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PERSISTENT BENEFICIAL EFFECTS OF A NOVEL NONANTI-COAGULANT HEPARIN (GM 1892) ON CARDIOVASCULAR AND HEPATIC FUNCTIONS AFTER TRAUMA-HEMORRHAGE AND RESUSCITATION

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John Paul Kepros

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PERSISTENT BENEFICIAL EFFECTS OF A NOVEL NONANTICOAGULANT HEPARIN (GM1892) ON CARDIOVASCULAR AND HEPATIC FUNCTIONS AFTER TRAUMA-HEMORRHAGE AND RESUSCITATION

By

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ABSTRACT

PERSISTENT BENEFICIAL EFFECTS OF A NOVEL NONANTICOAGULANT HEPARIN (GM1892) ON CARDIOVASCULAR AND HEPATIC FUNCTIONS AFTER TRAUMA-HEMORRHAGE AND RESUSCITATION

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Although a nonanticoagulant heparin, GM1892, improves cardiovascular and hepatocellular functions immediately after hemorrhage and resuscitation, it is not known whether this effect persists. To study this, rats underwent trauma and hemorrhage. The rats were then resuscitated with crystalloid plus GM1892, conventional heparin, or normal saline. At 24 hours after resuscitation, cardiac index, stroke volume, and total peripheral resistance were determined. SGOT, liver water content and hepatic microvascular blood flow were also determined. TNF, IL-6 and PGE_2 were measured to investigate the mechanism of the action of GM1892.

The results indicate that cardiovascular function remains depressed at 24 hours after resuscitation. Treatment with GM1892 or conventional heparin, however, significantly improves this function and reduces hepatic damage and edema. Furthermore, the mechanism of the beneficial effects of GM1892 appears to be downregulation of proinflammatory cytokine (TNF, IL-6) production. Thus, GM1892, which, unlike conventional heparin, has no significant anticoagulant activity, appears to be useful for maintaining cardiovascular function and hepatic integrity following trauma-hemorrhage and resuscitation.

ACKNOWLEDGMENTS

I would like to acknowledge the hard work and help Irshad Chaudry, Ping Wang, Alfred Ayala, Rao Kareti, Mark Gorman, and Jim Harkema. I would also like to express my thanks and appreciation to Mary Morrison, Zheng F. Ba and the other members of the laboratory team without whose help this would not have been possible to complete. Finally, I would also like to express my thanks to Michele Hurrell for her patience throughout the writing of this thesis.

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INTRODUCTION

Historically, steady improvement has been made in the treatment of trauma patients. Primarily this has been in clarifying the problem (Simeone, 1963) and specifically in areas of fluid resuscitation (Shires, 1961), systematic management (Committee on Trauma, 1989), and nutrition (Dudrick, 1968). More recently, increased understanding of soluble mediators and improved animal models of hemorrhagic shock have led toward better understanding of shock at a cellular level including its effect of the immune system (Chaudry, 1993a). Along with this, corresponding areas of potential pharmacologic intervention have emerged (Harkema, 1992). This thesis will discuss the history of shock including the treatment and recent investigation of soluble mediators up to the present time. This will be followed by a study of the effects of a potential new therapeutic agent for the treatment of trauma and hemorrhagic shock.

HEMORRHAGIC SHOCK

History

Shock was first recognized in the early 1700's and the term *shock* was first used in 1743. Shock has also been referred to as a "rude unhinging of the machinery of life", and "a momentary pause in the act of death". Most of the observations on injured patients have been during wars and on the battlefield. Curiously, many illnesses including some war wounds were treated by phlebotomy. It was in 1872 that Samuel D. Gross first postulated that shock was a reaction to major injury (Sabiston, 1991).

Laboratory investigation of shock in the late 1800's showed that the likelihood of shock increased with the severity of the injury and prior hemorrhage impaired the ability of an animal to survive an injury (Sabiston, 1991). Experimental animals were found to have a decreased central venous pressure and administration of intravenous saline improved survival. The importance of the reduction of tissue damage first became evident during World War I, when splinting of femur fractures reduced the mortality from 70% to 10% for this injury. After World War I it was hypothesized that the systemic effects of shock were caused by toxic products from tissue damage. Further research showed that one of the toxic factors, the hydrogen ion was found to be responsible for metabolic acidemia and cardiovascular failure. This was countered by an experiment which showed internal hemorrhage would account for intravascular volume loss (Blalock, 1930); (Blalock, 1934).

During World War II, a dog model was used by Wiggers in the investigation of hemorrhagic shock and led to the development of the concept of irreversible shock (Wiggers, 1945). It was found that after a period of constant hypotension, the animal would decompensate and reinfusion of shed blood would not be sufficient to resuscitate the animal. Shires found that infusing large volumes of intravenous fluid would prevent renal failure and that this was superior to plasma or blood alone (Shires, 1964). It was

then discovered that an interstitital fluid deficit was responsible for the large amounts of fluid required for resuscitation. During this time period, a large amount of research was also performed by Moore on the effects of hemorrhage (Moore, 1965). On a cellular level, it was discovered that the cell membrane function changes resulting in movement of sodium and water into the cell. Attempts were made to localize the energy pathway in shock and the site of breakdown (Schumer, 1968). This method of aggressive fluid resuscitation resulted in many patients surviving an initial severe injury during the Vietnam conflict. The patients then developed a respiratory distress syndrome secondary to an interstitial edema. The invention of the pulmonary artery catheter revealed that this interstitial edema was secondary to an increased vascular permeability and not by high pulmonary artery pressures. Experiments showed that excluding one lung of an animal from circulation prevented damage and supported the theory of toxic factor involvement in the effects of hemorrhagic shock.

More recently, shock has been related to alterations in immune function which can result in sepsis (Abraham, 1991). It has also been hypothesized that the irreversibility seen in hemorrhagic shock may be caused by sepsis (Rush, 1989). Inflammatory cytokines are increased in shock (Abraham, 1988) and contribute to the immune depression. Translocation of bacteria from the intestine has also been thought to play a role in sepsis resulting from hemorrhagic shock (Alexander, 1990). A hypothesis for the mechanism of increased susceptibility to sepsis has been formulated using current information on shock and mediators (Ayala, 1994). With better ventilatory support, more patients would survive ARDS and were found to have failure of multiple organ systems (Baue, 1975). Multisystem organ failure has become the most feared result of circulating mediators in hemorrhagic shock (Anderson, 1990). Some evidence has been found pointing toward the intestine as the source of cytokines in hemorrhagic shock (Deitch, 1994). The gut has even been considered a central organ in the pathogenesis of hemorrhagic shock (Wilmore, 1988). The characteristics of different shock states are very similar and a common

pathophysiologic mechanism of generalized endothelial cell activation has been termed *systemic inflammatory response syndrome* (Bone, 1992).

Different animal models have been used to study the contribution of toxic factors to the effects of hemorrhagic shock (Bacalzo, 1971). Initially, fixed volume models were used in which a fixed volume of blood was removed from the animal to produce hemorrhagic shock. A major drawbacks of this model is that the amount of blood removed depends on the hydration state of the animal. Attempts to develop a fixed volume animal model with an untreated mortality of approximately 50% have been difficult (Collins, 1969). Anesthesia also causes problems in animals models because most anesthetic agents depress cardiac function to some degree and also decrease the metabolic demands of the body thereby altering the response to hemorrhage. Because of the beneficial effect of heparin on animals undergoing hemorrhage, the ideal model would also be nonheparinized. Cardiac output has been measured with radiolabeled microspheres, thermodilution, electromagnetic flowmetry, and Fick oxygen method. Microspheres require blood sampling and provide only a limited, interrupted measurement of cardiac output. Thermodilution is difficult to perform accurately in small animals such as the rat because of the difficulty in controlling the temperature of the injectate. Electromagnetic flowmetry requires thoracotomy. Indocyanine green measurement with a fiberoptic catheter allow repeated accurate measurements of cardiac output without blood sampling and also allows measurements of hepatocellular function. For experimental purposes trauma can be broken down into two separate events, the initial tissue trauma, and hemorrhage. Many animal models have been described throughout the history of the study of hemorrhagic shock (Chaudry, 1993b). Because heparin maintains microvascular patency during and after hemorrhage, it is recommended that a clinically relevant model not be heparinized (Rana, 1992). Taking into account reproducibility, predictability, and expense, one animal model in particular, a non-heparinized, non-anesthetized, fixed pressure rat model has been developed and used extensively (Chaudry, 1989). With this

model it has been possible to assess changes in cardiac output, hepatocellular function, and circulating blood volume successfully as sensitive indicators of the pathological effects on organs and homeostasis during as well as following the onset of hemorrhage (Wang, 1991a). A nonheparinized model is used in the present study because of the reported beneficial effects of heparin on animals which have undergone hemorrhagic shock (Rana, 1992).

Events of Hemorrhagic Shock

Hemorrhagic shock results from a loss of circulating blood volume which leads to decreased perfusion of the nonvital tissues and vital organs. Anaerobic metabolism soon follows from decreased perfusion of the large nonvital tissue cell mass. Hormonal mediators such as catecholamines are secreted and baroreceptors are stimulated producing increased peripheral resistance.

The cellular response to hypoperfusion results in anoxia and cellular starvation. Subsequently, anoxia limits the amount of substrate available to the cell. Severe and prolonged hemorrhage produces a cascade of events which can ultimately lead to increased susceptibility to sepsis, multiorgan failure and death. The mechanism for the immune suppression is felt to be multifactorial and includes early systemic mediators, decreased ATP levels, Ca^{++} alterations, and inflammatory mediator release (Chaudry, 1993a). The immediate hemorrhage is closely followed by regional hypoxia and alteration in cellular functions. Directly or indirectly this results in the increased release of tumor necrosis factor (TNF) from macrophages and this is followed by increased PGE_2 synthesis. PGE_2 depresses macrophage and lymphocyte functions finally resulting in increased susceptibilty to sepsis (Chaudry, 1993a). Interleukin-6 is also elevated during shock and may be useful as a marker of severity of shock (Damas, 1992).

Tumor necrosis factor in large doses has been found to reproduce the clinical syndrome observed in shock (Tracey, 1986) as well as being an important mediator of the

inflammatory response cascade (Harkema, 1992). Prostaglandin E_2 has been shown to have important properties of immunosuppression and inflammation (Stephan, 1988). Hemorrhage has been shown to decrease the ability of macrophages to present antigen (Ayala, 1992).

There is evidence for prolonged depression in microvascular blood flow following hemorrhage and resuscitation (Wang, 1989) as well as cardiac function (Wang, 1991a), and hepatocellular function (Wang, 1990a) which persist despite fluid resuscitation.

Most mechanisms for the complication of multiple system organ failure from hemorrhagic shock include cytokine release from macrophages (Border, 1988). The mechanism of defective antigen presentation has also been studied (Ertel, 1991). Translocation of intestinal bacteria continues to be examined as one of the most likely origins of infectious agents in sepsis seen after trauma (Border, 1987). In animal models, post-shock cultures were normal rat enteric flora (Koziol, 1988). In fact, some have considered the gastrointestinal tract to be the "motor" of multiple system organ failure. (Meakins, 1986). Susceptibility to infection has also been found to persist despite fluid resuscitation and antibiotics (Livingston, 1987). Despite this work, there are still many questions regarding the precise events that relate hemorrhage to multiple organ failure (Carrico, 1993).

Fluid Intervention in Hemorrhagic Shock

Fluid resuscitation is the primary therapy treatment for hemorrhagic shock. During acute hemorrhagic shock, crystalloid infusion restores cardiac preload and helps to prevent further ischemic damage and acute renal failure. Crystalloid resuscitation alone, however, does not restore or maintain immune function (Ayala, 1992), cardiac output, hepatocellular function, microvascular blood flow, or glomerular filtration rate following such conditions (Wang, 1991a). Small-volume, slow-rate saline infusion may produce physiologic benefits that are independent of arterial pressure (Lilly, 1992).

Colloid solutions are useful in prehospital treatment of patients with hemorrhagic shock where it is not possible to give large amounts of intravenous fluids. Colloid administration provides a greater intravascular volume expansion than crystalloid for the same volume but this effect is transient as shock is associated with an increased permeability of the vascular endothelium and their is leakage of the colloid into the interstitial space (Velanovich, 1989). Colloid solutions combined with hypertonic saline is also much more effective than crystalloid solutions (Holcroft, 1984).

Administration of blood for the treatment of hemorrhagic shock is controversial. Healthy animals have been shown to be able to tolerate a hematocrit as low as 15% as long as there is adequate intravascular volume (Wilkerson, 1988). The decision to give blood is usually determined by the patency status of the patient's coronary arteries. The heart requires approximately 50% of the oxygen delivered to it and is the first organ to become ischemic with a falling hematocrit.

MEDIATORS

Hemorrhage is responsible for the release of chemical mediators into the bloodstream that affect the response to shock. A variety of mediators are released including complement, hormonal mediators, cytokines (Fong, 1990), and eicosanoids.

Both the classic and alternative pathways can activate the complement cascade. This activation generates anaphylatoxins which affect the hemodynamic system. C3a stimulates histamine release, increases capillary permeability and causes smooth muscle contraction. C5a also increases capillary permeability, stimulates degranulation of mast cells and is also a chemotactic factor (Feldbush, 1984).

Hormonal mediators released in response to hemorrhage are primarily released acutely and are directed at compensating for the loss of intravascular volume. Some of the hormones released include cortisol, anti-diuretic hormone (ADH), epinephrine, norepinephrine, and aldosterone. Endorphines are also released in response to hemorrhage from the pituitary gland (Moore, 1965). Trauma stimulates increased secretion of CRF, ACTH, and cortisol. The result is increased cortisol levels. The effects of cortisol are on many organs. Cortisol appears to maintain euglycemia during stress by its action on the liver. Cortisol does inhibit immunologic and inflammatory responses. It inhibits the function of lymphocytes, monocytes, and polymorphonuclear cells. Antidiuretic hormone has osmoregulatory, vasoactive, and metabolic effects. Catecholamine secretion results in increased secretion of glucagon and deccreased secretion of insulin. The hemodynamic effects of catecholamines include vessel constriction, an increase in heart rate and myocardial contractility. Catecholamines may also be useful is assessment of overall severity of an injury (Davies, 1984).

Cytokines are a class of proteins which include the interleukins, tumor necrosis factors, transforming growth factors and colony stimulating factors. Cytokines are biologically active at very low concentrations and are synthesized by many different types of cells in amounts that are dependent on severity and location of the stimulus. Beneficial effects of cytokines include antimicrobial functions, wound healing, and activation of immune responses (Sheppard, 1989). If cytokines are present systemically in high concentrations, however, they can produce hypotension, tissue damage and a state that is very similar to shock (Nuytinck, 1988). Tumor necrosis factor is produced by activated macrophages and causes neutrophil activation and increased vascular permeability. It also causes hypotension, lactic acidosis and pulmonary failure (Tracey, 1986). TNF also is involved in the production and release of leukotrienes and prostaglandin E_2 (Aggarwal, 1985). TNF induces ARDS when infused into animals (Ferrari-Baliviera, 1989). TNF has also been shown to produce shock and tissue injury when given alone (Tracey, 1986).

Free radical injury also is involved in shock. After an episode of ischemia, elevated hypoxanthine and xanthine levels may develop and form the superoxide radical which can lead to cell and endothelial damage. Other reported effects of free radical injury include microvascular permeability, granulocyte adhesion and arteriolar vasoconstriction. (Parks, 1983.)

Eicosanoids have many diverse effects on the physiologic and immune systems (Fletcher, 1979). Prostaglandins and leukotrienes are the primary metabolites of arachidonic acid that are involved in shock. These metabolites are released from macrophages in response to hemorrhage (Bankhurst, 1981). Prostaglandin levels are also elevated in response to endotoxin shock (Anderson, 1982). Leukotrienes have also been implicated as mediators in tissue trauma (Denzlinger, 1985). Thromboxane is stimulated by regional ischemia which occurs in hemorrhagic shock (Lelcuk, 1984). Prostaglandin E_2 also deprsses antigen presenting cell function of peritoneal macrophages (Stephan, 1988).

PHARMACOLOGIC THERAPY IN SHOCK

Different therapeutic agents have potential for use in hemorrhagic shock. These agents are directed at the various factors which appear to contribute to the pathologic processes involved in shock. With optimization of systematic management and fluid resuscitation, recent efforts have been on intervening at the cellular level with pharmacologic agents (Harkema, 1992). Certain pharmacologic agents such as cyclooxygenase inhibitors, platelet activating factor antagonists, calcium antagonists, cytokine antibodies or antagonists, may be needed during and following resuscitation to restore the immunological functions, as well as improve organ function (Harkema, 1992). Preheparanization of animals produces various beneficial effects, such as improvement of cell and organ function as well as survival time of experimental animals following adverse circulatory conditions (Rana, 1992). Administration of conventional heparin following trauma-hemorrhage and resuscitation, significantly improves organ function and microcirculation (Wang, 1994a). ATP-$MgCl_2$ has been used to correct the depression of adenosine triphosphate (ATP) production (Chaudry, 1981). Pentoxifylline may be effective in improving microcirculation or downregulating neutrophil response (Coccia, 1989). Pentoxifylline may function by suppressing TNF gene transcription which gives some insight and direction for the development of other chemical therapeutic agents (Doherty, 1991). Calcium channel antagonists may be effective in restoring calcium homeostasis within the cell. In animal models, calcium channel blockade has been shown to improve myocardial and cellular function (Hess, 1983). Diltiazem may exert its effect by resoring cytokine and interferon-γ synthesis (Meldrum, 1991). Monoclonal antibodies to inflammatory cytokines or their receptors appear to have some beneficial effects. A receptor antagonist to IL-1 has been found to improve survival after endotoxemia in animals (Alexander, 1991). Cytokine administration may also be of benefit in desensitization to the negative influence of cytokines. Because of the critical role of

cytokines in hemorrhagic shock different applications have emerged (Dinarello, 1993). As mentioned, TNF has been found to reproduce the clinical effects seen in shock. It is often thought of as the primary mediator in shock and thus blocking of TNF has been a primary goal of many investigators because of the potential clinical value. Specific blockade of TNF with neutralizing antibodies or soluble receptors reduces mortality and severity of disease. The results are similar for blockade of IL-1 (Dinarello, 1993). TNF induced mortality is also reversed with administration of cyclooxygenase inhibitors (Fletcher, 1993). Administration of anti-TNF antibodies has been found to prevent shock in animals that are bacteremic (Tracey, 1987). Interferon-γ may be helpful in reducing the spread of infection and decreasing the susceptibility to sepsis (Harkema, 1992).

Administration of eicosanoids or their inhibitors may be beneficial by improving systemic or regional blood flow as well as a number of other possible mechanisms (Almqvist, 1982). Administration of prostacyclin and thromboxane A_2 has been found to moderate postischemic renal failure (Lelcuk, 1985). Dietary administration of n-3 fatty acids has been shown clinically to reduce wound infections, shorten hospital stay , and reduce mortality. Free radical scavengers may be effective by reducing the amount of oxygen free radicals which have been implicated in the damage caused by ischemic injury. Coenzyme Q_{10} may protect against cellular damage by preserving energy content, preventing oxygen-radical injury and stabilizing membrane structure and function. Agents which may protect against dysfunction in the peripheral vascular regulation (adrenergic agents, angiogensin-converting enzyme inhibitors) have also been shown to have beneficial effects. Naloxone, which is an opiate antagonist, reverses some of the detrimental effects produced by endogenous endorphin release (Curtis, 1980). One of the proposed pharmacologic agents that may be useful in hemorrhagic shock is a chemically modified heparin (GM1892). Heparin and chemically modified heparin will be discussed below.

HEPARIN AND CHEMICALLY MODIFIED HEPARIN (GM1892)

Heparin

Heparin had initially been used as an agent in small animal models to maintain the patency of small venous and arterial cannulas used in hemorrhage models. Administration of heparin prior to hemorrhage has been shown to improve cardiac output, hepatocellular function and renal function. (Wang, 1990b). Heparin was found to have beneficial effects on animal survival after hemorrhage but could not be used in clinical situations because its anticoagulant effect precludes its use in trauma patients (Rana, 1992). Sternbergh et. al. demonstrated, however, that the anticoagulant effect and the beneficial effects in animals were independent (Sternbergh, 1993). Preheparanization of animals prior to hemorrhagic shock has also been found to protect the microvasculature (Rana, 1992). Heparin has been shown to improve survival in animal models of gram-negative septic shock (Griffin, 1990).

Heparin is released from basophilic granules of mast cells and leukocytes during inflammatory reactions (Dziarski, 1992). During these conditions it has been found to have several immunomodulatory effects. Lymphocytes have been found to have binding sites for heparin (Parish, 1985). At clinically utilized doses it enhances the proliferative responses of murine T cells in mixed lymphocyte reaction. It also enhances the generation of cytotoxic T lymphocytes against allogeneic cells and histocompatible tumors (Dziarski, 1989). It also plays a role in the modulation of immunologic function (Lider, 1989).

Several of the other nonanticoagulant properties include modulation of smooth muscle cell proliferation (Clowes, 1977), angiogenesis (Folkman, 1989), platelet function (Gregorius, 1976), and acute inflammation (Carr, 1979). There is evidence to show that heparin reduces vascular permeability (Hobson, 1988). It also has membrane stabilizing effects (Srinivasan, 1968) as well as increasing superoxide dismutase production which may decrease the toxic effects of free radicals (Karrison, 1988). Heparin may also increase cGMP levels by increasing endothelial EDRF release (Sternbergh, 1993).

The exact mechanism of the beneficial effects of heparin, however, is unknown. Heparin and modifed forms of heparin have been shown to inhibit complement activation in-vivo (Weiler, 1992). It has been hypothesized that the strong negative charge of heparin and the increased release of tumor necrosis factor-binding protein by heparin could be responsible for their beneficial effects (Lantz, 1991). The mechanism may be related to the function of the endothelium which is preserved in preheparinized animals (Wang, 1993). The highly negatively charged heparin molecule may also attach to red blood cells and keep them dispersed in low blood flow situations (Chopra, 1967).

The elimination curve of conventional heparin is biphasic consisting of a rapidly declining alpha phase with a half life of 10 minutes and after the age of 40, a slower beta phase, indicating uptake in organs. The is no relationship between anticoagulant half-life and concentration half-life indicating that there might be some protein binding of heparin (Physicians Desk Reference, 1995).

GM1892

There are many different forms of nonanticoagulant heparin that have been developed including low molecular weight heparins and other modified forms. GM1892 is a nonanticoagulant heparin that was developed by Glycomed (Alameda, CA). GM1892 is composed of the same natural saccharide residues found in heparin and is prepared from the parent low molecular weight heparin (LMW) by selective O-desulfation using alkaline conditions. This modification results in the loss of specific sulfate groups important to antithrombin III (AT III) binding and reduces the overall charge density of the molecule. It has a molecular weight of 4-5 KDa and approximately 2% of the anticoagulant activity of conventional heparin as measured by the activated partial thromboplastin time (Figure 1). It has approximately 60% of the antiplatelet activity of conventional heparin and no growth factor activity. The ability of splenocytes to release IL-2 and IL-3 in response to mitogen is markedly improved in hemorrhaged animals treated with GM1892 or

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conventional heparin compared to saline-treated animals. The capacity of splenic and peritoneal macrophages to release IL-6 is also restored in the hemorrhaged animals that receive GM1892 or conventional heparin (Zellweger, 1995).

Although GM1892 has been shown to improve hepatocellular function, cardiac output, and microcirculation acutely (up to 4 hours) after trauma-hemorrhage and resuscitation in rats (Wang, 1994a), it is not known whether this agent produces persistent beneficial effects on organ function.

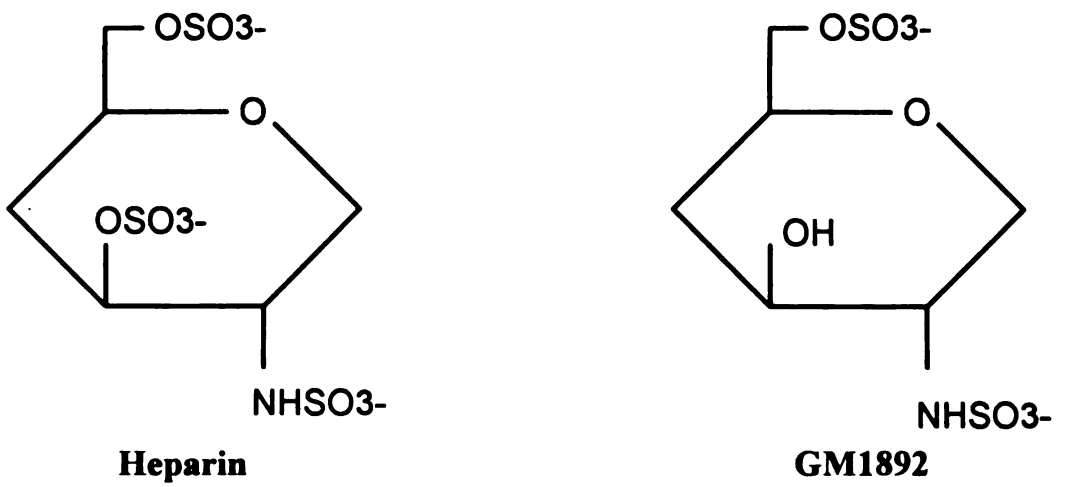

Figure 1. Chemical structures of heparin and GM1892. GM1892 is prepared by selective O-desulfation using alkaline conditions.

HYPOTHESIS

Administration of GM1892 improves cell and organ function in a chronic trauma/hemorrhage model by improving microcirculation and by downregulating proinflammatory cytokine (TNF, IL-6) synthesis /release.

SPECIFIC AIMS

1. To determine GM1892 improves cardiovascular and hepatocellular function in a chronic trauma/hemorrhage model.
2. To determine the mechanism of action of GM1892.
3. To determine if administration of GM1892 improves survival in animals subjected to trauma hemorrhage and resuscitation.

MATERIALS AND METHODS

Experimental design

Eight animals were used for each sham group, saline group (control group), chemically modified heparin (GM1892) group, and heparin group. The animals in the saline, GM1892, and heparin groups underwent trauma- hemorrhage and resuscitation as described below. Twenty four hours after trauma-hemorrhage and resuscitation the animals were anesthetized and heart rate, blood pressure were measured. Cardiac output and hepatocellular function were measured using indocyanine green clearance. Blood samples were obtained for TNF, interleukin-6 (IL-6), PGE_2 , and aspartate aminotransferase (AST) also known as serum glutamic-oxaloacetic transaminase (SGOT). Major organs including the lungs, liver, kidneys, intestines, spleen and heart were removed and weighed prior to and after dessication to quantitate tissue edema (Wang, 1992).

The mechanism of action of GM1892 was investigated by examining the possible sites of action. Laser Doppler flowmetry was used to assess differences in surface microcirculation of liver, kidneys, small intestine, and spleen between treated animals and controls (Wang, 1990c). A bioassay for TNF described below was used to quantitate TNF activity. The results of this were compared with the results of an ELISA quantitating the total amount of antigenic TNF which detects bioactive and non-bioactive TNF thus allowing an estimation of the presence of an inhibitor.

Animal model of trauma and hemorrhage

A nonheparinized model of trauma-hemorrhage and resuscitation in the rat was used and which has been described previously (Chaudry, 1989). Male Sprague-Dawley rats weighing 275-325 gm, were fasted overnight before the experiment but were allowed water ad libitum. After methoxyflurane anesthesia, the animals underwent a 5 cm ventral midline laparotomy to induce tissue trauma before hemorrhage. The abdominal incision was then closed in 2 layers. Both femoral arteries were cannulated with polyethylene

(PE)-50 tubing for bleeding or measurement of mean arterial pressure (MAP) and heart rate (HR). MAP and HR were continuously monitored with a femoral catheter connected to a manometer. The rats were bled to a mean arterial pressure (MAP) of 40 mmHg and maintained at that pressure until 40% of the shed blood volume was returned as Ringer's lactate (RL). This is the point or irreversibility in Wigger's model. The rats were then resuscitated with 4X the volume of the maximal bleedout with RL over 60 minutes via the femoral venous catheter. The animals received a single dose of GM1892 (7 mg/Kg), heparin (7 mg/Kg), or an equivalent amount of normal saline intravenously in a bolus dose at this time. Maximal bleedout was determined as the volume of blood withdrawn following which the animal cannot maintain the MAP of 40 mm Hg unless some fluid was returned to the intravascular space. Prehemorrhage and posthemorrhage hematocrit were also measured. The shed blood was not returned to the animal. Control animals received the same operation but were administered normal saline only instead of a pharmacologic agent. Sham operated animals underwent the same surgical procedure but were not hemorrhaged. All experiments were performed in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This experiment was approved by the Michigan State University committee on the care and use of animals.

Heart rate

Heart rate was determined by counting the number of pulsations of the blood/saline interface in the cannulation tubing connecting the femoral artery to the manometer. The number of pulsations was counted for a 15 second interval and the result multiplied by 4 to give the number of beats per minute.

CO measurement

Cardiac output was determined by indocyanine green (ICG) dilution. A fiberoptic catheter was placed in the left carotid artery to the level of the thoracic aorta (Figure 2). A polyethylene catheter was placed in the right internal jugular vein. A carefully measured quantity of ICG dye was injected into the internal jugular vein and the ICG concentrations were measured continuously with the fiberoptic catheter and recorded digitally on a computer disk through a data aquisition program (Figure 3).

The dose used for the calculation of cardiac output was the same dose as that used for the measurement of hepatocellular function. The smallest dose (0.05 mg) gave the best results for cardiac output as larger doses would result in higher blood concentrations near the saturation level of the in-vivo hemoreflectometer. Dimensional analysis confirms that the dose (mg) divided by the area under the curve (mg/L/min) gives the correct units for cardiac output (L/min)(Figure 4). The cardiac output was then converted into a cardiac index by dividing by the body weight of the rat.

The usual method or measurement is to place the fiberoptic catheter from the in-vivo hemoreflectometer in the right sided carotid artery leaving it rest in the aorta. Because of the small amount of distance between the junction of the carotid artery and the inominate artery and the aortic valve, however, small movements in the rat can cause movement of the fiberoptic catheter resulting in inaccurate measurements of ICG concentrations. Because of this problem, it was decided to place the catheter in the left sided carotid artery with the tip in the descending aorta. Using this method, there is approximately 4 cm or more of room for the tip of the catheter. This creates a larger distance for the tip of the catheter to be effective.

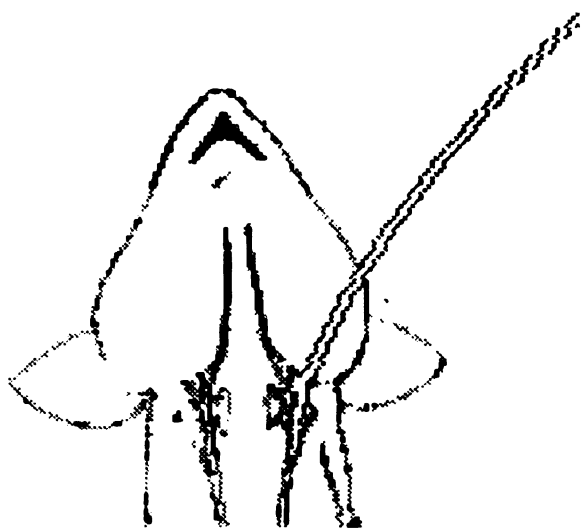

Figure 2. In-vivo hemoreflectometer catheter placement. Twenty four hours following hemorrhage and resuscitation, a fiberoptic probe from an in-vivo hemoreflectometer was placed in the left carotid artery approximately 4 cm to place the tip in the descending aorta.

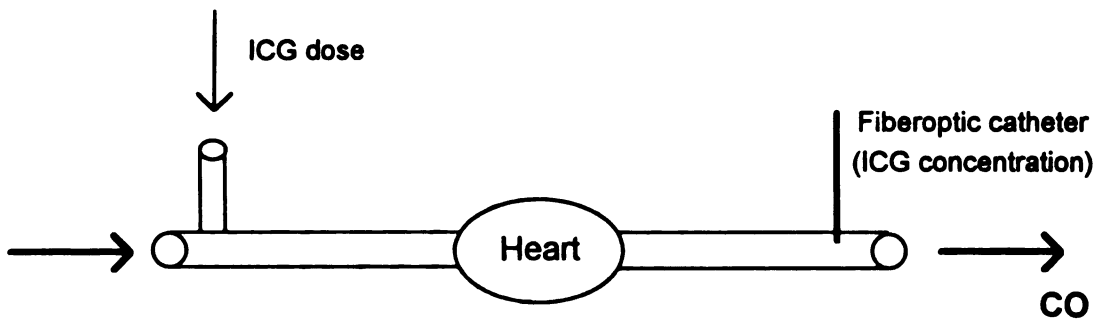

Figure 3. Schematic diagram of cardiac output measurement. An ICG dose is given in the venous circulation close to the heart. The ICG concentration is then monitored with a fiberoptic catheter in the aorta. The cardiac output is determined from the area under the cardiac output curve.

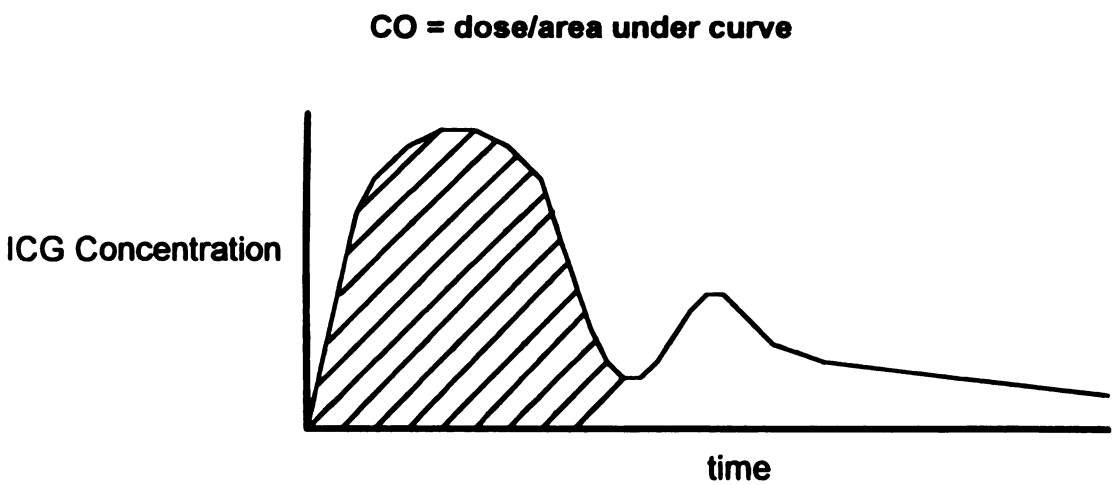

Figure 4. Cardiac output curve using a small dose of indocyanine green (0.05mg).

Determination of hepatocellular function

Indocyanine green clearance was described in 1968 as being a nontoxic accurate method of determining hepatocellular function (Bradley, 1968). It has since been shown to be an accurate indicator of hepatic blood flow (Burczynski, 1987). Another advantage of indocyanine green clearance is that it can also be used to determine circulating blood volume concurrently (Busse, 1990). Indocyanine green is metabolized exclusively by the liver and its metabolism reflects the hepatic blood flow and hepatocellular function (Cherrick, 1960).

After a 24 hour recovery period for the animals, they were anesthetized with pentobarbital and the right jugular vein was cannulated with PE-50 tubing. The left carotid artery was isolated and a fiberoptic catheter was passed down the carotid artery to the level of the aortic arch for the continuous measurement of ICG concentration. Three doses of ICG (0.05, 0.10, 0.25 mg per rat) were administered with the smallest dose first. After administration of the ICG, the concentration was recorded with an in-vivo hemoreflectometer. For each dose, the nonlinear regression equation was calculated for the relationship between ICG concentration and time according to the polynomial equation

$$[ICG] = \exp(a + bt + ct^2)$$

where [ICG] is the ICG concentration in milligrams per liter, t is the time in minutes, a, b, and c are coefficients unique to each measurement. (Hauptman, 1991). V_0 which is the initial rate of change of ICG concentration was calculated by

$$V0 = b \exp(a)$$

An explanation of the derivation of this is given in the discussion section below. The initial velocity of clearance was converted from milligrams per liter per minute to milligrams per kilogram per minute by

$$V_0 \text{ (mg/Kg/min)} = V0\text{(mg/L/min)} \times EBV/BW$$

where V0 is the initial velocity of clearance in milligrams per kilogram per minute, BW is the body weight in kilograms, and EBV is the estimated blood volume in liters, calculated according to

$$EBV = (mg\ ICG\ injected)/exp(a)$$

The reciprocal of the dose (1/D) was plotted against the reciprocal of the velocity of clearance (1/V0), and a regression equation was calculated

$$1/V_0 = (1/dose)(slope) + 1/V_m$$

In this formula, the y-intercept represented 1/Vmax or the reciprocal of the theoretical maximal velocity of clearance of ICG at an infinite dose of ICG (Figure 5).

Vm represents the transport maximum or the theoretical dose at which the there would be an infinite rate of indocyanine green clearance (Hauptman, 1989). An estimate of the hepatic blood flow (EHBF) can be obtained by the following formula

$$EHBF = V_0/[ICG]_0$$

Where $[ICG]_0$ is the concentration of ICG at time = 0. Mathematically this reduces to

$$V_0/[ICG]_0 = b\ exp(a)/exp(a) = b$$

OR

$$EHBF = b$$

The EHBF may be inaccurate because it assumes an extraction ratio of 1 for ICG. In reality, it is probably closer to 0.5 or possibly even 0.3 for rats in shock. Despite this limitation, this gives an intuitive sense for the significance of the value of the coefficient b. Reduced indocyanine green clearance has been found to be an indicator of early sepsis (Wang, 1991b) despite microvascular hyperperfusion (Wang, 1991c).

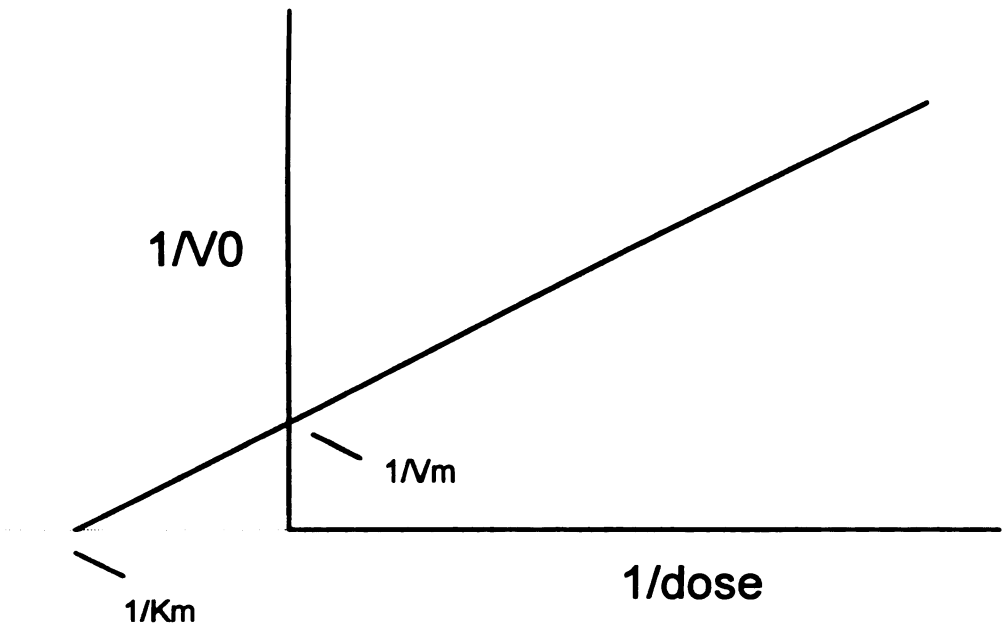

Figure 5. Calculation of Vm and Km from the double reciprocal plot of 1/dose vs. $1/V_0$.

Assessment of microvascular blood flow

Surface blood flow was determined by laser Doppler flowmetry. This is a measurement which is performed by placing a fiberoptic probe from the monitor on the surface of each organ to be measured during laparotomy. The probe directs laser light into the tissue, illuminating a volume which contains both red blood cells and stationary tissue cells. Light energy (photons) is randomly scattered by both cell types. Photons scattered by moving cells are Doppler shifted. Return fibers on the cable transmit the a portion of the scattered light which is then converted into electronic signals by a photodetector in the monitor. The results are read in arbitrary units and are processed by the monitor (Laserflow Operator Manual).

Cytokine Measurement

Blood samples were drawn from the rat and placed in small plastic blood containers. TNF activity was determined by assessing plasma samples for WEHI 164 subclone 13 cytotoxicity (Eskandari, 1990). Total TNF was quantified by antigenic assessment with TNF ELISA (Genzyne corp.). IL-6 activity was determined with IL-6 dependent 7TD1 B-cell hybridoma cells (Ayala, 1991). The relative unit value of plasma activity per milliliter of TNF and IL-6 was determined by a comparison of the curves produced from dilution of the experimental plasma to those generated by dilution of murine TNF standard and a human recombinant IL-6 standard.

Other Measurements

Serum GOT was measured using a kit commercially available from Sigma company. The procedure utilizes the following reaction:

Aspartic Acid + α-ketoglutarate —GOT—> Oxalacetic Acid + Glutamic Acid

The oxalacetic or pyruvic acid formed in this reaction is reacted with 2,4-dinitrophenylhydrazine. The color intensity of the resulting phenylhydrazones is

proportional to the transaminase activity. Colorimetric measurement was then performed on the mixture and represents the enzyme activity (Sigma Diagnostics technical manual).

PGE_2 was measured using a kit commercially available from Dupont. The technique uses a radioimmunoassay. A constant, limiting amount of antibody is added to a mixture of the sample (or standard) and a fixed amount of radiolabeled antigen (tracer). As the amount of antigen (PGE_2) is increased, the amount of tracer bound to antibody decreases.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Student-Newman-Keuls tests were used and the differences were considered significant at p value < 0.05. An ANOVA on ranks was used if the data did not have a Gaussian distribution or the equal variance test was not passed. The chi squared test was used for mortality analysis. Sigmastat^R was used as a computerized statistical package. All results are given as mean ± SEM. *$p<0.05$ versus sham, #$p<0.05$ vs saline.

RESULTS

Cardiovascular system

The heart rate of the saline treated group was significantly higher than the sham group. The heart rate of both the GM1892 and heparin treated animals was significantly less than the saline treated group and not significantly different than the sham group (Figure 6). The mean arterial pressure, however, was significantly decreased in all animals that underwent trauma-hemorrhage and resuscitation despite treatment (Figure 7). The cardiac index as measured with indocyanine green dilution was significantly decreased in the saline treated group compared to the sham group but was restored in both the GM1892 and heparin treated groups (Figure 8). The calculated stroke volume was significantly decreased in the saline group compared to the sham group but restored in the GM1892 and heparin groups to sham levels (Figure 9). The calculated total peripheral resistance was significantly increased in the saline group compared to the sham group but decreased to sham levels in both the GM1892 and heparin groups (Figure 10).

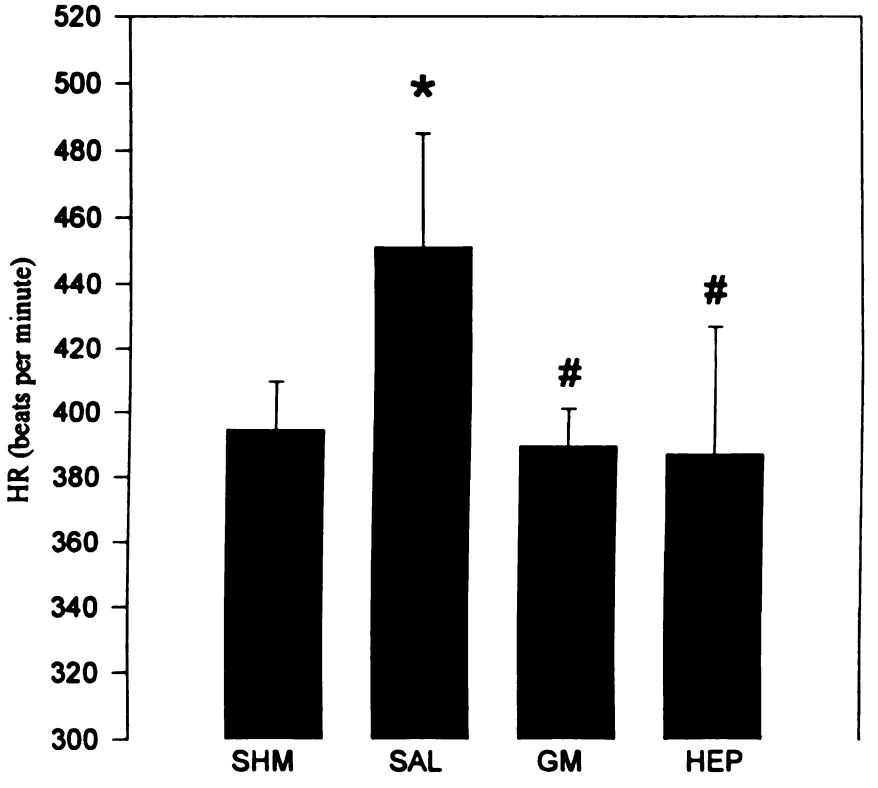

Figure 6. At 24 hours rats were anesthetized and a catheter placed in the carotid artery. Heart rate (HR) was measured with a manometer as described under "Materials and Methods". *$p<0.05$ versus sham, #$p<0.05$ vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

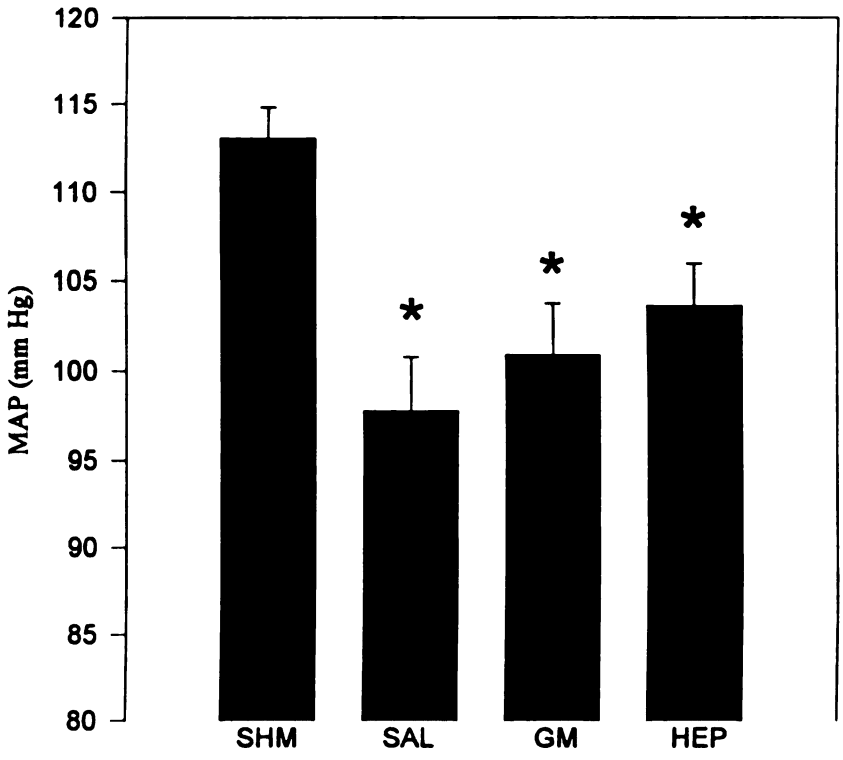

Figure 7. At 24 hours rats were anesthetized and a catheter was placed in the carotid artery. Mean arterial pressure (MAP) was measured using a manometer as described under "Materials and Methods". *$p<0.05$ versus sham. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

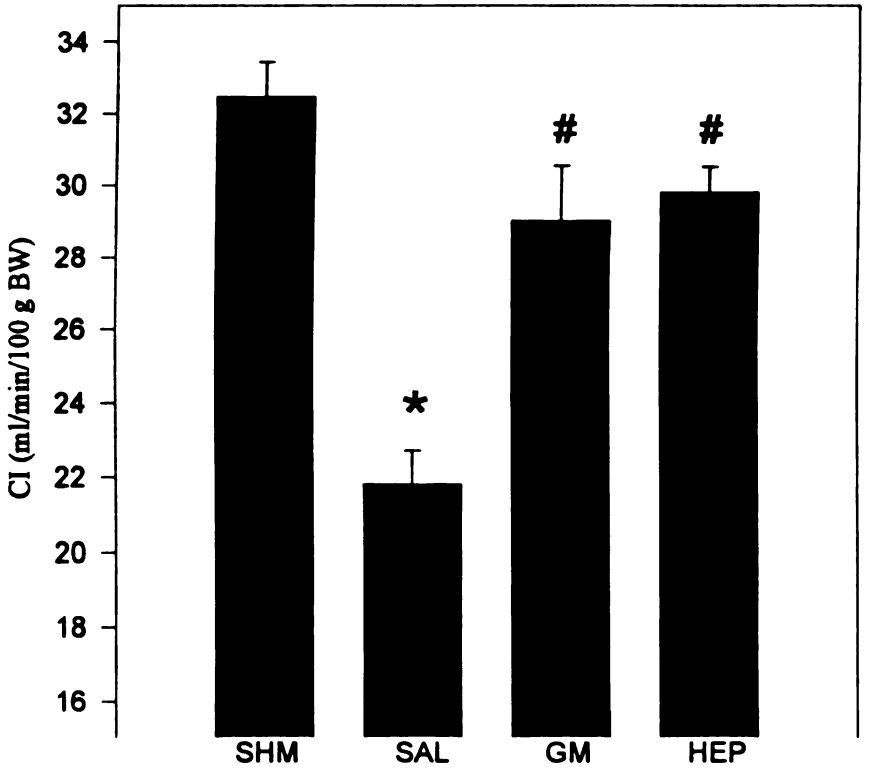

Figure 8. At 24 hours after trauma-hemorrhage and resuscitation, a fiberoptic catheter was placed in the left carotid artery to the level of the descending thoracic aorta. Cardiac index was determined by indocyanine green dilution as described in "Materials and Methods". *$p<0.05$ versus sham, #$p<0.05$ vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

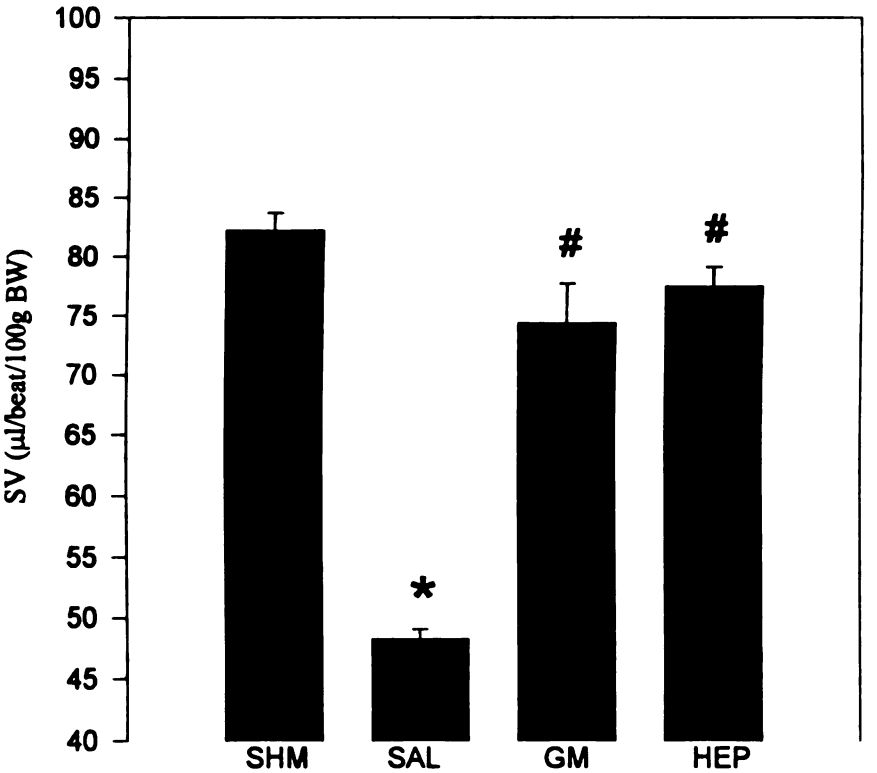

Figure 9. Stroke volume was calculated using cardiac index and heart rate. SV=CI/HR. *p<0.05 versus sham, #p<0.05 vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

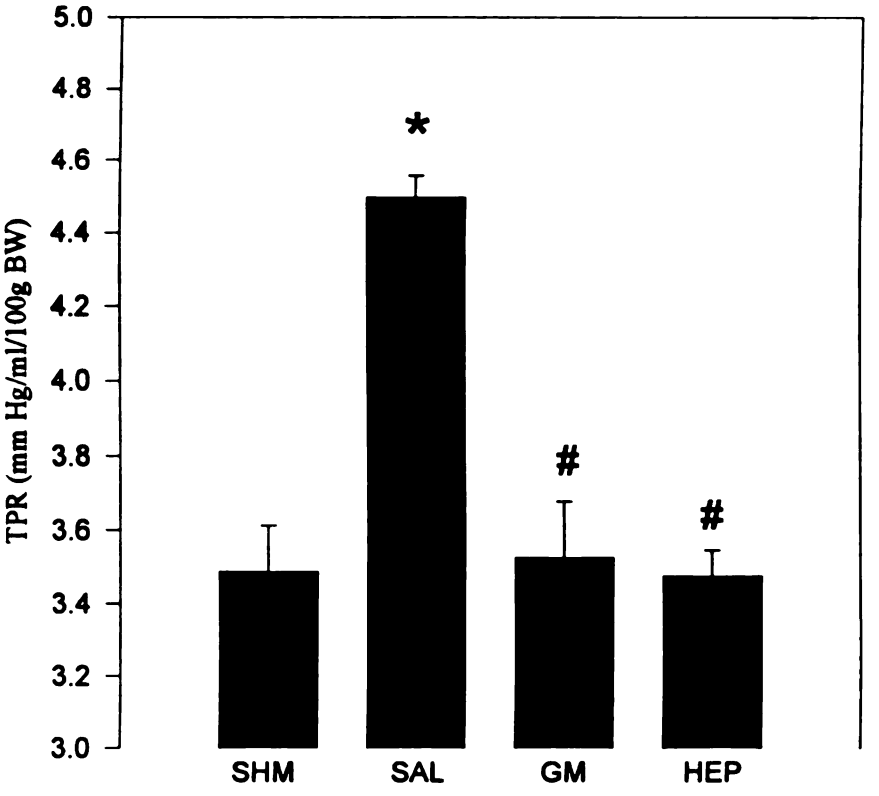

Figure 10. Total peripheral resistance was calculated value using CI and BP measurements. TPR = MAP/CI. *p<0.05 versus sham, #p<0.05 vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

Hepatocellular function

V_0 as measured with the 0.25 mg indocyanine green dose was significantly decreased in the saline group compared to the sham group. V_0 in the GM1892 and heparin treated groups, however, was not significantly different than in the sham group (Figure 11). The GOT level was significantly increased in the saline group compared to the sham group. The levels in both the GM1892 and heparin groups were decreased from the saline group and not significantly different than the sham group (Figure 12).

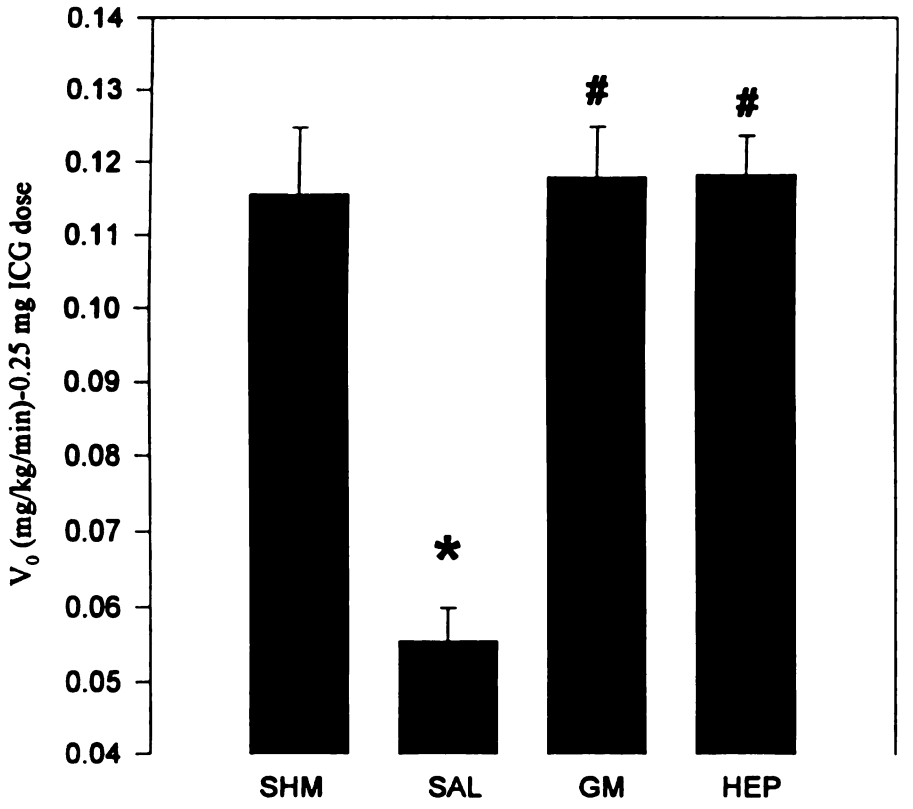

Figure 11. V_0 at 24 hours after trauma-hemorrhage and resuscitation. A fiberoptic catheter was placed in the left carotid artery to the level of the descending thoracic aorta. V_0 was determined by indocyanine green dilution as described under "Materials and methods". *$p<0.05$ versus sham, #$p<0.05$ vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

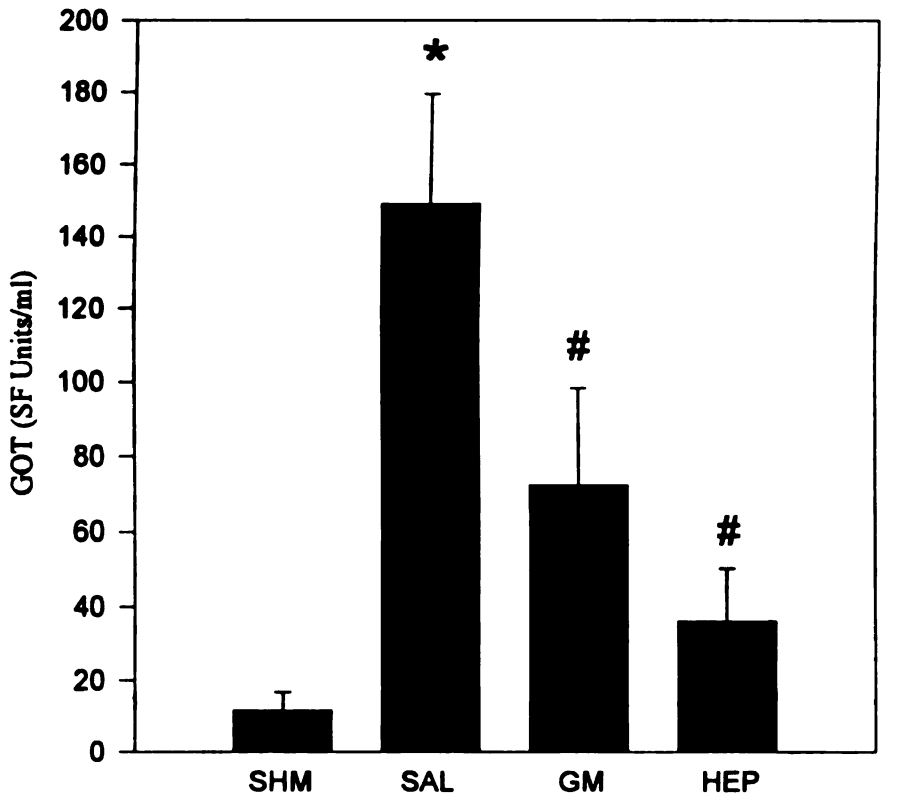

Figure 12. Glutamic oxalacetic transaminase (GOT) at 24 hours after trauma-hemorrhage and resuscitation. Plasma samples were obtained and frozen. GOT was determined by colorimetry as described under "Materials and Methods". *$p<0.05$ versus sham, #$p<0.05$ vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

Tissue water content

Table 1 shows the tissue water content of the organs examined by weighing prior to and after dessication. The water content of the heart was significantly increased in all animals subjected to trauma-hemorrhage and resuscitation despite treatment. The water content of the liver was significantly increased in saline treated animals but decreased to sham levels in the GM1892 and heparin treated groups. The water content of the lungs was significantly higher in the saline and GM1892 groups but the heparin group was not significantly different that the sham group (Figure 12). The kidney water content was significantly increased in the saline only treated group but decreased to sham levels in the GM1892 and heparin treated groups. The water content of the spleen was significantly increased in all the animals that underwent trauma-hemorrhage and resuscitation and significantly less in the GM1892 group compared to the saline group. The water content of the intestine was significantly higher in the animals that underwent trauma-hemorrhage and resuscitation but significantly lower in the heparin group compared to the saline group.

Table 1. At 24 hours after hemorrhage and resuscitation, small sections of organs were removed. The weight of the organs was measured prior to and after dessication as described in "Materials and Methods". Measurements are percent. *p<0.05 versus Sham; #p<0.05 vs Saline.

Tissue Water Content (%)

	Sham	Saline	GM1892	Heparin
HEART	76.8 ± 0.57	79.7 ± 0.55*	78.7 ± 0.41*	78.9 ± 0.67*
LUNGS	79.5 ± 1.81	82.8 ± 0.73*	81.9 ± 0.54*	80.0 ± 0.87#
LIVER	68.6 ± 1.71	75.5 ± 0.68*	73.2 ± 0.51*#	73.8 ± 0.59*#
KIDNEY	71.2 ± 2.24	75.7 ± 1.04*	73.5 ± 1.26	73.3 ± 0.59
SPLEEN	73.7 ± 0.87	79.3 ± 0.73*	78.1 ± 0.61*#	79.3 ± 0.57*
INTESTINE	74.3 ± 0.82	76.5 ± 0.983*	77.6 ± 0.61*	79.9 ± 0.89*#

Microvascular blood flow

Table 2 shows the microvascular blood flow of the abdominal organs examined. The blood flow of the liver was significantly decreased in the saline group and heparin compared to the sham group. The liver blood flow was significantly higher in the GM1892 and heparin treated groups compared to the saline group. The kidney blood flow was significantly less in all the animals that underwent trauma-hemorrhage and resuscitation compared to the sham group. The kidney blood flow in the GM1892 and heparin groups was also significantly increased compared to the saline group. The splenic blood flow and intestinal blood flow was significantly decreased in all the animals subjected to trauma-hemorrhage and resuscitation. It was also significantly improved in animals treated with either GM1892 or heparin.

Table 2. At 24 hours after hemorrhage and resuscitation, the animal was anesthetized. A laparotomy was performed and the microvascular organ surface blood flow was measured using laser Doppler flowmetry as described in "Materials and Methods". Measurements are in arbitrary units. *p<0.05 versus Sham; #p<0.05 vs Saline.

Microvascular Blood Flow (Arbitrary Units)

	Sham	Saline	GM1892	Heparin
LIVER	40.7 ± 1.59	25.4 ± 1.24*	37.9 ± 1.89#	29.8 ± 0.91*#
KIDNEY	80.4 ± 1.60	45.8 ± 0.94*	60.7 ± 1.36*#	58.5 ± 1.38*#
SPLEEN	35.8 ± 1.06	25.3 ± 0.54*	28.0 ± 0.83*#	29.9 ± 0.72*#
INTESTINE	90.5 ± 1.26	58.2 ± 1.05*	81.9 ± 1.71*#	81.8 ± 1.69*#

Mediator analysis

Plasma IL-6 levels were significantly elevated in the saline group compared to the sham group. IL-6 levels were reduced to sham levels in the GM1892 and heparin treated groups (Figure 13). Plasma TNF levels as measured by the bioassay method were significantly elevated in the saline group compared to the sham group. TNF levels were also reduced to sham levels in the GM1892 and heparin treated groups (Figure 14). Plasma TNF levels as measured by ELISA were elevated in the saline and heparin treated groups but significantly over the sham or GM1892 treated groups (Figure 15). PGE_2 was significantly elevated in the saline group compared to the sham group but reduced to sham levels in the GM1892 and heparin treated groups (Figure 16).

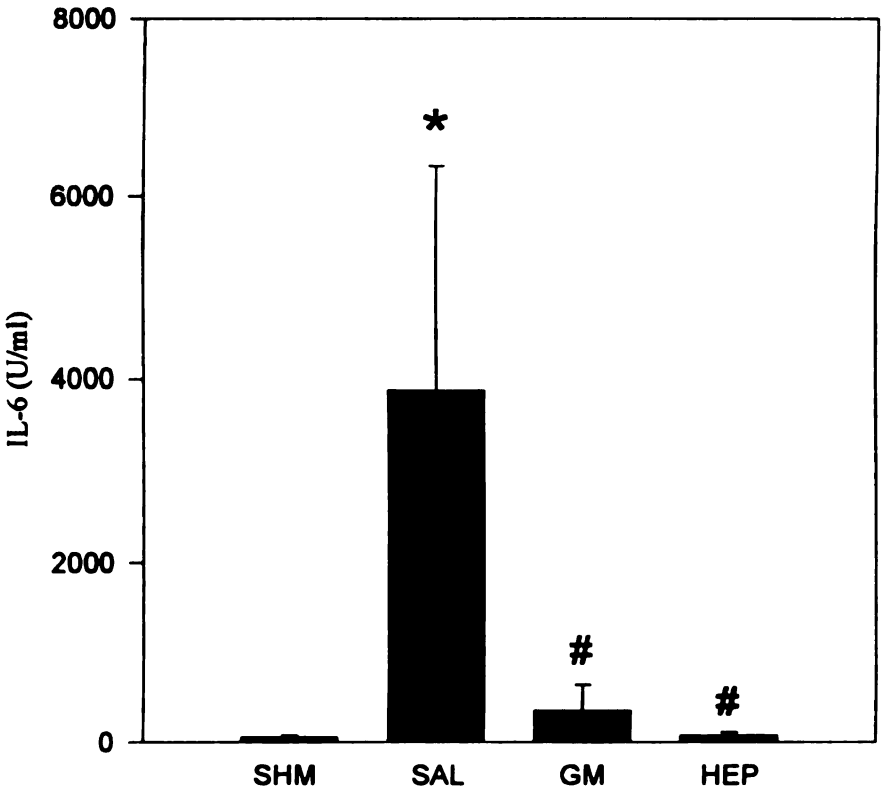

Figure 13. IL-6 was measured by 7TD1 cell bioassay from frozen plasma samples taken at 24 hours after trauma-hemorrhage and resuscitation as described in "Materials and Methods". *$p<0.05$ versus sham, #$p<0.05$ vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

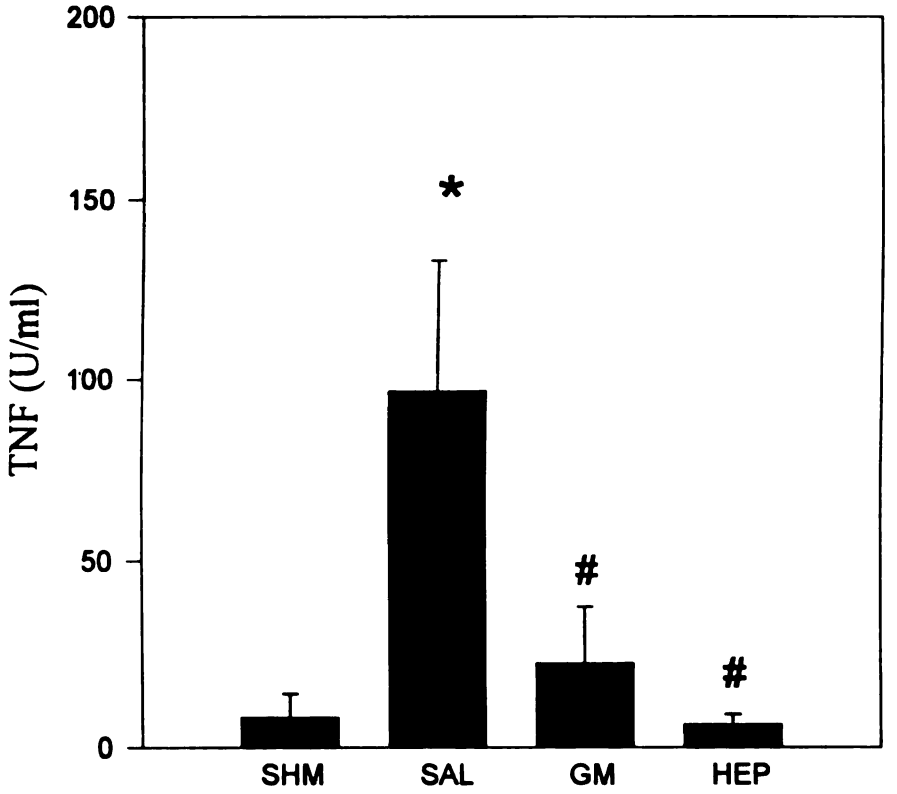

Figure 14. TNF-a was measured by WEHI-164 clone 13 bioassay from frozen plasma samples taken at 24 hours after trauma-hemorrhage and resuscitation as described in "Materials and Methods". *$p<0.05$ versus sham, #$p<0.05$ vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

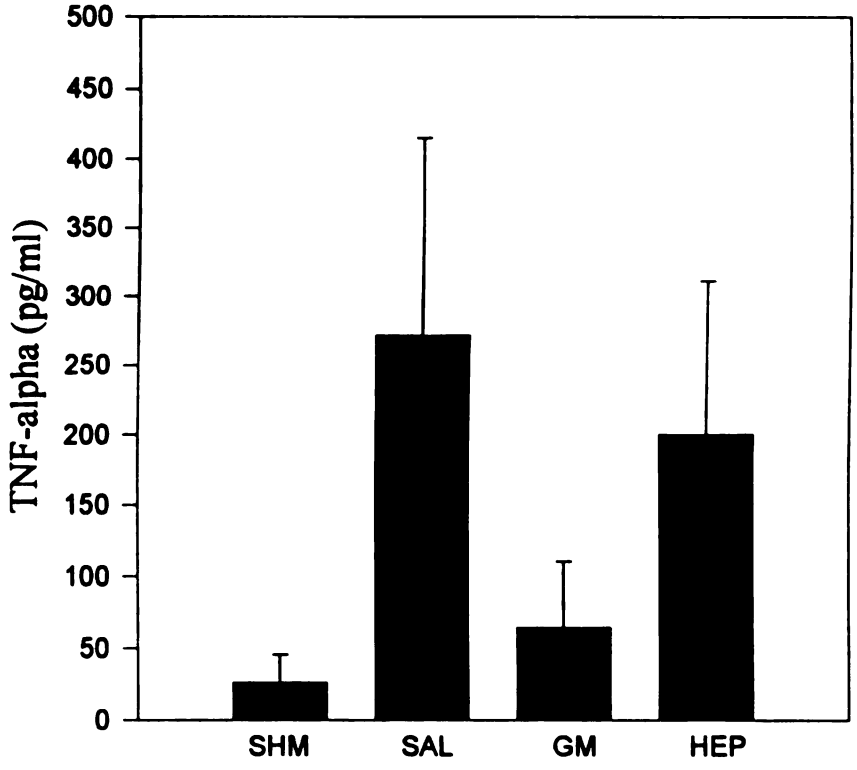

Figure 15. TNF as measured by enzyme linked immunoabsorbant assay (ELISA) at 24 hours after trauma-hemorrhage and resuscitation. The differences between the groups were not statistically significant.

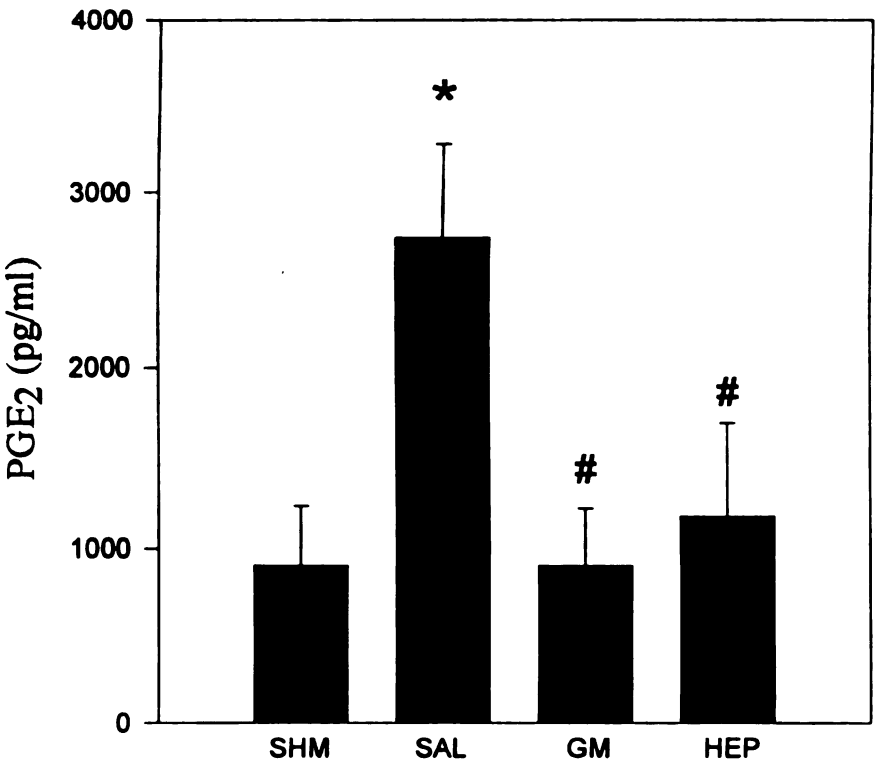

Figure 16. PGE_2 at 24 hours after trauma-hemorrhage and resuscitation. *p<0.05 versus sham, #p<0.05 vs saline. SHM-sham group, SAL-saline group, GM-GM1892 group, HEP-heparin group.

Mortality

There was a trend toward increased survival in GM1892 treated animals compared with saline treated animals in a small mortality study (N=32). The mortality in the saline treated group was 56% compared to 38% survival in the GM1892 treated group (Figure 17). Because of the large number of animals required to show a significant difference at this mortality rate, this study was abandoned.

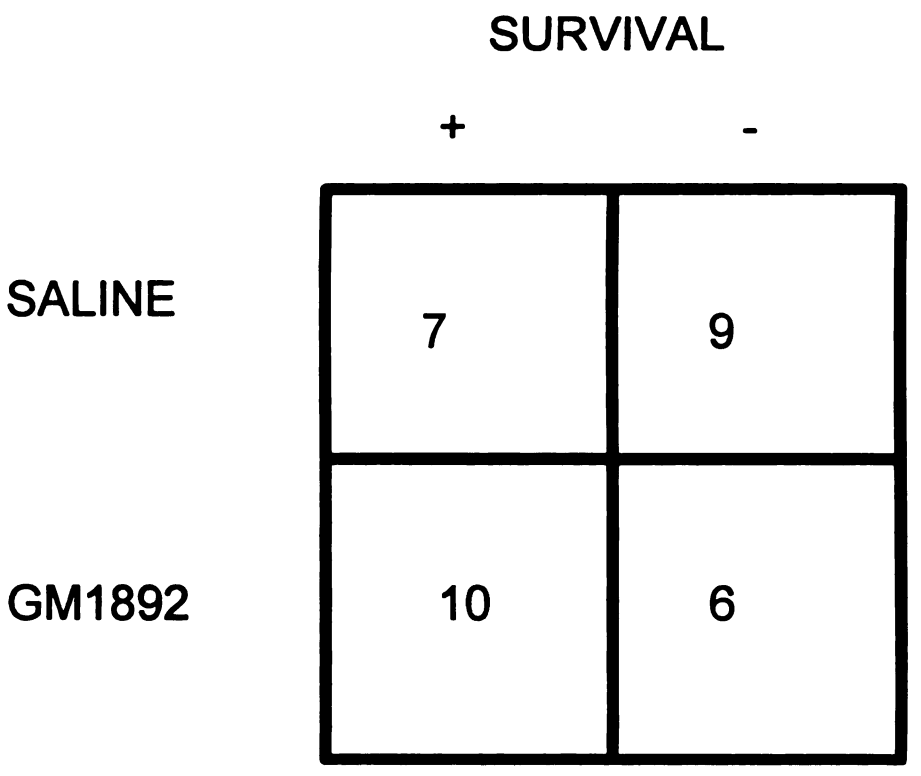

Figure 17. Mortality of saline treated animals and GM1892 treated animals after trauma-hemorrhage and resuscitation. Overall mortality of the saline treated group was 56% vs. 38% for the GM1892 treated group.

DISCUSSION

The development of intravenous fluid resuscitation was a tremendous advance in the therapy of hemorrhagic shock. However, because fluid resuscitation alone does not prevent many of the effects of circulating mediators in shock, pharmacologic agents have been developed and studied in the desire to obtain this effect. Many of these agents are available commercially and many are not. Conventional heparin was discovered to have beneficial effects independent of its anticoagulant activity which led to the development of modified heparins and GM1892. Previous animal studies with GM1892 have shown that it improves hepatocellular function, cardiac output, and microcirculation after trauma-hemorrhage and resuscitation acutely (up to 4 hours) in rats (Wang, 1994a). Prior to this current study, however, it was not known whether GM1892 produced sustained beneficial effects on organ function. The mechanism of action had also not been investigated.

Because of the importance of cardiac function and hepatic function to overall animal survival, these two organs were focused on during the course of the laboratory work. This study showed that cardiac function is improved by administration of GM1892. Heart rate was decreased by approximately 13% representing decreased compensation for a depressed stroke volume. Although the MAP was not significantly different in any of the hemorrhaged animals, there was a trend toward an increased MAP in the treated groups supporting an improvement in cardiac function. The most significant cardiac paramater that showed improvement is the cardiac index which increased by approximately 32% in animals treated with GM1892. Similarly, by calculation, the stroke volume increased by 57% reflecting the improvement of both the heart rate and cardiac index. The compilation of these results show a definite improvement in cardiac function.

The data for the hepatocellular function is less clear but still convincing. The initial rate of clearance of ICG (V_0) decreased by approximately 110% in the saline group compared to the sham group but restored in animals treated with GM1892. Although the

V_0 is dependent on both hepatocellular function and hepatic blood flow, the fact that the cardiac index increased by only 32% suggests that the improvement in V_0 is due to some other factor. Most likely this other factor is an improvement in hepatocellular function. Serum GOT, commonly used clinically to assess hepatocellular damage was decreased by approximately 53% in animals treated with GM1892 compared to saline treated animals. This most likely represents hepatocellular damage secondary to ischemia from the decreased perfusion of the organ from damaged microcirculation or directly from circulating mediators. Liver water content was decreased in animals treated with GM1892 compared to saline treated animals. GM1892 appears to decrease the hepatocellular damage caused by hemorrhagic shock which results in an improvement in hepatocellular function.

The cytokine data strongly suggests cytokine release as a critical part of the mechanism of action of GM1892. Plasma IL-6 levels were decreased by approximately 90% and plasma TNF levels were decreased by 78% in animals treated with GM1892. Prostaglandin levels were also decreased by the administration of GM1892. Plasma PGE_2 levels were decreased by approximately 65% in animals treated with GM1892 compared to saline treated animals. Plasma TNF as measured by ELISA was decreased in animals treated with GM1892 compared to saline treated animals but this effect was not statistically significant. If there had been no trend toward decreased levels TNF levels in the GM1892 and heparin groups it would have suggested the presence of a TNF binding protein or inhibitor. The present data with some decrease in TNF levels in the GM1892 and heparin treated group, however, lends support to an improvement in microcirculation as a possible mechanism. Another possibility is that there is a TNF inhibitor present in heparin but not in GM1892.

A mortality study was initiated that compared the survival of hemorrhaged animals treated with saline compared to the survival of hemorrhaged animals treated with GM1892. The mortality rates were different between the two groups but not significantly.

Although this trend would be likely to continue with more animals, such a large number of animals (>100) would be required to show a statistical difference that this study was abandoned.

The rat trauma-hemorrhage model used is reproducible, has a predictable outcome, and is similar to the clinical situation of trauma followed by hemorrhage. This model also involves multiple organ dysfunction which is often followed by organ failure and late mortality. The model uses unanesthetized animals to avoid the complicating effects of anesthesia. Anesthesia lowers the metabolic demands of organs and tissues dampening the effects of the trauma and hemorrhage. A fixed pressure model is also desirable because fixed volume models depend on the state of hydration of the animal. Fixed pressure models are physiologically similar and thus give a higher degree of reproducibility. A nonheparinized model is also required because of the complex effects heparin has on hemorrhaged animals as mentioned above. The trauma induced prior to hemorrhage is significant and alone produces immune function depression (Stephan, 1987). The addition of an initial trauma also makes the model more clinically relevant.

Crystalloid solution (RL) was used exclusively in this model for resuscitation. Shed blood was not transfused. Previous investigators have concluded that the critical systemic oxygen transport and fractional oxygen extraction are independent of hemoglobin concentration down to hemoglobin levels of at least 40% of normal (Heusser, 1989). Because the hemoglobin concentration in this study was approximately 50% immediately after resuscitation and at 24 hours after resuscitation, it is unlikely that the decrease in systemic hematocrit could cause any of the significant abnormalities that were identified.

GM1892 has been studied in the same rat model acutely after resuscitation (Wang, 1994a). In that study, the HR was found to be decreased during the entire study period. In contrast, in the present study the HR was increased in animals that underwent trauma and hemorrhage although less so in animals treated with GM1892. Another difference is that the SV was not significantly reduced at 2 hours after trauma and hemorrhage

although it was at 24 hours in the present study. TPR was found to be decreased in animals treated with GM1892 in both studies. The hepatocellular function was evaluated by the V_m which was not used in the present study. Microvascular blood flow was similar in the organs measured (liver, kidney, spleen, intestine) acutely and at 24 hours. The protective effect of GM1892 was also similar.

Potential mechanisms of action of GM1892

The improvement in most of the measured parameters was similar between GM1892 and conventional heparin suggesting a similar mechanism. The mechanism of action of GM1892 was investigated by measuring cytokine levels at 24 hours after trauma and hemorrhage. Microvascular blood flow was increased and proinflammatory cytokines were also decreased in those animals suggesting improvement in microcirculation as a possible mechanism. This is supported by studies indicating that modulation of activated neutrophils by heparin may decrease vascular injury (Greischlag, 1992). There is also an improvement in the splenocyte response to mitogen and the capacity of splenic and peritoneal macrophages to release IL-6 is restored in the hemorrhaged animals that receive GM1892 or conventional heparin.

The half life of GM1892 is unknown but is most likely similar to the half life of conventional heparin. The is no relationship between anticoagulant half-life and concentration half-life indicating that there might be some protein binding of heparin (Physicians Desk Reference, 1995). This does not appear likely in GM1892, however, because the levels of TNF were decreased in both the bioassay and the ELISA. Animals that undergo trauma-hemorrhage and resuscitation develop decreased microcirculatory blood flow which leads to cytokine and other mediator release leading to complications such as edema, multiple organ failure and death. Animals treated with GM1892, however, have an improved microcirculation which attenuates the mediator release both acutely and at 24 hours leading to decreased release of mediators, decreased complications and possibly to improved survival (Figure 18).

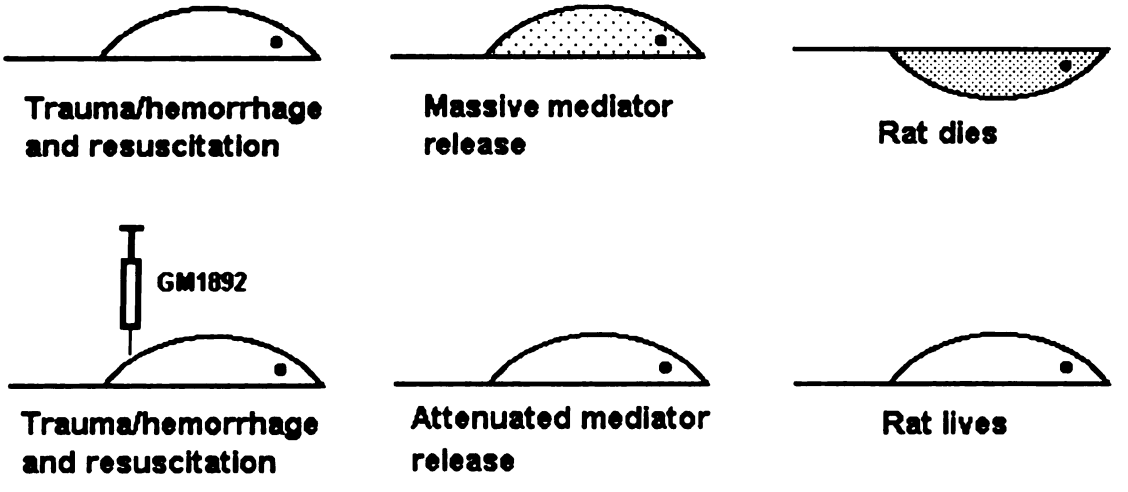

Figure 18. Summary of the mechanism of action of GM1892. Animals in both groups undergo trauma-hemorrhage and resuscitation. Animals treated with GM1892 have an attenuated mediator release leading to lower cytokine levels at 24 hours and possibly to improved survival.

Other potential mechanisms may be related to heat shock proteins, protamine or antithrombin III (AT III). Heat shock proteins may play a role, specifically, a 90-kDa stressprotein (HSP90), which has been found to have heparin and antibody-binding domains (Itoh, 1993). The use of protamine sulfate (a heparin antagonist) in patients has been associated with circulatory collapse because of depressed left ventricular function. Incubation with heparin before protamine addition prevents the negative effects of protamine on myocyte function (Hird, 1994). In one study, more severely injured adult trauma patients had low AT III levels at some time during hospitalization (Miller, 1994). AT III is potentiated in its anticoagulation effect by heparin although as mentioned earlier, GM1892 does not have this effect.

Other potential mechanisms of action could be similar to the mechanism of action of heparin which has been studied to a greater extent. There is a modulation of acute inflammation including modulation of smooth muscle cell proliferation, stabilization of cellular membranes, and inhibition of complement activation. These could lead to preservation of the vascular endothelium and decreased aggregation of platelets. There are also some vascular effects which may be involved in the mechanism proposed above. There may be an increased production of EDRF as well as modulation angiogenesis and modulation of platelet activation and aggregation leading to improvement of red blood cell flow to ischemic tissue in low flow situations such as shock. This protection of the microvasculature appears to be the most probable mechanism of action.

Potential clinical uses

GM1892 would most likely have potential clinical uses in patients with any form of ischemia and reperfusion injury where circulating mediators are thought to play a role. The clinical case most similar to the model used in this study is a trauma patient with acute hypovolemic shock secondary to hemorrhage who is resuscitated with crystalloid and has the bleeding controlled. These patients undergo a dramatic systemic release of cytokines resulting in multiple organ failure including ARDS and prolonged hospitalization in the intensive care unit. GM1892 may attenuate the mediator release thus decreasing the risk for multiple organ failure, sepsis and possibly decreasing the hospital stay. Other potential uses would be for transplant patients given donor organs with prolonged warm ischemia time, patients with peripheral vascular disease or coronary artery disease with tenuous blood flow requiring bypass, and vascular free flaps which have had a period of ischemia.

Limitations and future studies

There are several limitations to this study. The dosing regimen used was a single 7 mg/kg dose. This was effective but the effects of multiple dosing or continuous dosing are unknown. Other dosing regimens may provide more consistent blood levels and provide better results. Measurment of plasma drug levels (heparin, GM1892) would also be helpful in determining adequate availability. In this study, a single time point (24 hours after resuscitation) was used for measurement of TNF and IL-6. Serial cytokine measurements may provide more insight into the pattern of cytokine production and release in relation to trauma, hemorrhage, resuscitation, and recovery. Similarly, continuous monitoring of any or all of the physiologic parameters may provide more information about what organs are affected by the injury and the medication. More detailed studies regarding the effect of GM1892 on the immune system would be helpful to determine if the mechanism of action is similar to that of heparin. Finally, a life table

analysis of mortality with more frequent observations (hourly) would provide more information and decrease the sample size required for significant results.

CONCLUSION

These results indicate that cardiovascular and hepatic functions remain depressed at 24 hours after hemorrhage and resuscitation. Treatment with GM1892 or heparin, however, significantly improves these functions and reduces hepatic edema. Thus, GM1892, which unlike conventional heparin, has no significant anticoagulant activity, appears to be a useful adjunct for maintaining cardiovascular function and hepatic integrity following hemorrhage and resuscitation, even at 24 hours after the insult. Downregulation of proinflammatory cytokines by GM1892 may be the mechanism responsible for the beneficial effects of this agent following trauma-hemorrhage and resuscitation.

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