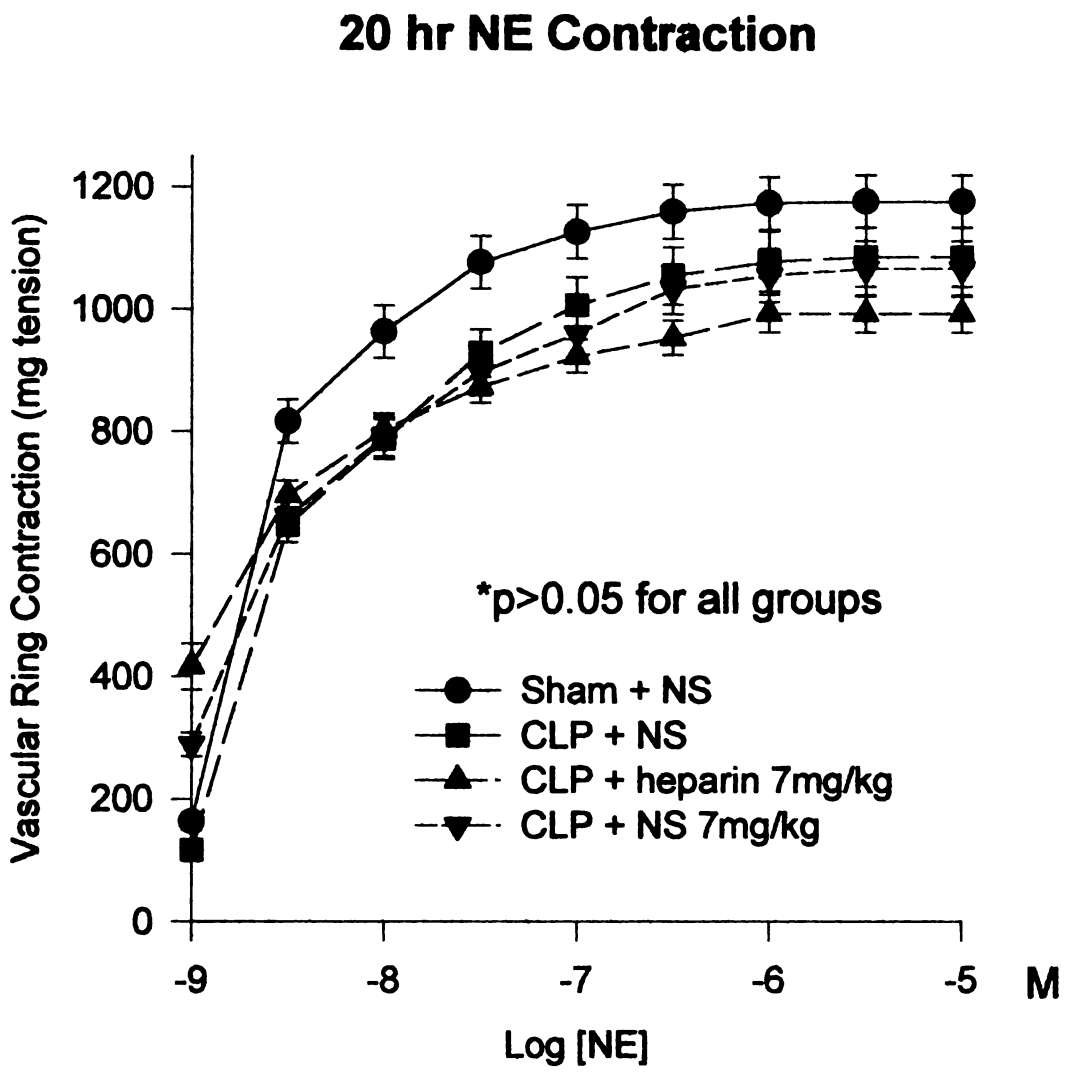

Figure 3. Cumulative dose-response relationship to norepinephrine in aortic rings isolated twenty hours after onset of sepsis from animals that underwent sham operation or CLP with either normal saline, unfractionated heparin or GM1892 at 7 mg/kg. There were seven to nine rats in each group with one ring from each animal. Values are means +/- SEMs and compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

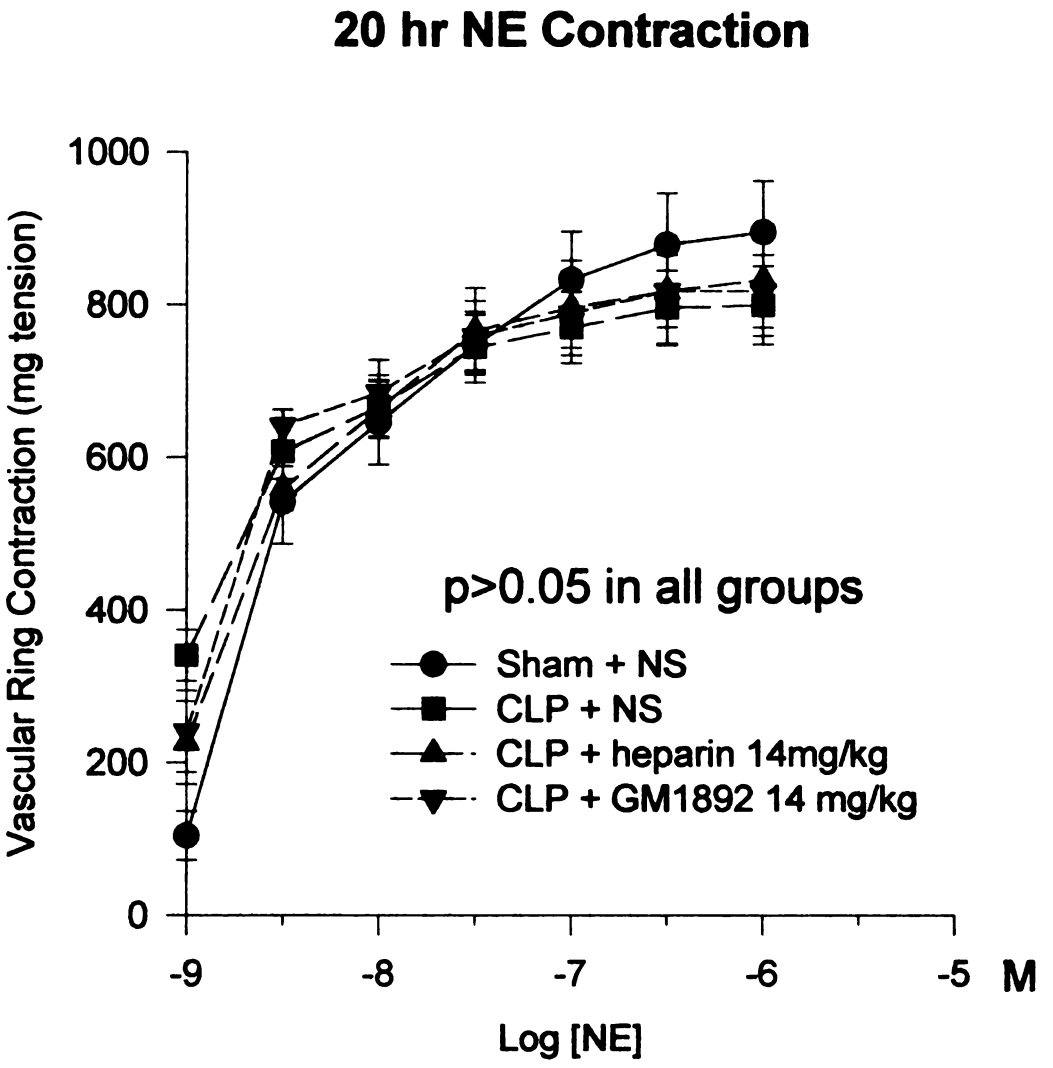

Figure 4. Cumulative dose-response relationship to norepinephrine in aortic rings isolated twenty hours after onset of sepsis from animals that underwent sham operation or CLP with either normal saline, unfractionated heparin or GM1892 at 14 mg/kg. There were seven to nine rats in each group with one ring from each animal. Values are means +/- SEMs and compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

Alterations in ACh-Induced Vascular Relaxation:

The cumulative ACh-induced vascular relaxation dose-response curves are illustrated in Figures 5-8. In the groups evaluated 5 hours post operation, the ACh-induced vascular ring peak and near-peak relaxation was significantly greater in the sham + NS, CLP + GM1892 14mg/kg and CLP + heparin 14mg/kg BW treated groups compared to the CLP + NS group. However, at 5 hours post operation, the CLP + 7mg/kg BW GM1892 and CLP + 7mg/kg BW heparin groups did not exhibit any significant difference in relaxation compared to the CLP + NS group. At 20 hours post operation, there was a significantly greater amount of ACh-induced relaxation in the sham + NS group compared to the CLP + NS, and the 7 mg/kg BW heparin and GM1892 treated groups. However, in contradiction, there was no significant differences between the sham + NS and other groups at 20 hours post operation in the block that included treated rats receiving 14 mg/kg BW of GM1892 or heparin.

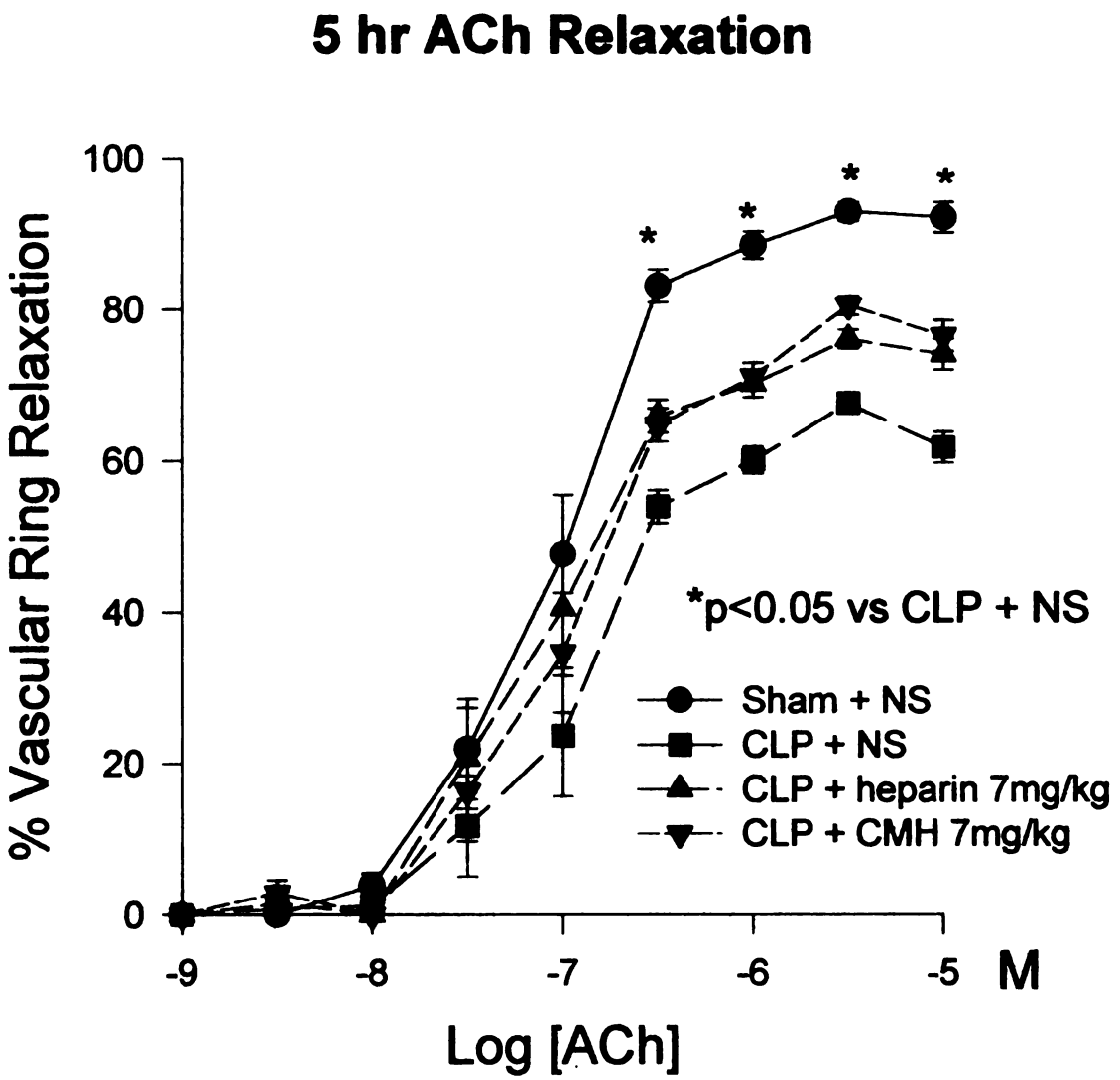

Figure 5. Cumulative dose-response relationship to acetylcholine administration in aortic rings that were precontracted with $2x10^{-7}$ M of NE. Animals were sacrificed 5 hours post induction of sepsis; dosage of treatment was 7 mg/kg. There were seven to nine animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the percent change in relaxation from a baseline at the NE-induced precontracted state. Values are means +/- SEMs and were compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin Squareroot transformation t normalize percentages.

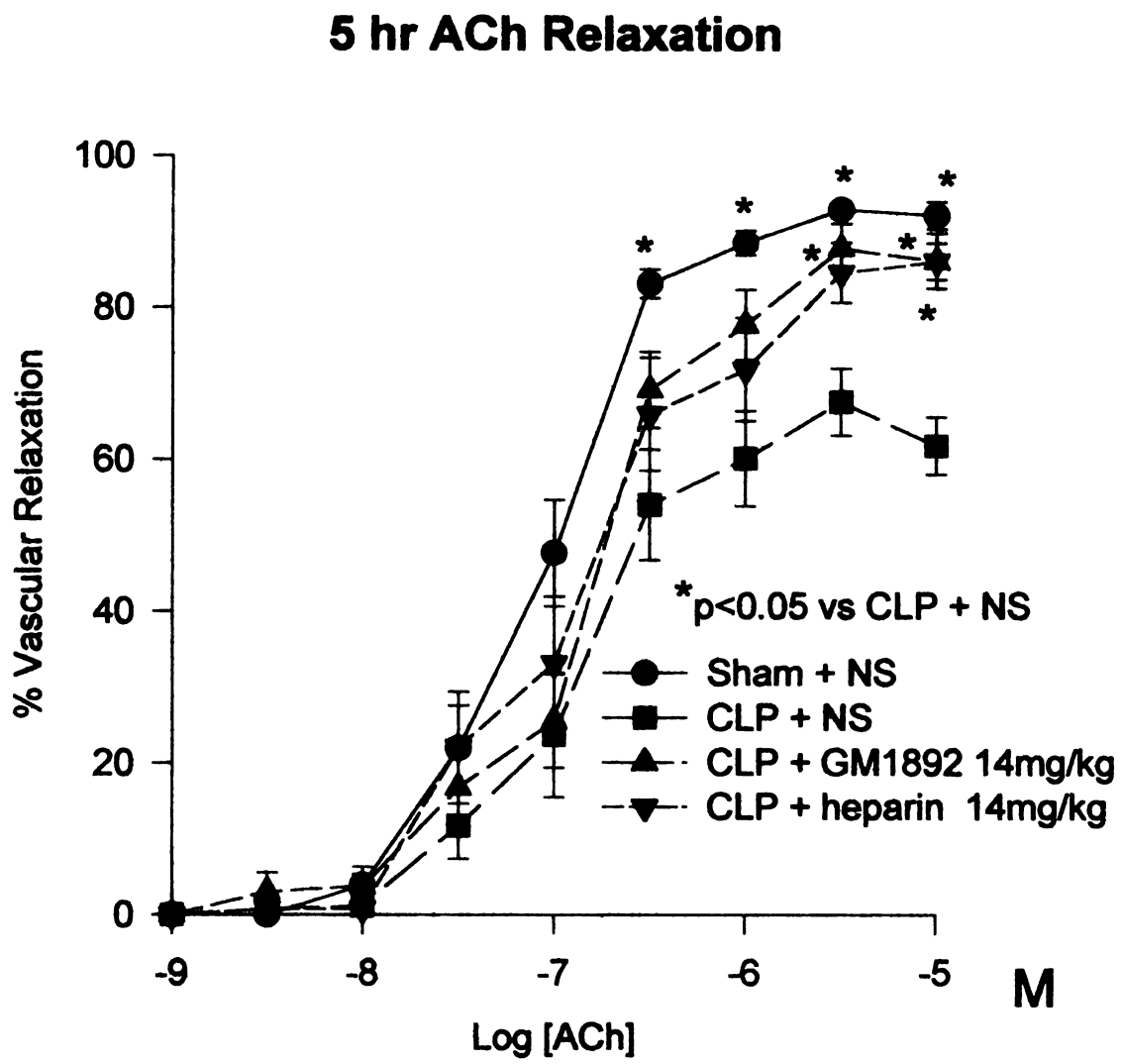

Figure 6. Cumulative dose-response relationship to acetylcholine administration in aortic rings that were precontracted with $2x10^{-7}$ M of NE. Animals were sacrificed 5 hours post induction of sepsis; dosage of treatment was 14 mg/kg. There were seven to nine animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the percent change in relaxation from a baseline at the NE-induced precontracted state. Values are means +/- SEMs and were compared by one-way analysis of variance and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

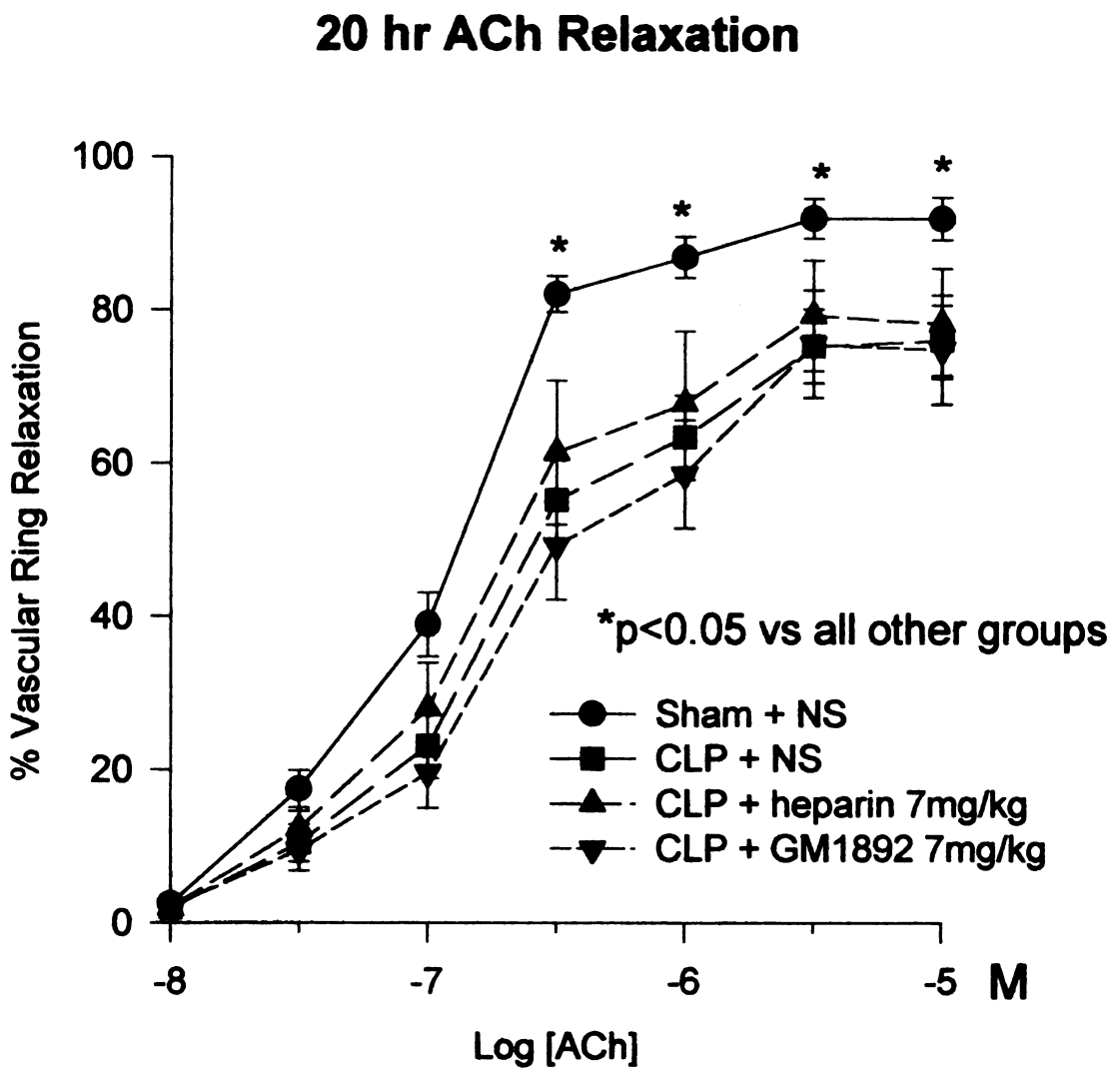

Figure 7. Cumulative dose-response relationship to acetylcholine administration in aortic rings that were precontracted with 2x10-7 M of NE. Animals were sacrificed 20 hours post induction of sepsis; dosage of treatment was 7 mg/kg BW. There were seven to nine animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the NE-induced precontracted state. Values are means +/- SEMs and were compared by one-way analysis of varience and Student-Newman-Keuls post-hoc test after ArcSin squareroot transformation to normalize percentages.

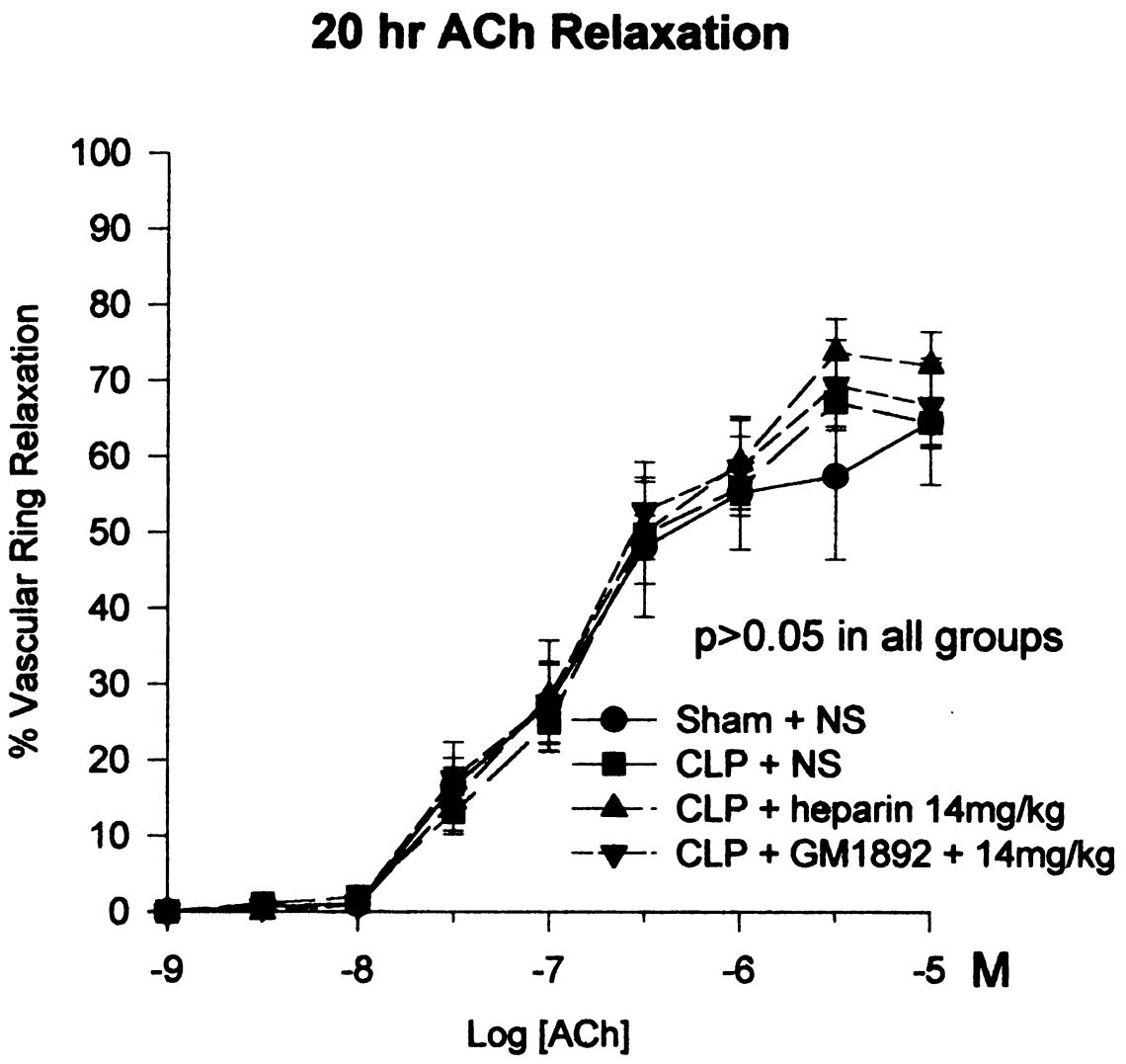

Figure 8. Cumulative dose-response relationship to acetylcholine administration in aortic rings that were precontracted with $2x10^{-7}$ M of NE. Animals were sacrificed 20 hours post induction of sepsis; dosage of treatment was 14 mg/kg BW. There were seven to nine animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the NE-induced precontracted state. Values are means +/- SEMs and were compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

NTG-Induced Vascular Contraction Curve:

The cumulative NTG-induced vascular relaxation dose-response curves are illustrated in Figures 9-12. The cumulative NTG-induced vascular dose-response relaxation curves were not found to be significantly different between any of the treatment groups at 5 hours or 20 hours post operation.

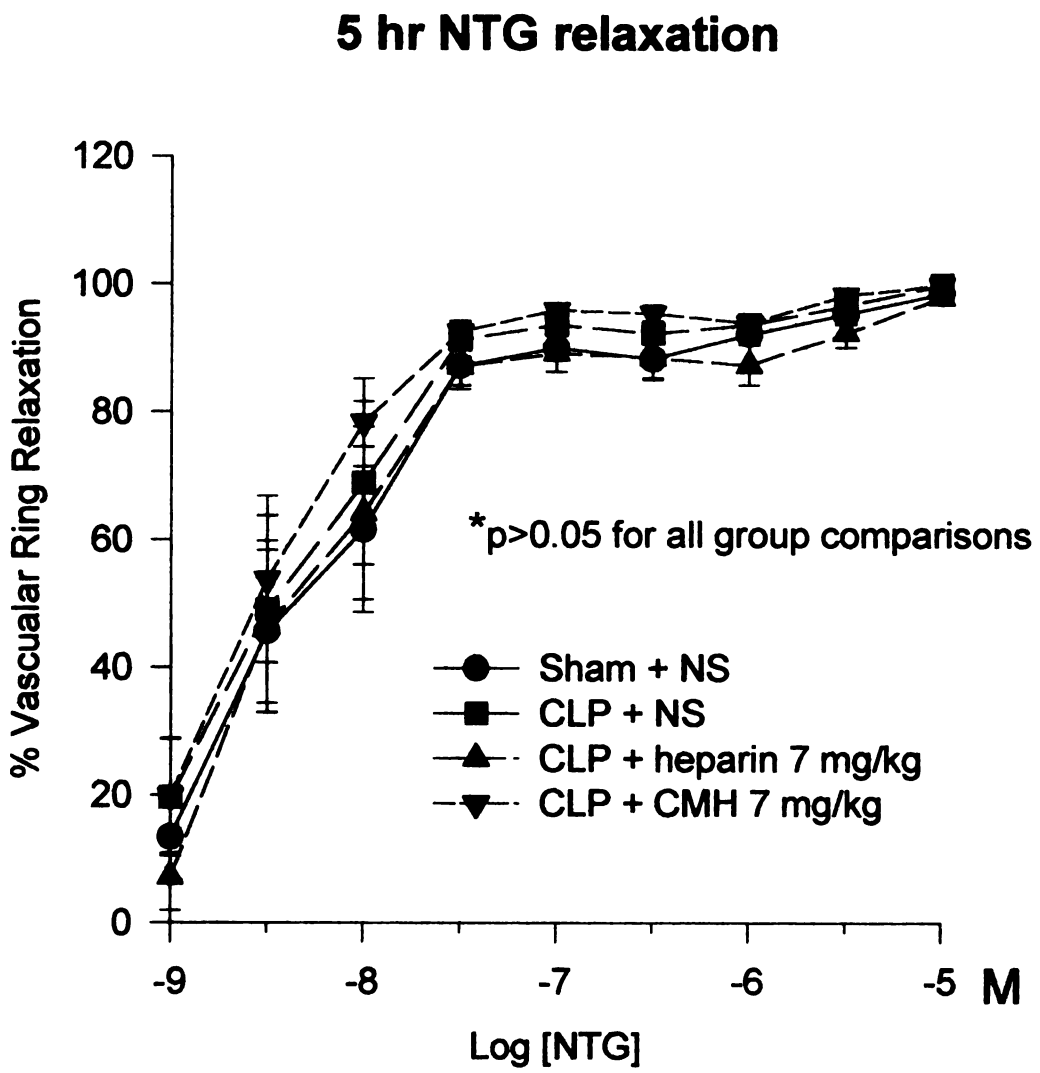

Figure 9. Cumulative dose-response relationship to nitroglycerin administration in aortic rings that were precontracted with $2x10^{-7}$ M of NE. Rings were harvested 5 hours after onset of sepsis. GM1892 and heparin were given at 7 mg/kg BW in treatment groups. There were sex to eight animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the NE-induced precontracted state. Values are means +/- SEMs and were compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin Squareroot transformation to normalize percentages.

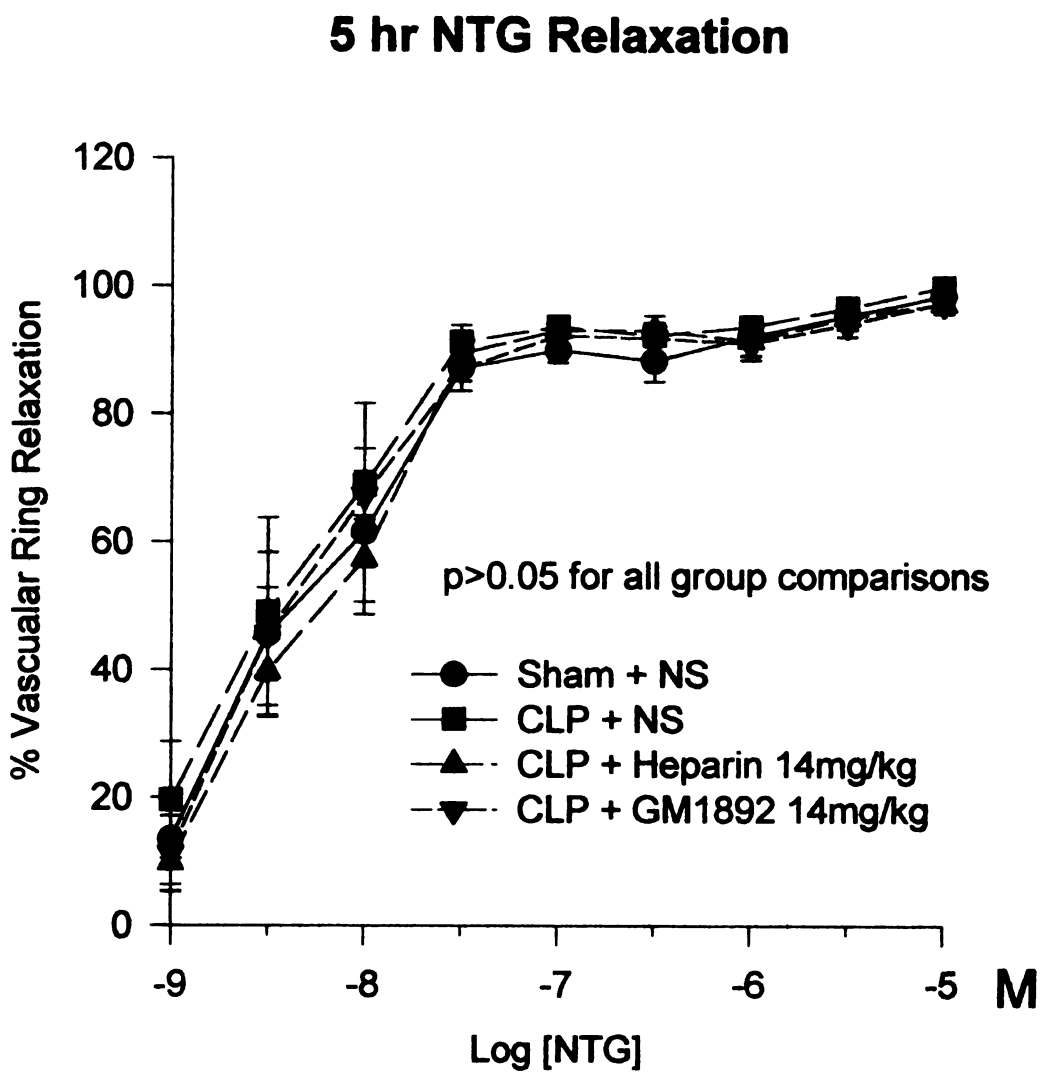

Figure 10. Cumulative dose-response relationship to nitroglycerin administration in aortic rings that were precontracted with $2x10^{-7}$ M of NE. Rings were harvested 5 hours post onset of sepsis. Gm1892 and heparin were given at 14 mg/kg BW in treatment groups. There were six to eight animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the NE-induced precontracted state. Values are means +/- SEMs and compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

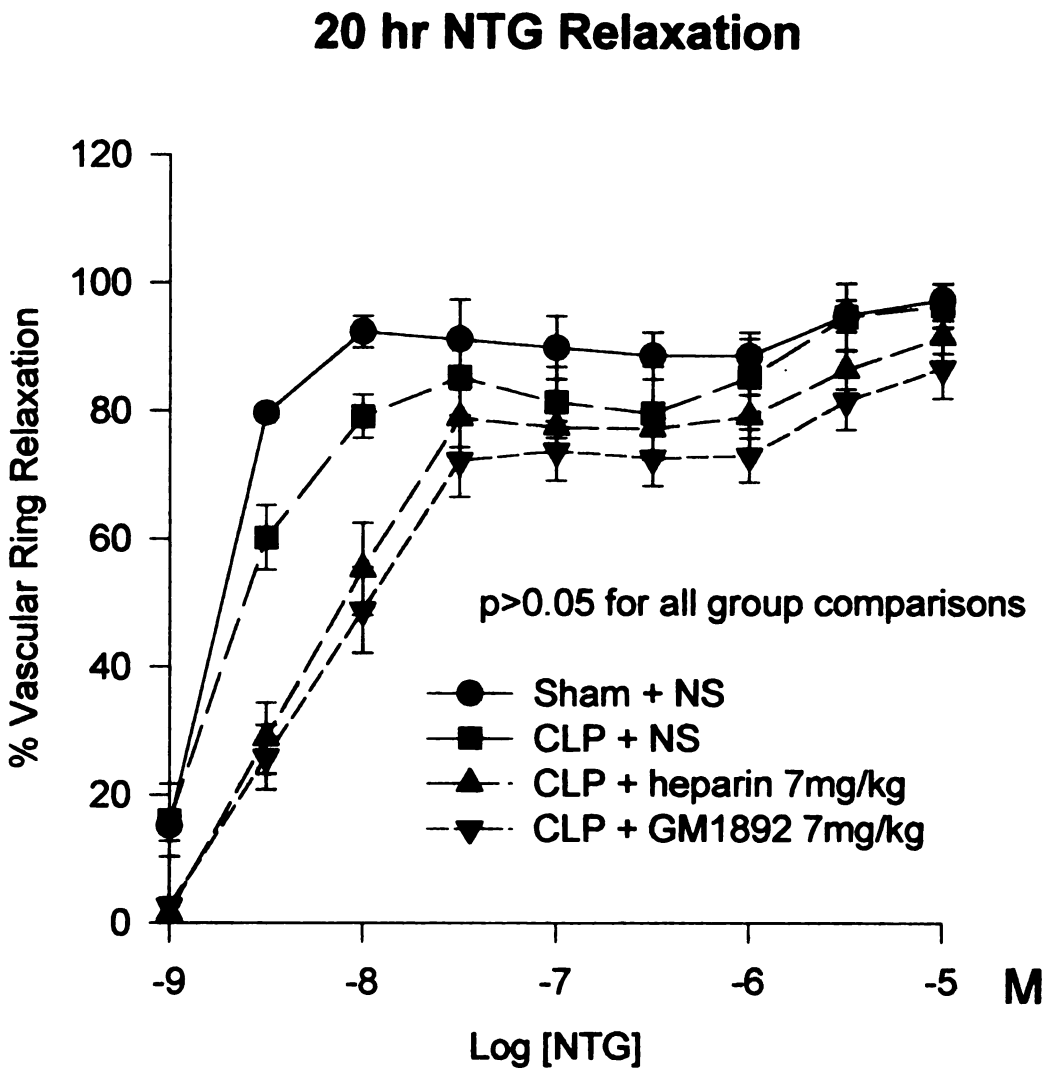

Figure 11. Cumulative dose-response relationship to nitroglycerin administration in aortic rings that were precontracted with $5x10^{-8}$ M of NE. Rings were harvested 20 hours post onset of sepsis. GM1892 and heparin at 7 mg/kg BW were given in treatment groups. There were only 2-3 animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the NE-induced precontracted state. Note: the NE precontraction dose differed from other blocks in the study and was changed thereafter due to excessive relaxation.

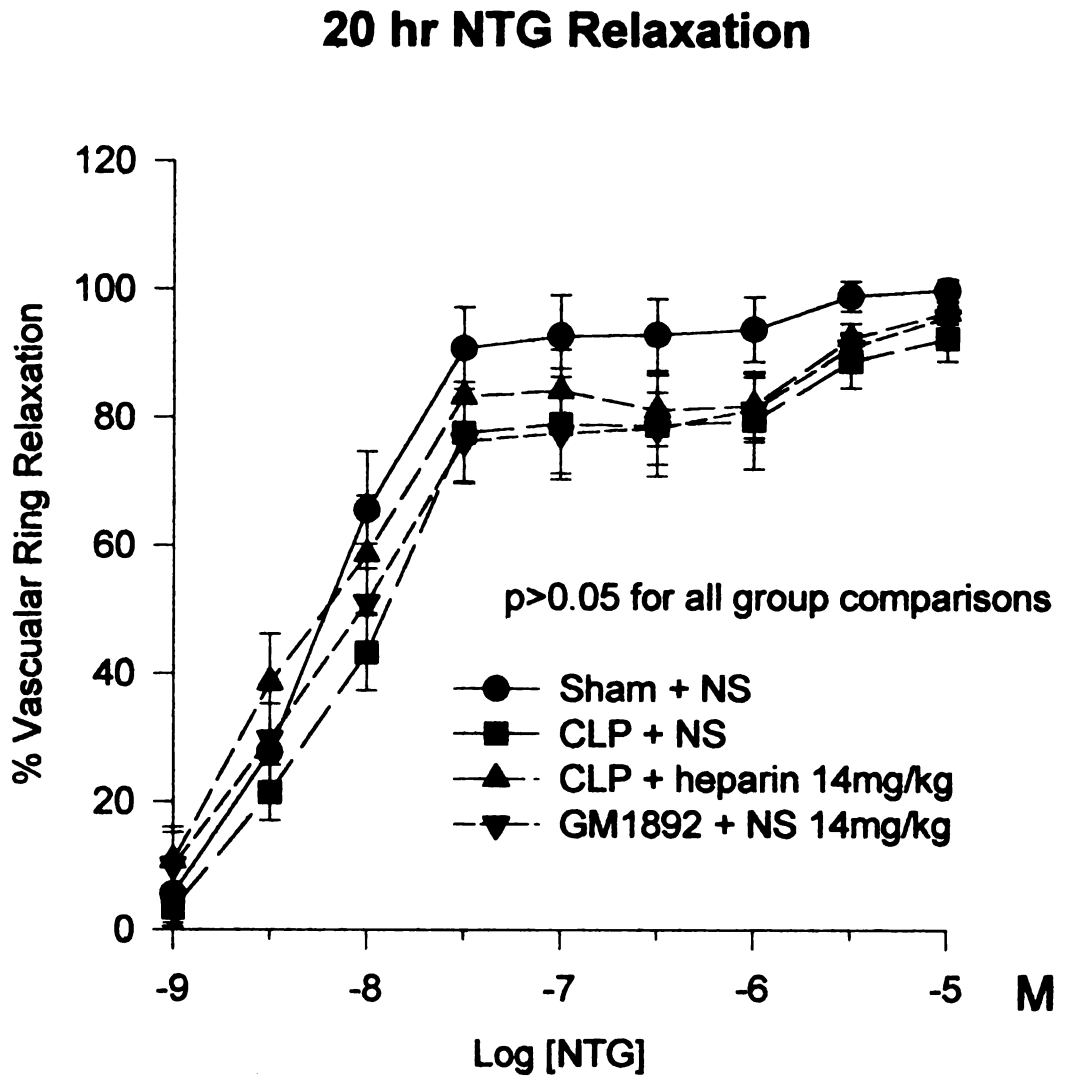

Figure 12. Cumulative dose-response relationship to nitroglycerin administration in aortic rings that were precontracted with $2x10^{-7}$ M of NE. Rings were harvested 20 hours post onset of sepsis. GM1892 and heparin were given at 14 mg/kg BW in treatment groups. There were six to eight animals in each group with one aortic ring per animal. The data is presented as the percent change in relaxation from a baseline at the NE-induced precontracted stated. Values are means +/- SEMs and compared by One-Way ANOVA and Student-Newman-Keuls Post-hoc test after Arcsin squareroot transformation to normalize percentages.

Urine Output:

The groups that were sacrificed and studied 5 hours post operation were found to have urine outputs as depicted in Figure 13. There was a significantly higher urine output in the sham + NS group than all other groups. The groups that were sacrificed and studied 20 hours post operation were found to have the following urine outputs as depicted in Figure 14. There was a significantly higher urine output in the sham + NS group than all other groups.

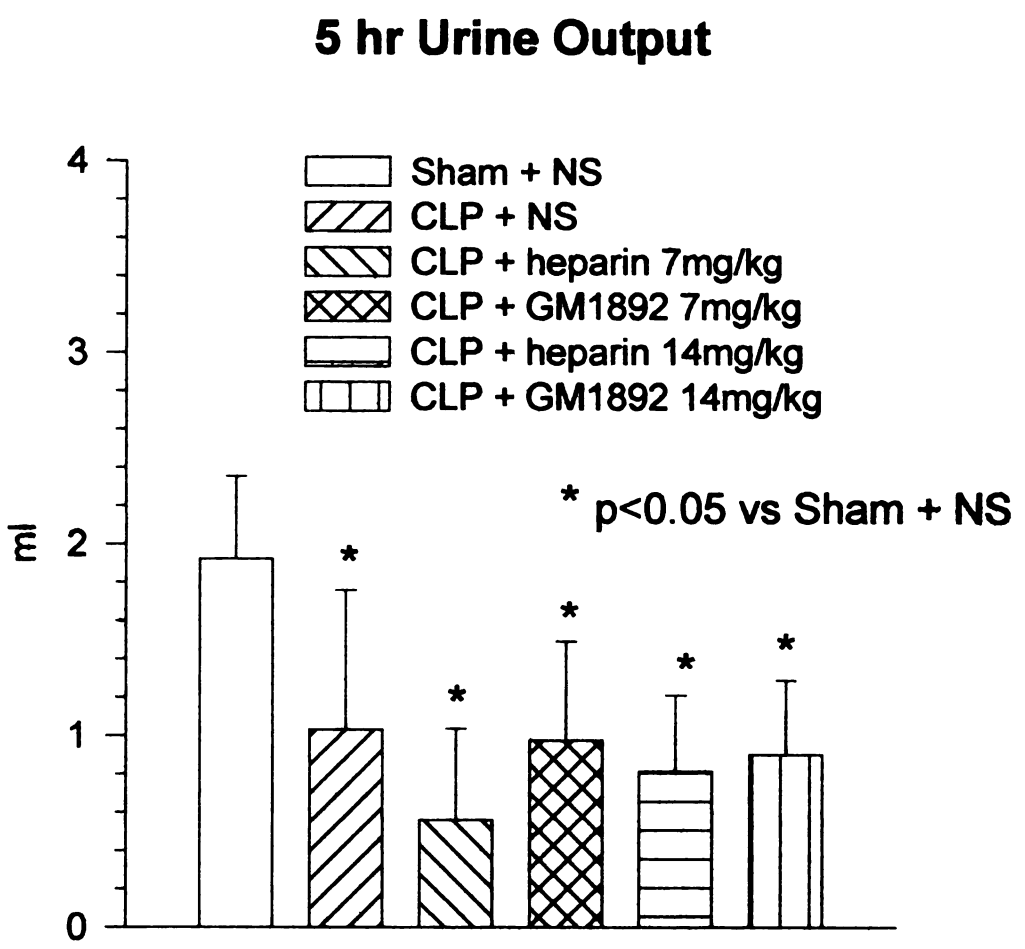

Figure 13. Total cumulative urine output over 5 hours post operation. Statistical analysis by One-Way ANOVA with Student-Neuman-Keuls Post-hoc test. All groups were significantly different from sham + NS. However, no other groups were significantly different.

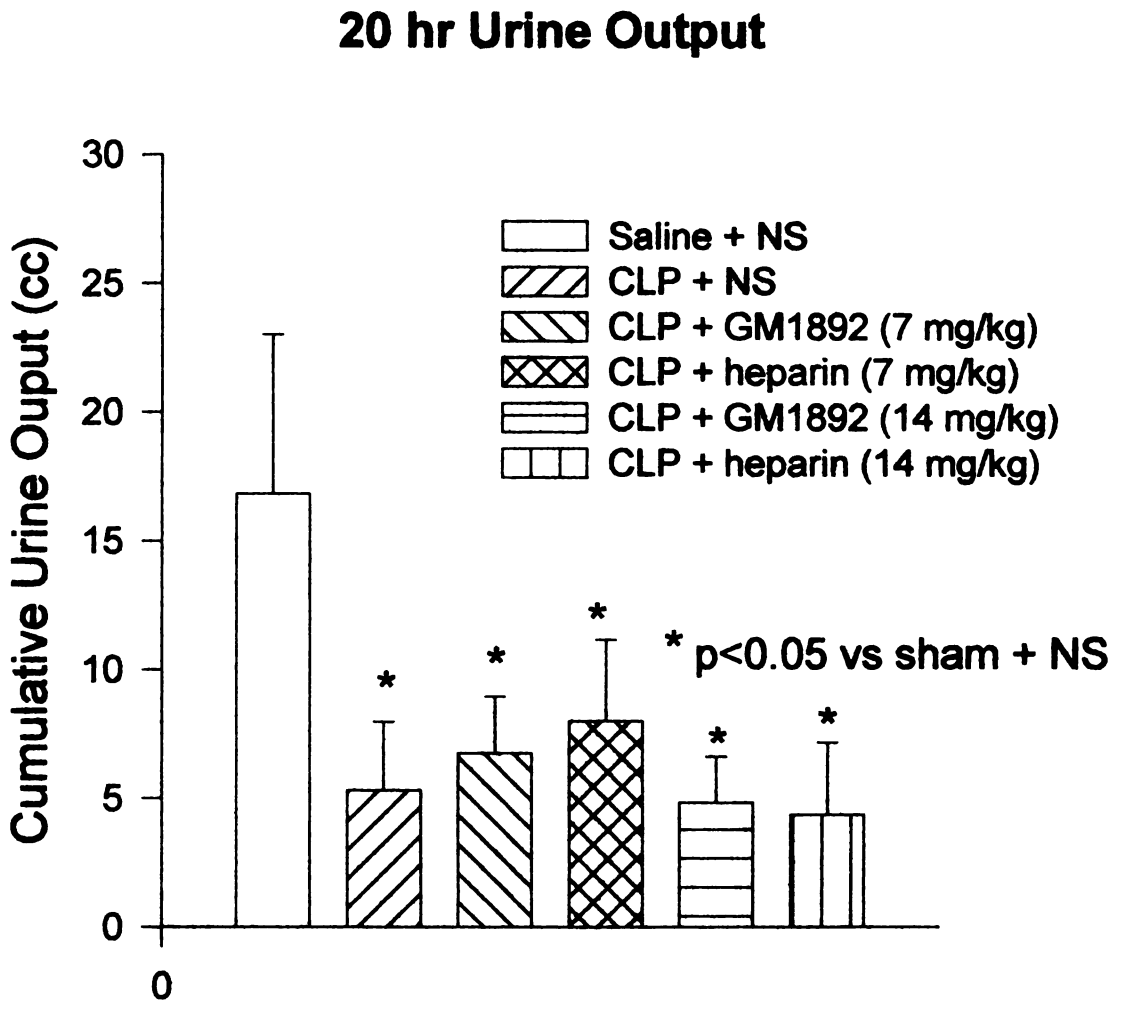

Figure 14. Total cumulative urine output over 20 hours post induction of sepsis. Statistical analysis by One-Way ANOVA with Student-Newman-Keuls Post-hoc test. All groups were significantly different from sham + NS. However, no other groups were significantly different.

Ring Weight:

The groups sacrificed at 5 hours after operation were found to have ring weights as depicted in Figures 15 and 16. There was no significant statistical differences between groups noted. The groups sacrificed at 20 hours after operation were found to have ring weights as depicted in Figures 17 and 18. There were no significant statistical differences between groups.

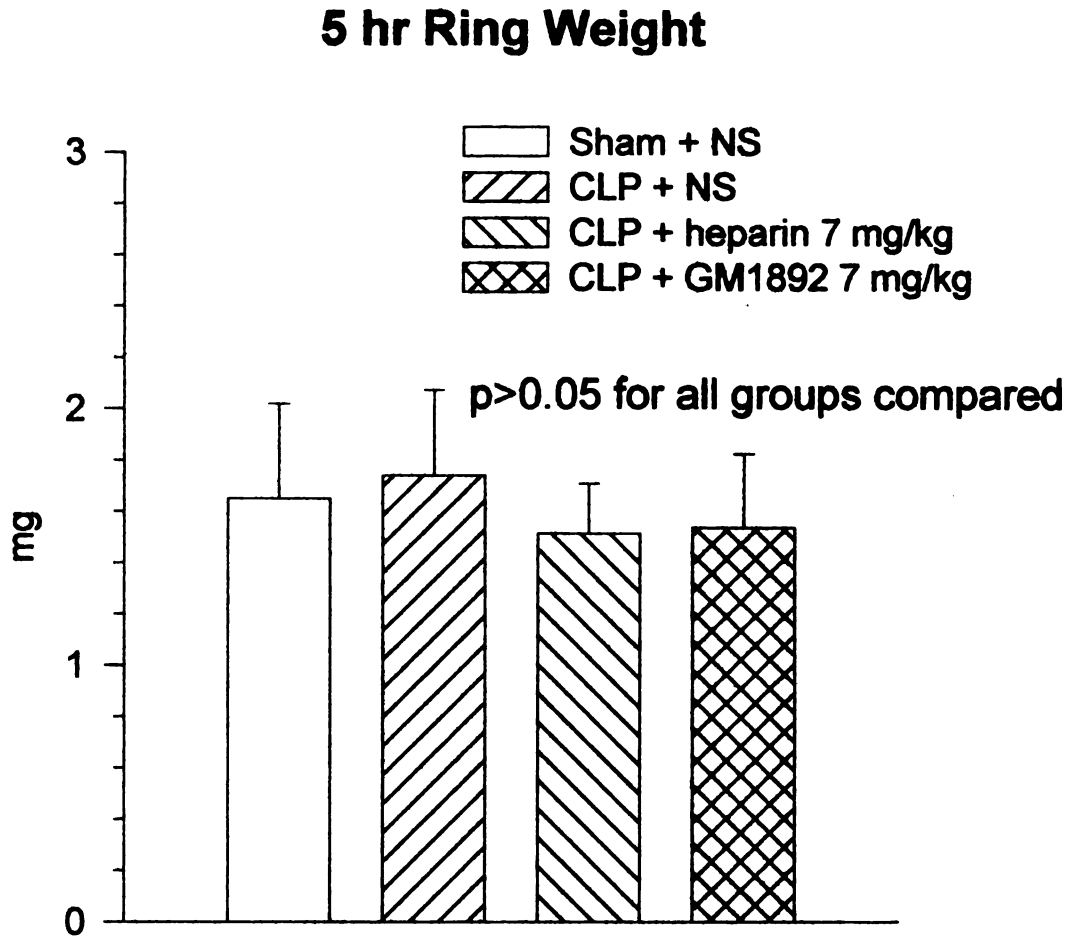

Figure 15. Ring weight in vessels harvested 5 hours after operation including GM1892 and heparin at 7 mg/kg BW dose. Statistical analysis was by One-Way ANOVA with Student-Newman-Keuls Post-hoc test. Rings were blotted once with tissue paper and weighed immediately after removal from waterbath.

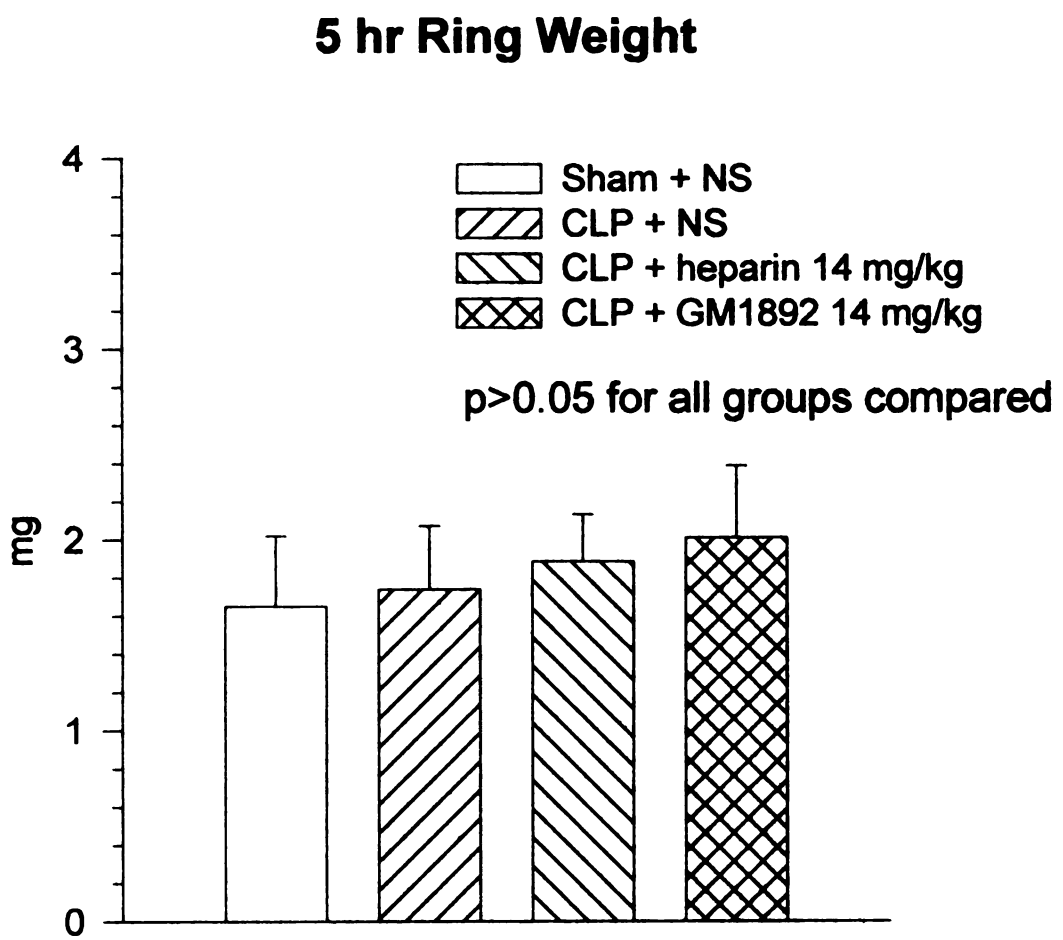

Figure 16. Ring weight in vessels harvested 5 hours after operation including GM1892 and heparin at 14 mg/kg BW dose. Statistical analysis was by One-Way ANOVA with Student-Newman-Keuls Post-hoc test. Rings were blotted once with tissue paper and weighed immediately after removal from waterbath.

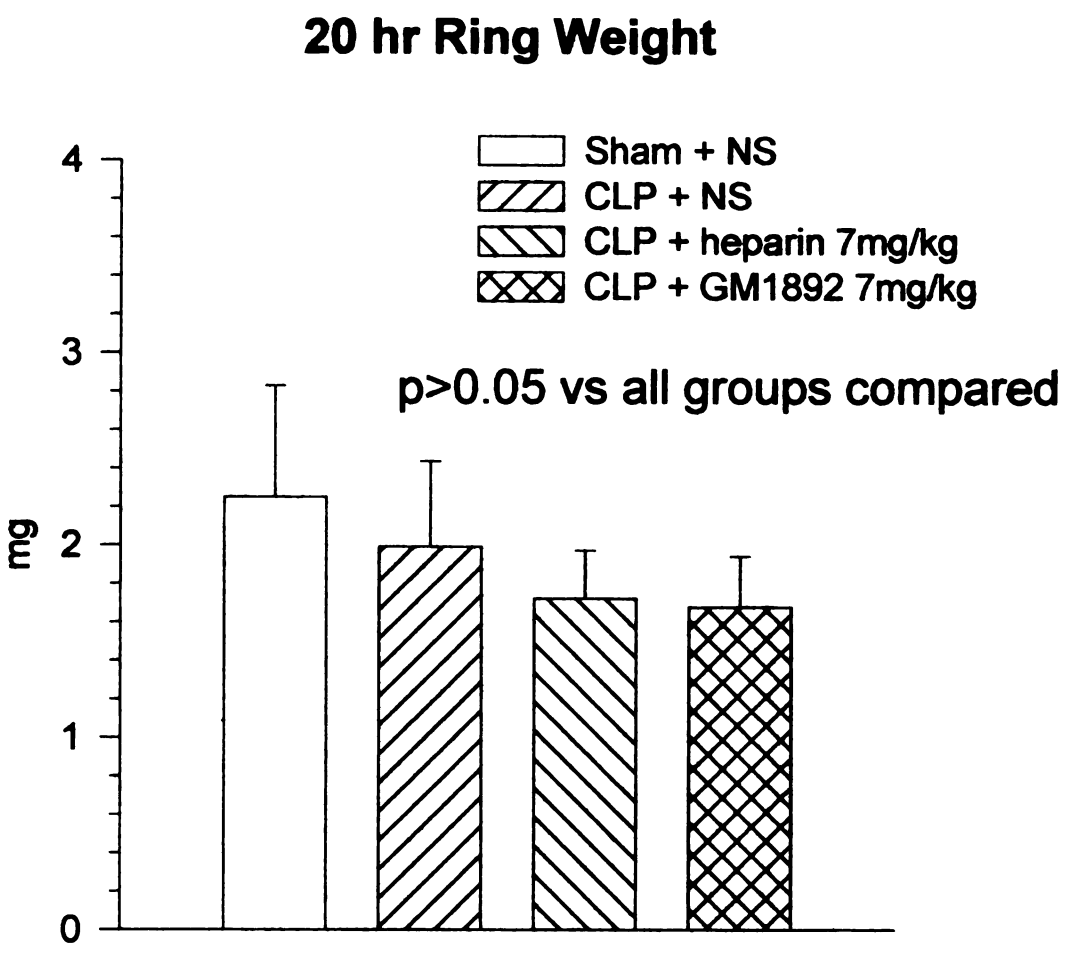

Figure 17. Ring weight in vessels harvested 20 hours after operation including heparin and GM1892 at 7 mg/kg BW dose. Statistical analysis was by One-Way ANOVA with Student-Newman-Keuls Post-hoc test. Rings were blotted once and weighed immediately after removal form waterbath with tissue paper.

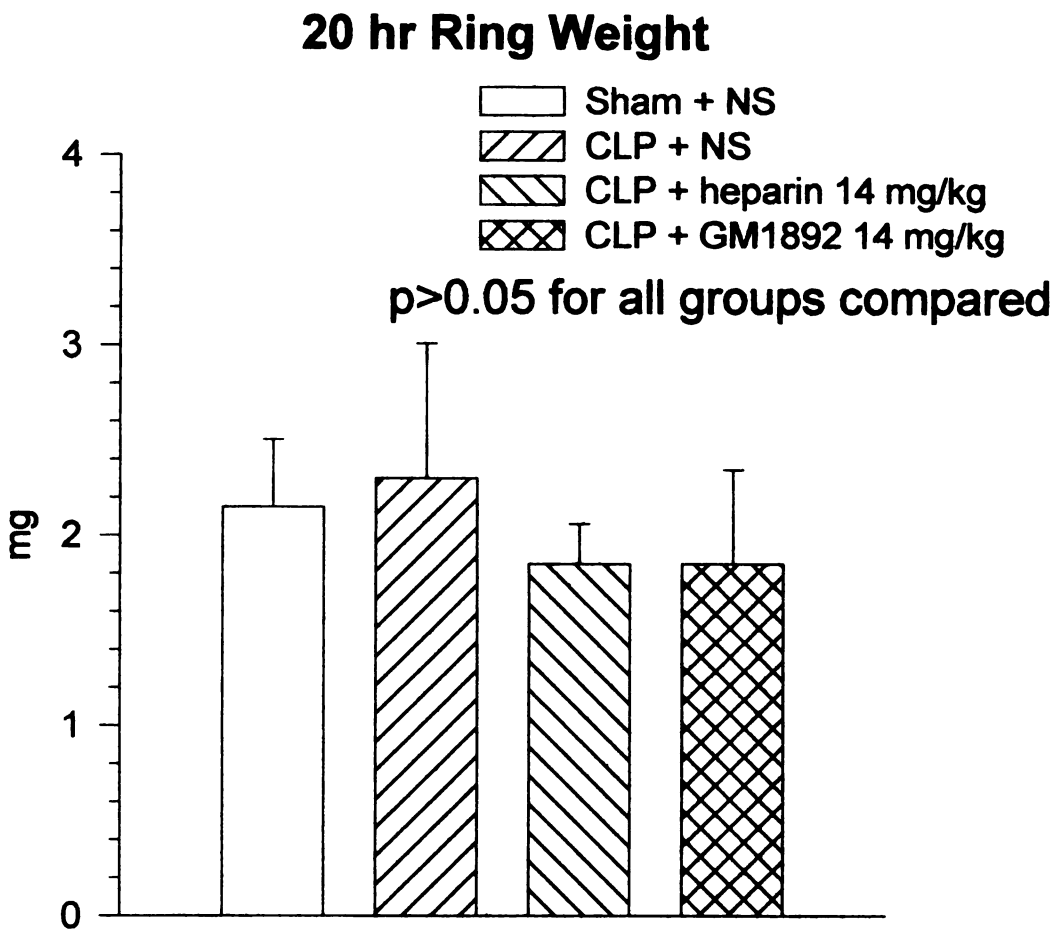

Figure 18. Ring weight in vessels harvested 20 hours after operation including heparin and GM1892 at 14 mg/kg BW dose. Statistical analysis was by One-Way ANOVA with Student-Newman-Keuls Post-hoc test. Rings were blotted once and weighed immediately after removal form waterbath with tissue paper.

DIC STUDY

Hematocrit:

There was a significant difference between the CLP + NS group and the sham + NS group, as the CLP + NS group was significantly decreased compared to the sham + NS group. There were no other significant differences. There was, however, a trend toward lower hematocrit counts in all the different CLP groups compared to the Sham + NS group. This is depicted in Figure 19.

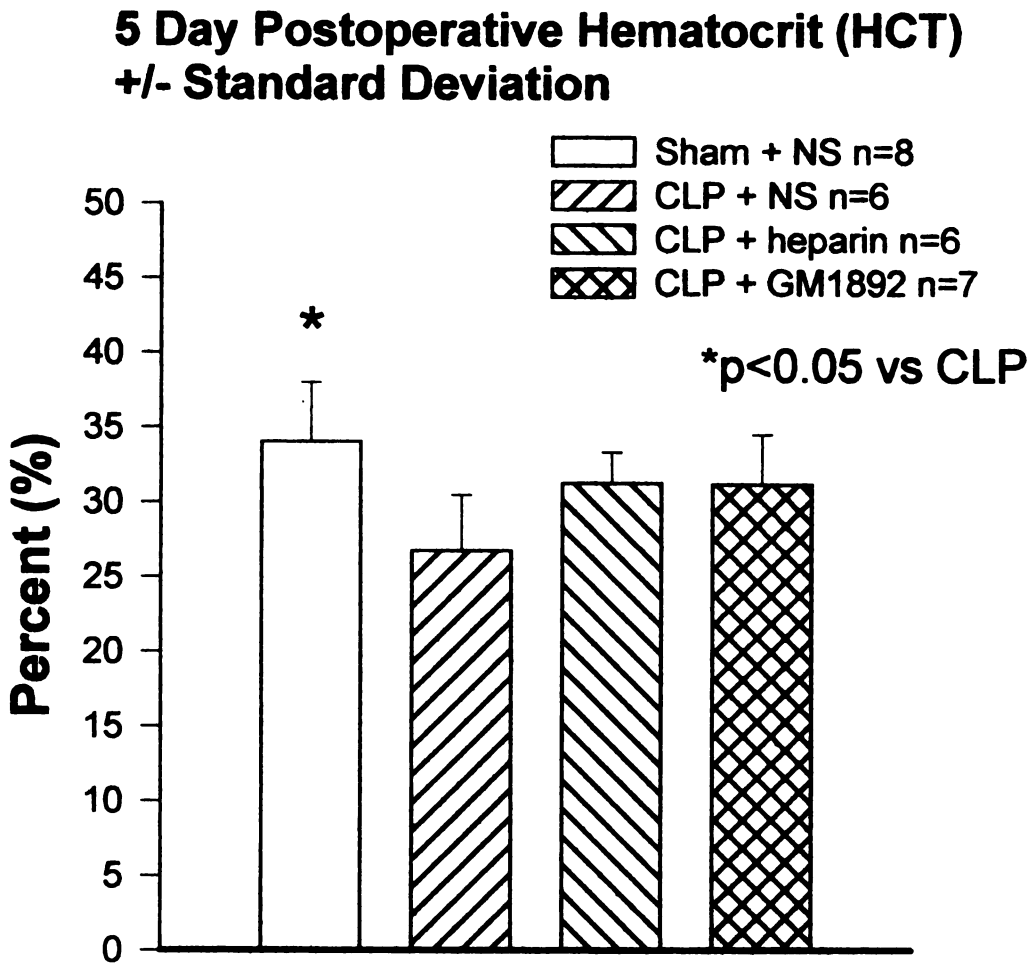

Figure 19. The hematocrit level is compared in all groups 5 days after operation and postsurgical continuous infusion of normal saline. Statistical analysis revealed the CLP + NS group had a significantly lower hemoglobin than did the sham + NS, but there were no other significant differences between groups noted. Means +/- the standard deviation were compared using One-Way ANOVA and the Student-Newman-Keuls Post-hoc test.

White blood cell count:

CLP + GM1892 was significantly greater than sham + NS. However, there were no other statistically significant differences. A great amount of variability was noted in the CLP + NS group that negated any significant difference even though a trend toward a lower WBC count was seen compared to the groups treated with GM1892 or heparin. This is depicted in Figure 20.

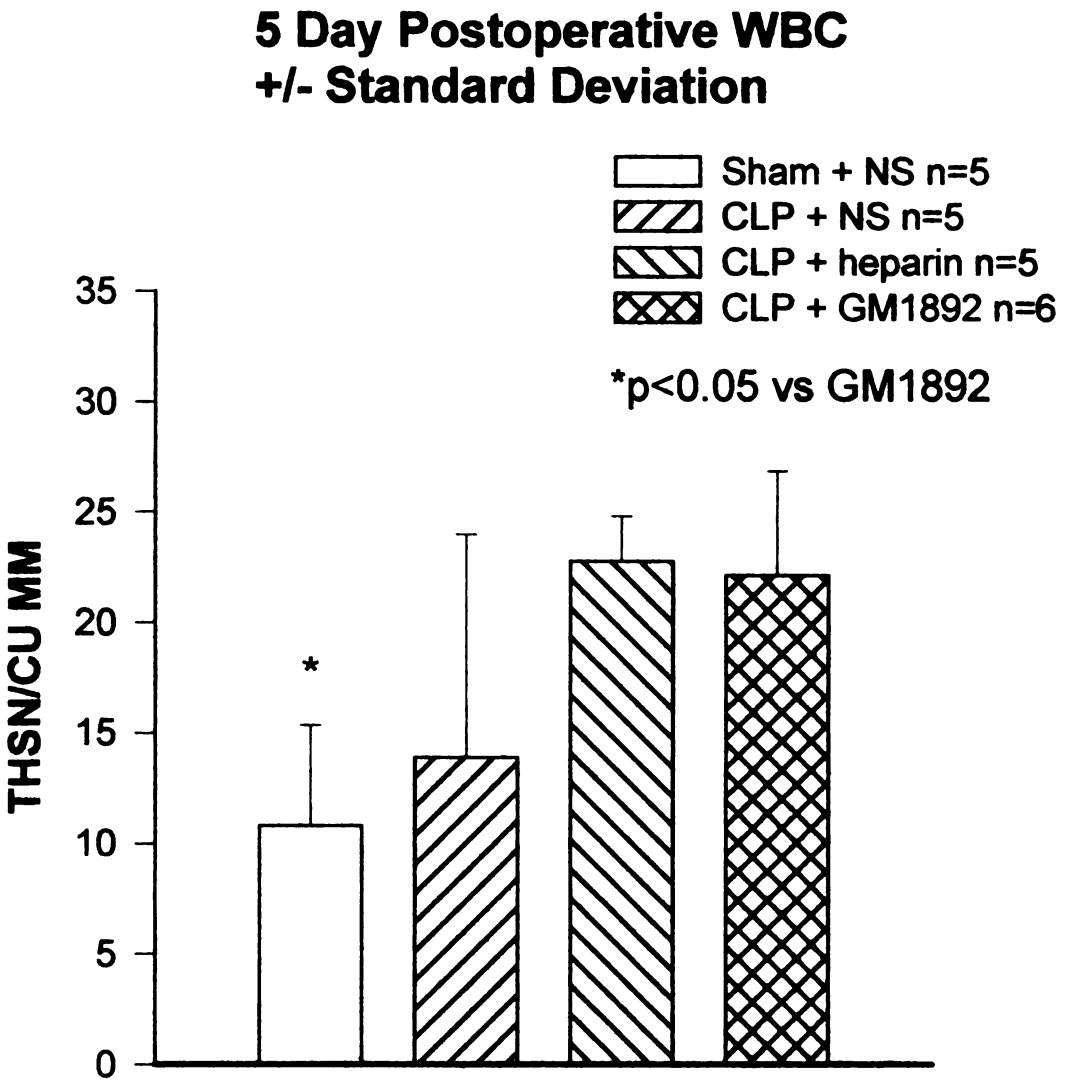

Figure 20. The white blood cell count is compared in all groups 5 days after operation and postsurgical continuous infusion of normal saline. Statisical anaylsis revealed that only the sham level was significantly lower than was the sham. No other groups differed significantly. Means +/- the standard deviation were compared using One-way ANOVA and the Student-Newman-Keuls Post-hoc test.

Platelet count:

Sham + NS, CLP + GM1892, CLP + heparin were all found to be significantly greater than CLP + NS. Furthermore, there was no significant statistical difference between the Sham + NS, CLP + GM1892 and CLP + heparin groups, and these groups were within normal limits of rat platelet levels. This is depicted in Figure 21.

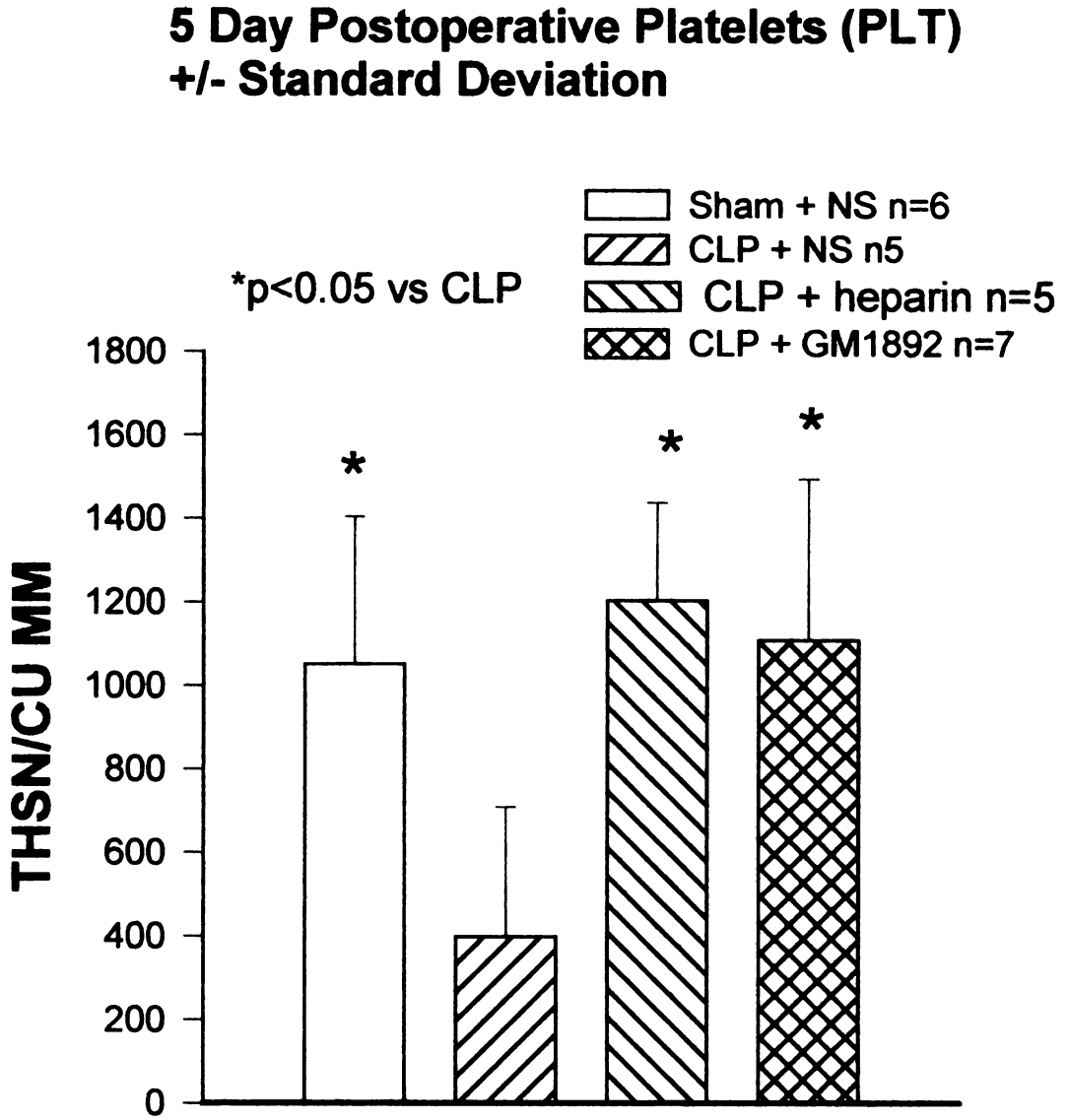

Figure 21. The platelet count is compared in all groups 5 days after operation and postsurgical continuous infusion of normal saline. Statistical analysis revealed that only the CLP + NS groups was significantly reduced compared to the treatment groups. Statistical analysis was performed by One-Way ANOVA and the Student-Newman-Keuls Post-hoc test.

Protime:

There was a significantly prolonged protime in the CLP + NS group compared to all other groups. The sham + NS, CLP + GM1892, and CLP + heparin groups were not significantly different from each other, and they were within ranges considered normal for rats. This is depicted in Figure 22.

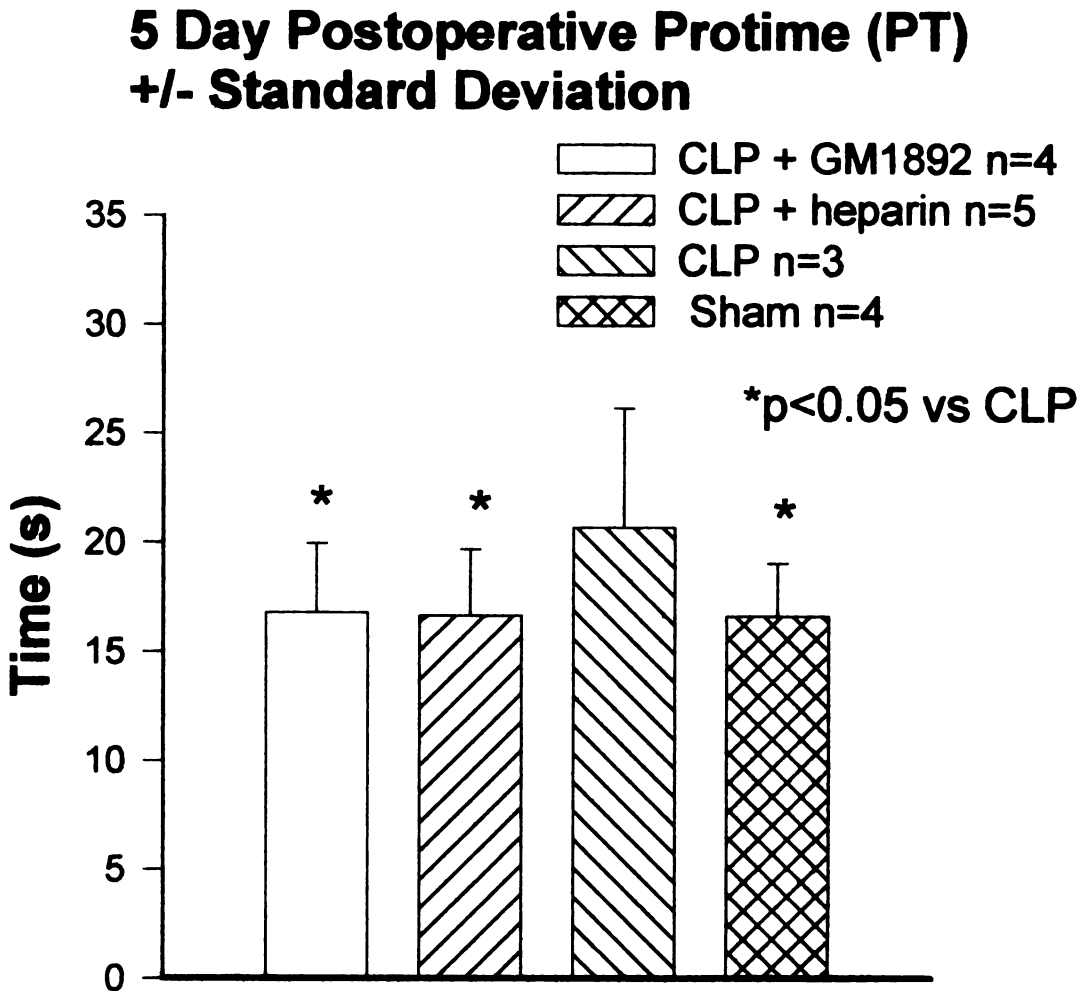

Figure 22. The protime is compared in all groups 5 days after operation. Statistical analysis revealed that all groups differed significantly from the CLP + NS group. No other significant differences amoung groups were noted. Statistical analysis was performed by One-Way ANOVA and the Student-Newman-Keuls Post-hoc test.

activated Partial Thromboplastin Time:

There was a significantly prolonged protime in the CLP + NS group compared to all other groups. The sham + NS, CLP + GM1892, and CLP + heparin groups were not significantly different from each other, and they were within ranges considered normal for rats. This is depicted in Figure 23.

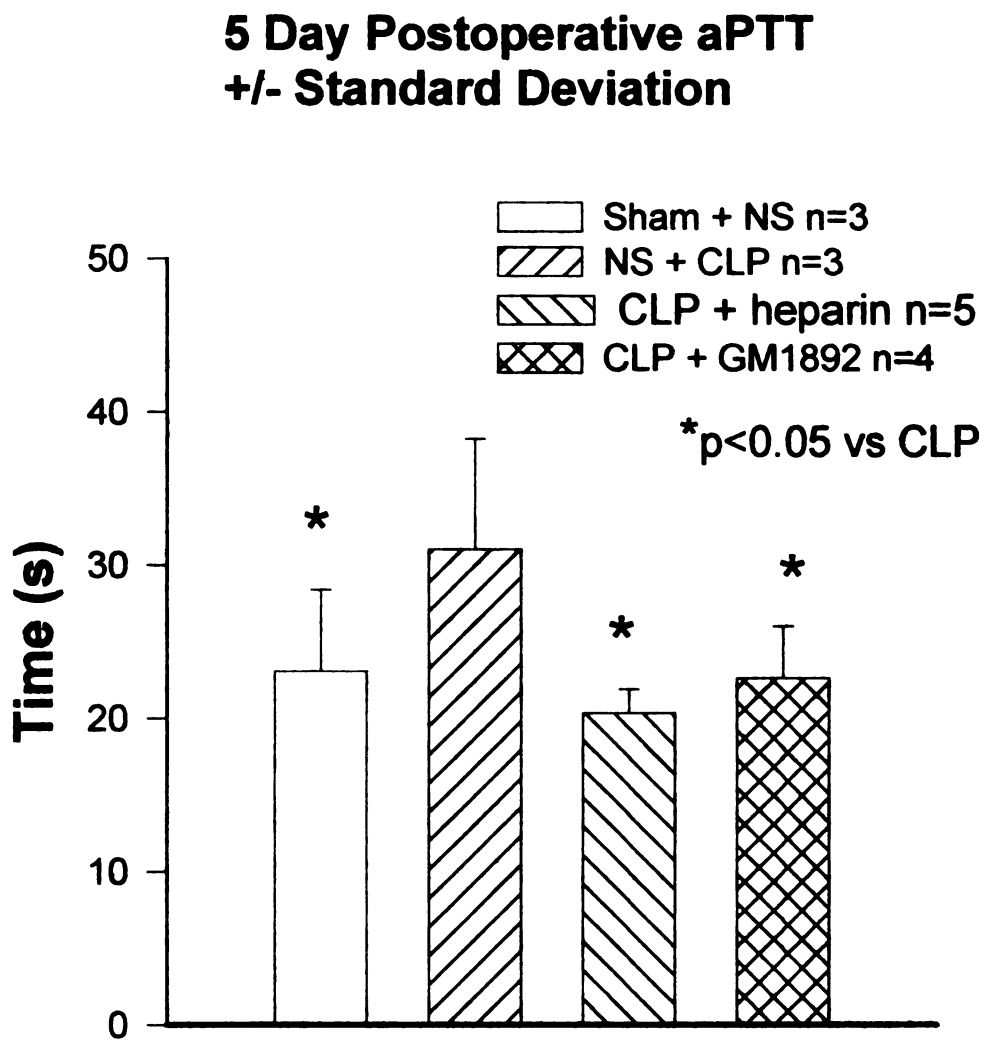

Figure 23. The protime is compared in all groups 5 days after operation. Statistical analysis revealed that the CLP + NS group mean aPTT was significantly prolonged and differed significantly from all other groups. No other significant differences among groups were noted. Statistical analysis was performed by One-Way ANOVA and the Student-Newman-Keuls Post-hoc test.

DISCUSSION

Acute and Chronic Models of Sepsis:

In this thesis, GM1892 was evaluated for its effectiveness in diminishing the sequellae of sepsis, including both vascular endothelial cell dysfunction and DIC. In order to do this, modifications of the CLP model that diminished the severity of sepsis were utilized to achieve optimal DIC parameters. Thus, two different models were used to study GM1892 effects during sepsis: an acute model of sepsis for EC study and a chronic model for study of DIC. The modifications incorporated for the DIC study included: continuous fluid resuscitation with about 1.5 times maintenance fluids and continuous administration of the treatment medications over 5 days. The traditional model of CLP-induced sepsis consists of fluid resuscitation with a one-time dose of 3ml/100 mg BW immediately after operation and results in a high mortality--usually greater than 50% after 24 hours.

These modifications were based upon a previous study (Yang and Hauptman, 1994), which showed that conventional heparin significantly reduced DIC parameters and improved survival. The advantage of using this model was the reduction in the uncertainty of the proper dosing of heparin in a chronic model

that would reasonably improve DIC without resulting in significant anticoagulation.

DIC, although shown to be present early in animal sepsis, takes more than a day to become clinically evident. Therefore, the model allows long-term survival for DIC to develop because the animals receive a continuous infiltration of NS at 3 ml/hr until sacrifice 5 days after operation. This rate used to allow for adequate volume resuscitation during the third spacing of fluid from peritonitis.

Acetylcholine Relaxation Response:

Our results have shown that at 5 and 20 hours after the onset of sepsis, there is a significant decrease in cNOS enzyme activity in the untreated septic group (see Figure 6). This is consistent with at least three other studies. First, work in our lab has shown that at 5 hours and later after onset of sepsis, cNOS is decreased (Wang, 1994a). Similarly, Parker et al. have shown that at 4 hours after onset of sepsis in guinea pigs, induced by administration of Escherichia coli endotoxin, there is a decrease in cNOS activity (Parker, 1994). Finally, another study found that E. coli endotoxin administration (4mg/kg) results in a decreased release of NO by cNOS in guinea pigs 16 hours after onset of sepsis (Myers, 1995). Furthermore, this study differentiated cNOS from iNOS, revealing that iNOS was not a factor and was similar in septic and control groups. In light of these findings, we evaluated

whether GM1892 and heparin were able to prevent sepsis-induced endothelial cell dysfunction and reduced cNOS activity.

To this end we demonstrated that GM1892 and heparin preserved cNOS activity at sham levels, 5 hours post sepsis, using a dose of 14mg/kg; however, there was no preservation of endothelial function at 5 hours post sepsis using 7mg/kg. Moreover, at 20 hours post sepsis, neither 7mg/kg nor 14 mg/kg was effective in preventing decreased NO production. Previous studies in our lab have shown that in a hemorrhage-shock model 7 mg/kg of conventional heparin is able to preserve cNOS activity.

The reason for this variation in effective doses in the different models is unclear. It may be that intrinsic differences between the models could account for this difference in outcomes. For instance, a greater amount of heparin degradation may occur in the sepsis model than in the hemorrhage model necessitating administration of more heparin to preserve endothelial cell function.

There is some indirect evidence for this supposition. In sepsis there is a higher incidence of disseminated intravascular coagulation (DIC) than in hemorrhage. For instance, one study found that 50% of 126 septic human patients developed significant signs and symptoms of DIC (Hernandez et al., 1989). Furthermore, rat and rabbit septic models have been shown to induce DIC so uniformly as to be utilized as DIC models (Yang and Hauptman, 1994).

DIC appears to result in significant endothelial cell damage by causing a vast amount of platelet cell degranulation and neutrophil activation. This leads to a profound amount of oxygen free-radical production and very extensive vascular endothelial cell damage--a greater amount than what initially occurs after the hemorrhage-shock model. Endothelial cell damage by inflammatory mediators that are released during sepsis has been shown to result in the leakage of intracellular contents. Indirect evidence for this process comes from a study showing that interleukin 1 or endotoxin increases the release of von Willebrand factor from human endothelial cells (Schorer, 1985). Furthermore, this study found that the release of von Willebrand factor was not associated with increased synthesis; although, it was associated with decreased intracellular levels. It is postulated this loss of von Willebrand factor from "leakage" may be the mechanism by which inflammatory mediators are able to cause focal areas of endothelium to be procoagulant and proinflammatory. It is not known whether the release of von Willebrand factor is a direct effect of inflammatory mediators or an indirect effect secondary to inflammatory mediator-induced cell damage.

Heparin administration results in a significant amount of heparin being taken up by endothelial cells. Thus, it may be that endothelial cells, sequestering heparin and von Willebrand's factor, may loose their sequestered heparin just

as they loose von Willebrand factor. This leads to the conclusion that a greater amount of heparin may be necessary in the septic model than in the hemorrhage model to maintain endothelial cell function.

Another similar possibility is that oxygen-free radicals may be the cause of the heparin dosage discrepancy present between the sepsis model and the hemorrhage model. Again, due to the extensive activation of platelets, it is possible that NO is being neutralized and inactivated by its interaction with free-radicals resulting in a quicker depletion of NO. Thus, again more heparin would be necessary to maintain NO levels consistent with those in the hemorrhage-shock model.

Even though high-dose GM1892 and conventional heparin were able to protect vascular endothelial cell function at 5 hours after onset of sepsis, there was no protection afforded at 20 hours in either low or high-dose treatment groups. The etiology for treatment ineffectiveness in late sepsis is uncertain, but some important considerations should be addressed that may explain this lack of therapeutic efficacy. First, there may be confounding variables interfering with the vascular ring studies at 20 hours that are not present in the 5 hour groups. For instance, Wurster et al. have shown that blood vessel rings harvested during early sepsis (10 hours) exhibited diminished ex-vivo contraction to NE and KCL that was restored by denudation of the endothelium; however, at 35 hours after sepsis this decreased responsiveness to NE and KCL

was not reversed by denudation (Wurster, 1994). They concluded that different mechanisms during early and late sepsis were responsible for this effect and that at 35 hours direct vascular smooth muscle cell dysfunction may have been present. No groups were evaluated at 20 hours after sepsis. Thus, in our study, although speculative, this same effect could have interfered with normal precontraction, altering the effects of acetylcholine-induced relaxation. This may have diminished the magnitude of the difference noted between treated and untreated septic animals enough to diminish any statistically significant differences.

It is however, also very likely that the ability of GM1892 and conventional heparin to maintain vascular endothelial cell function was diminished at 20 hours because of the greater amount of tissue damage that occurs at 20 hours (compared to 5 hours) of sepsis resulting in greater endothelial cell dysfunction. Many have begun to believe that the effective treatment of sepsis must include definitive intervention during the early stages of sepsis, otherwise an inevitable progression toward tissue damage, cell death and ultimate demise will occur. Thus, even though GM1892 and conventional heparin are capable of preserving endothelial cell function initially, it may be that during late sepsis a greater amount of cell damage occurs than these agents are capable of attenuating. In effect, the irrevocable progressive nature of sepsis, in which no one drug has proven a cure for the

syndrome, is evidence to this possibility. For example, in late sepsis multi-system organ failure occurs and is associated with DIC and a large amount of vascular endothelial cell damage and destruction, which leads to progressive organ involvement.

Specifically, Wang has shown (unpublished observation) that heparin treated septic rats had essentially normal endothelial cell histology using electron microscopy to evaluate the morphological changes that occur after sepsis with and without heparin treatment. However, in the untreated animals, significant histologic changes showing significant damage was present.

Norepinephrine Relaxation Response:

In our study we also evaluated the ability of the vascular rings to contract in response to norepinephrine at 5 and 20 hours after the onset of sepsis. We recorded the contraction in terms of tension (mg tension). The results showed no significant difference between any of the groups, including shams and CLPs, at low dose and high dose treatment at 5 or 20 hours. This is in conflict with two previous studies: first, Wurster et al. showed there was a significant reduction in the norepinephrine-induced contraction in endothelium-intact vascular rings in CLP groups at both 10 and 35 hours (Wurster, 1994). Similarly, another study found that NE-induced contraction in vascular rings was diminished after sepsis compared to shams (McKenna, 1986). There is no obvious

explanation for this discrepancy in results. This discrepancy is difficult to understand in that virtually the same methods and equipment were utilized in both this study and in the study by Wurster et al. It will probably not be explained until further experimentation reveals which outcome is reproducible and clarifies these inconsistencies.

Nitroglycerin induced contraction:

Concerning the nitroglycerin findings, it should be noted that the 7 mg/kg study groups at 20 hours was completed under a different dosing regimen than were all the other groups. Early during experimentation, NTG-induced relaxation of the norepinephrine precontracted rings was inadequate. Thus, a smaller dose of norepinephrine (5×10^{-8}) was used to precontract the rings, which resulted in a greater amount of relaxation than was desired, which caused an inadequate dosing-response curve to be formed for adequate evaluation of the groups. Thus, these results have a different shape than the other NTG relaxation curves and a greater amount of variation.

Another difficulty which persisted throughout the entire ring study was the lack of consistency in results. There are many sources of variation to be considered. First, the variability in the stock solutions was a dilemma. It appeared to be evident to this researcher that the potency of the NE varied with each batch. The problem was eliminated by the production of a quantity sufficient to be stored and frozen

for all the animals in the study. This was adhered to during the greater portion of the experiment upon the realization of this phenomenon. Second, the vascular ring baths are not completely amenable to obtaining completely consistent results. For instance, the baths were marked at the level where 20 ml of Kreb's solution would be placed into the chamber. However, this was by gross visual determination only. Thus, the ability to accurately place the necessary amount for 20 ml was limited. Furthermore, the clips on the tubing leading to the drainage system were inevitably shifted slightly with each use. This again made the chamber 20 ml mark less accurate as the greater volume in the tubing included at varying lengths made the volume determination less certain. Another source of inconsistency was the pressure transducers and the Grass polygraph. There was a significant amount of baseline drift that shifted over time resulting in difficulties with consistency. All these variables resulted in variation which made it more difficult to show true between group variation and eliminate experimental variation to the degree necessary to show significant between groups differences.

DIC Study Discussion:

In this experiment we were able to demonstrate a significant difference between the CLP group without treatment and the CLP groups with treatment in regards to the platelet count, protime, and activated partial thromboplastin time.

This is consistent with the findings of other experiments (Yang and Hauptman, 1994). This study shows that DIC was attenuated by the use of GM1892 and conventional heparin. Unfortunately, based on these results one cannot make an absolutely definitive diagnosis of DIC, although the results are highly suggestive of this. This is because decreases in the PT, aPTT, and platelets are consistent with both primary and secondary hemolysis. If this were a primary hemolysis, then plasminogen activator could provoke the same clinical picture. Unfortunately, the fibrinogen level and fibrin split products assays were not felt to be accurate based on preliminary findings. These inconsistent results were never able to be eliminated from the assays, and these assays were therefore not included in the final results. Furthermore, the only true assay to differentiate primary from secondary hemolysis is the D-dimer test (Bick and Baker, 1991). The D-dimer is the cross linked antithrombin III to activated factor XIII during DIC and was found to be 93.7% sensitive and was found to be positive in less than 20% of those who had other coagulatory disorders but not DIC. Unfortunately, the D-dimer test is an antibody test that lacks specific homology for rats resulting in the incompatibility of the test on rats.

Thus, while our results suggest that DIC was attenuated, further studies are necessary before a definite conclusion can be made. For instance, refinements in the performance of FSP and fibrinogen test may allow their utilization since others have previously performed these tests on rat serum.

Further preliminary studies to familiarize oneself with the fibrin split products test via Staphylococcus clumping factor method should to be performed to ensure uniform results, as it is a visual assay graded into discrete categories which requires practice to perform accurately. Also, fibrinogen levels have been accurately obtained before should be reproducible.

SUMMARY AND CONCLUSIONS

Alpha-agonist induced vascular ring contraction with NE showed there is no significant difference between any of the groups studied within blocks regarding contractility and that sepsis did not diminish the ability of the vascular smooth muscle to contract compared to sham animals at 5 or 20 hours post sepsis.

Our results did demonstrate that at 5 hours after onset of sepsis with CLP there is vascular endothelial cell dysfunction in which EDRF is reduced compared to non-septic sham animals. However, at 20 hours there were inconsistent findings: one of the two blocked studies found reduced EDRF in untreated septic animals compared to the sham group, while the other study did not.

When given 1 hour after the onset of sepsis, treatment of septic animals with high dose (14mg/kg BW) GM1892 and heparin maintained EDRF at sham group levels when sacrificed 5 hours after the onset of sepsis. Low dose treatment (7mg/kg BW) with GM1892 and heparin did not maintain EDRF at sham group levels at 5 hours after sepsis. In the 20 hour study block that showed sepsis-induced reduction in EDRF compared to sham animals, neither low dose or high dose treatment maintained EDRF at sham group levels.

Vascular endothelial independent relaxation with NTG in endothelial rings harvested 5 and 20 hours after the onset of sepsis revealed no intrinsic smooth muscle dysfunction present in treated and untreated septic animals compared to shams as all groups had similar responses.

The DIC study showed that GM1892 and conventional heparin attenuated DIC parameters compared to untreated septic animals, including the maintenance of platelets, normal protimes and activated partial thromboplastin times.

In conclusion, GM1892 is able to maintain vascular endothelial cell function after early sepsis when given at high doses. It also diminishes DIC parameters in chronically septic rats. It appears to be acting without significant anti-coagulant effects and therefore, conventional heparin also must be acting to preserve endothelial cell function by mechanisms other than just its anti-coagulatory properties. Further evaluation of GM1892's ability to stimulate EDRF should be evaluated to confirm its effects, such as endothelial cell culture studies. Further studies to determine GM1892's mechanism of action, such as its ability to stimulate cell proliferation, should be evaluated too. Finally, survival studies to determine if GM1892 improves mortality should be performed to determine if its specific effects on endothelial cells affect overall survival as does conventional heparin.

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