Immunemodulatory effects of hypertonic saline in hemorrhagic shock: in vivo alterations of neutrophil-endothelial dynamics and vascular permeability result in attenuated tissue injury

José L. Pascual Lopez

Submitted June 2004

Supervisor: Nicolas V. Christou

Department: Surgery
Division: Experimental Surgery
McGill University, Montreal, Canada

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for a doctoral degree

Your file Votre référence
ISBN: 0-494-12925-5
Our file Notre référence
ISBN: 0-494-12925-5

Abstract (English)

Multiple organ dysfunction is the most common cause of late mortality in trauma patients. Despite adequate resuscitation, hemorrhagic shock may progress to a state of profound systemic inflammation where the polymorphonuclear neutrophil (PMN) plays a key role. Resuscitation with hypertonic saline (HTS) may modulate the host inflammatory response in hemorrhagic shock.

A murine hemorrhagic shock model evaluated by cremaster intravital microscopy demonstrated significant in vivo attenuation of neutrophil rolling and adhesion to endothelium (EC) immediately after resuscitation with HTS, as compared to Ringer's lactate (RL). Concurrently, macromolecular leakage from the same post capillary venules was 45% lower in HTS animals.

To better simulate clinical conditions, the model was transformed to recreate two-hit conditions by subjecting resuscitated animals to a subsequent mimicked pulmonary infection. Attenuated neutrophil adhesion to endothelium in HTS animals persisted 5 and 22 hours after resuscitation. Additionally, compared to RL, HTS resuscitation reduced neutrophil lung sequestration (by the myeloperoxidase assay) and neutrophil lung transmigration (by histologic analysis) one day after resuscitation. HTS resuscitation also tended to improve cremaster and lung histologic injury a day after resuscitation conferring a 50% survival advantage for that time interval.

To determine if reductions in tissue injury were due to the ability of HTS to functionally block neutrophil adhesion to endothelium, another variation to the two-hit model was developed. Two additional groups were added to evaluate if supplementation of standard fluid resuscitation with anti adhesion monoclonal antibodies (anti-CD11b and anti-ICAM-1) would reproduce the effects of HTS alone. Although early EC/PMN interactions and 24-hour lung PMN accumulation were similarly attenuated by either HTS alone or RL with anti adhesion blockade, only HTS alone definitely reduced early in vivo macromolecule leakage, and one day lung histologic injury.

Hemorrhagic shock resuscitation with hypertonic saline reduces neutrophil activation and interactions with microvascular endothelium resulting in diminished lung PMN sequestration persisting well beyond the initial resuscitation phase. Yet the anti-adhesive effects of HTS are not essential for HTS-mediated reductions in tissue injury and organ dysfunction. Hypertonic resuscitation may prove to be an immunomodulatory therapy useful in critically ill trauma victims, the precise mechanisms of which need further elucidation.

Abstract (French)

Les patients hospitalisés pour traumatismes multiples sont à risque de succomber au Syndrome de Défaillance Multiviscérale (DMV). Malgré une réanimation isotonique adéquate, le choc hémorragique peut déclancher un état d'inflammation systémique pouvant mener au DMV, où, croit-on, le polynucléaire neutrophile (PNN) joue un rôle clé. Le salin hypertonique (SHT) utilisé pour fins de réanimation amoindrit l'activation du PNN, tout en atténuant la production de médiateurs inflammatoires et sa libération d'intermédiaires toxiques.

Nous avons établi un modèle où des rongeurs en choc hémorragique ont été réanimés soit par un régime conventionnel de lactate de Ringer (LR), soit avec 4 ml/kg de SHT. À l'aide de microscopie intravitale on observe chez les animaux réanimés au SHT une forte baisse du taux de roulement et d'adhérence de PNNs à la paroi de vénules post-capillaires. De plus, la paroi vénulaire de ces mêmes animaux démontre une fuite macromoléculaire qui est moins de 50% celle de constatée avec le LR. La réanimation au SHT entraîne donc une diminution d'intéractions neutrophile-endothéliales (INE) associées avec une réduction de la perméabilité vasculaire.

Pour mieux simuler les conditions cliniques nous élaborons un modèle plus complexe avec double atteinte à l'appareil immunitaire en subjuguant les animaux réanimés à une infection pulmonaire quelques heures plus tard. Dans ces nouvelles conditions on remarque une persistance de la diminution des INE jusqu'a 22 heures après la réanimation avec le SHT. En outre, l'histologie et l'analyse de myéloperoxidase démontrent que le LR occasionne une séquestration considérable de neutrophiles au parenchyme pulmonaire lors du même délai, ce qui est réduit significativement par le SHT. Du reste, le SHT entraîne une réduction nette du taux de mortalité tout en améliorant le dommage histologique induit au tissu pulmonaire et crémastérique.

Nous apportons alors une nouvelle altération au modèle animal afin de mieux déterminer si le mécanisme responsable de l'avantage du SHT est principalement relié à ses effets anti-adhésifs sur les PNNs. Aux deux groupes initiaux de simple réanimation avec le LR ou le SHT on ajoute deux nouveaux groupes expérimentaux où ces mêmes régimes sont supplémentés d'anticorps bloqueurs du CD11b et de l'ICAM-1, récepteurs responsables pour l'adhésion du PNN à l'endothélium. Comme anticipé, les INE et la séquestration pulmonaire de PNNs sont atténuées par le LR supplémenté d'anticorps comme par le SHT seul. Toutefois, seul le SHT réduit définitivement la perméabilité vasculaire et le dommage pulmonaire histologique après 24 heures. Le LR même supplémenté d'anticorps contre l'adhésion semble, au contraire, augmenter le dommage pulmonaire après la réanimation.

La réanimation du choc hémorragique par salin hypertonique réduit l'activation du neutrophile et ses interactions avec l'endothélium. Ceci entraîne une résistance à la séquestration pulmonaire de neutrophiles qui persiste au-delà de la phase post-réanimatrice. Néanmoins, ces effets anti-adhésifs du SHT ne semblent pas être responsables de la réduction du dommage pulmonaire observé avec LR. En conclusion, le salin hypertonique pourrait servir d'immunomodulateur réduisant l'inflammation systémique et la dysfonction organique occasionnée chez certains multitraumatisés en choc hémorragique.

Table of Contents

Contribution of Authors
Manuscript #1

José L. Pascual researched, wrote and edited the manuscript for an invited presentation at The Fluid Resuscitation in Combat meeting of the Canadian Defence and Civil Institute of Environmental Medicine. Kosar A. Khwaja and Prosanto Chaudhury, co-workers in the laboratory, assisted with editing of the manuscript. Nicolas V. Christou was the research supervisor, providing direction and guidance as well as funding and educational opportunities, assisting with discussion and editing of the manuscript.

Manuscript #2

José L. Pascual performed all in vivo experiments, ex vivo dissections and researched, wrote and edited the manuscript. Lorenzo E. Ferri a co-worker in the laboratory taught José L. Pascual the intravital microscopy surgical technique and procedure, also assisting in the editing of the manuscript. Andrew J.E. Seely, Giuseppina Campisi and Prosanto Chaudhury, co-workers in the laboratory assisted with interpretation and discussion of the data and editing of the manuscript. Betty Giannias, chief technician of the laboratory helped with blinded quantification of neutrophil endothelial interactions as well as general technical questions. David C. Evans and Tarek Razek, trauma surgeons, assisted in the interpretation and discussion of data and editing of the manuscript. René P. Michel, pathologist, assisted with immunohistologic techniques and performed blinded cremaster section grading. Nicolas V. Christou, research supervisor, offered direction and guidance, assisting with the interpretation of data, discussion and editing of the manuscript.

Manuscript #3

José L. Pascual performed all in vivo experiments, myeloperoxidase assays, ex vivo dissections and researched, wrote and edited the manuscript. Kosar A. Khwaja and Lorenzo E. Ferri, co-workers in the laboratory assisted with interpretation and discussion of the data helping with manuscript editing. Betty Giannias, chief technician of the laboratory helped with myeloperoxidase assays and provided general technical help. David C. Evans and Tarek Razek, trauma surgeons, assisted in interpretation of the data and discussion and editing of the manuscript. René P. Michel, pathologist, provided assistance with lung section preparations, performed blinded histologic grading of lung sections and assisted in manuscript editing. Nicolas V. Christou, research supervisor, offered direction, guidance and funding; assisting with interpretation and discussion of the data and editing of the manuscript.

Manuscript #4

José L. Pascual developed the model and performed all in vivo experiments, myeloperoxidase assays, ex vivo dissections, ELISA protocols and researched, wrote and edited the manuscript. Lorenzo E. Ferri, Kosar A. Khwaja and Shea T. Chia, co-workers in the laboratory assisted with interpretation and discussion of the data helping with manuscript editing. Betty Giannias, chief technician of the laboratory helped with myeloperoxidase assays, ELISA protocols and provided general technical help. Renée Bernatchez helped with the development of histologic techniques and tissue processing. David C. Evans, trauma surgeon, assisted in interpretation of the data and discussion and editing of the manuscript. René P. Michel, pathologist, provided assistance with lung section preparations, performed blinded histologic grading of lung and cremaster sections and assisted in manuscript editing. Nicolas V. Christou, research supervisor, offered direction, guidance and funding; assisting with interpretation and discussion of the data and editing of the manuscript.

Manuscript #5

José L. Pascual developed the model and performed all in vivo experiments, myeloperoxidase assays, ex vivo dissections, and researched, wrote and edited the manuscript. Lorenzo E. Ferri, co-worker in the laboratory assisted with interpretation and discussion of the data helping with manuscript editing. Kosar A. Khwaja, a co-worker in the laboratory helped with ex-vivo PMN activation and flowcytometry analysis. Betty Giannias, chief technician of the laboratory helped with myeloperoxidase assays, flowcytometry PMN receptor determination and provided general technical help. David C. Evans, trauma surgeon, assisted in interpretation of the data and discussion and editing of the manuscript. René P. Michel, pathologist, provided assistance with lung and cremaster section preparations, and their blinded histologic grading and assisted in manuscript editing. Nicolas V. Christou, research supervisor, offered direction, guidance and funding; assisting with interpretation and discussion of the data and editing of the manuscript.

Introduction and Review of the Literature

1.1 Foreword

In recent years, institutions caring for sick patients have been looking after an increasingly ill population. Today's intensive care units commonly treat critically unstable patients, harboring various failing organ systems that, only decades ago, would have resulted in the patient's demise. Such patients are now surviving as a consequence of major advancements in intensive and post-anaesthsesia care[1,2]. Nonetheless, contemporary intensive care units around the world still contend with a significant mortality, much of which is attributed to multiple organ dysfunction syndrome (MODS), a progressive systemic inflammatory condition displaying various organ systems which fail in unison or in succession[3].

Resuscitated hemorrhagic shock, one of the conditions able to lead patients to MODS, has been studied and discussed in medical and scientific circles since antiquity[4]. Throughout history, major therapeutic advancements in the treatment and management of hemorrhagic shock were acquired in times of war and conflict, primarily since the 17th century[5]. A key immune system participant in the progression of hemorrhagic shock to MODS is the polymorphonuclear neutrophil (PMN), an essential constituent of the host immune response. Longstanding widespread scientific interest in neutrophil structure and function may be explained in part by the cell's paradoxical nature. On one hand, it is the first and most abundant member of the host's innate immune response and is indispensable in protecting the host from invading pathogens such as bacteria and fungi. Indeed, mild derangements in neutrophil function result in serious impairment to host immunity manifested clinically as recurrent life-threatening infections. On the other hand, the neutrophil may produce lethal tissue injury resulting in unrelenting systemic inflammation and organ dysfunction[6]. In the last half century, researchers have exposed several lines of evidence demonstrating the neutrophil's pivotal role in systemic

inflammation resulting in host tissue injury. It is in the microcirculation, and more importantly in the post capillary venule (PCV) that close interactions between neutrophils and endothelial cells (ECs) are believed to initiate the process of global inflammation.

Interestingly, research in the specific pathophysiology of resuscitated hemorrhagic shock has implicated the neutrophil as a prime accessory in host progression to MODS. Indeed, while fluid resuscitation is the only life-saving therapy of hemorrhagic shock, it may also contribute to worsening neutrophil mediated tissue injury. The nature and quantity of resuscitation regimens have been shown to considerably modulate deranged neutrophil function after resuscitated hemorrhagic shock. Hypertonic saline (HTS), a sodium chloride solution hypertonic and hyperosmolar to plasma is not only safe and efficacious in the resuscitation of shock, but additionally possesses immune modulating abilities shown to attenuate organ-level injury.

These immune altering qualities of HTS hold profound implications in the long and mostly unsuccessful search for the ideal treatment of hemorrhagic shock. Furthermore, these effects hold great promise in eventually reducing MODS-related mortality in resuscitated trauma victims. The present work will hopefully contribute to further elucidate the immunomodulating characteristics of hypertonic saline in shock, particularly through the exploration of its in vivo effects and the consequences of neutrophil mediated injury at the organ level.

1.2 Neutrophils, Endothelium and their Physiological Interactions

1.2.1 The Polymorphonuclear Neutrophil

The polymorphonuclear neutrophil is likely the most extensively studied cell of the host inflammatory response. It is the principal circulating phagocyte, a member of the innate immune system released first and most abundantly in response to host injury or insult. Rapidly detecting signs of tissue injury or microbial assault, the neutrophil

responds by moving away from its quiescent circulatory environment, crossing the vascular endothelial monolayer and migrating to the site of injury or invasion. Once there, the PMN may destroy foreign particles through phagocytosis or the release of an impressive assortment of cytotoxic mediators. Even mild derangements in neutrophil immune function may result in recurrent life-threatening illnesses.

On the other hand, neutrophil cytotoxic abilities may also cause inadvertent host tissue injury by the inappropriate intravascular release of byproducts capable of producing noxious effects remote from the area of injury or invasion. Thus, 'the double edge sword' nature of PMNs: essential for host defence but also capable of causing its demise.

The PMN originates from pluripotent progenitor stem cells that develop into the myeloid lineage; from promyeloblast, to myeloblast, to myelocyte, and to metamyelocyte[7]. These myelocytic differentiations occur in the bone marrow phase of the PMN life cycle which ends as the mature neutrophil is released into the circulation. Maturation of the neutrophil in the bone marrow phase takes twelve to sixteen days culminating in the release of 10^8 to 10^{11} cells into the circulation per day[7]. Myeloid differentiation is mediated by a variety of growth factors, most notably granulocyte/macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF). Alterations in these and other marrow growth factors results in variations in the production of PMNs allowing increases of more than ten fold during the host stress response[8]. After release from the bone marrow, the mature PMN enters the circulating phase, reversibly moving from the circulating and marginating pools. The PMN's circulating phase half-life is 6 to 9 hours. The much larger marginating PMN pool comprises the majority of intravascular PMNs which are 'stored' in the microcirculation of certain organs such as the lung, capable through its narrow capillary beds, to 'trap' passing neutrophils[9]. The marginated PMN pool is rapidly mobilized in response to host

injury or insult. In humans, neutrophils comprise more than 90% the circulating phagocytes and maintain a relatively stable count of 3000 to 4000 cells/mm^3 in physiologic conditions[10]. To enter the final 'tissue' phase, PMNs must undergo a series of interactions with endothelial cells (ECs) in order to move extravascularly.

1.2.2 The Endothelium

For a long time, the cellular lining of blood vessels or endothelium, was believed to be an inert bystander to the intricate events occurring in the circulatory and interstitial milieu. Extensive research on endothelial cell structure and function has revealed its far-reaching metabolic and homeostatic functions. The endothelial monolayer separating the vascular lumen from smooth muscle cells of the vessel wall is capable of transducing blood-borne signals, sense mechanical shear forces within the lumen, control vascular permeability, regulate vascular tone and blood flow and interact with other cellular elements of the circulation[11]. The endothelial cell's ability to react to various activating or inhibiting signals brings about its secretion of an assortment of mediators, cytokines and other substances that have a profound effect on local and systemic physiology and pathophysiology. Nitric oxide (NO), for example, is released by endothelial cells after activation by different stimuli and is a key mediator in the relaxation of muscular smooth muscle resulting in vasodilatation[12]. The effects of NO extend beyond regulation of vascular tone, and include inhibition of platelet aggregation and adhesion, scavenging of reactive oxygen species (ROS), maintenance of normal vascular permeability, inhibition of smooth muscle proliferation, stimulation of endothelial cell regeneration and attenuation of leukocyte adherence[13,14]. Endothelial derived prostaglandins such as prostacyclin (PGI2) are also important vasodilators involved in vascular injury and repair, similarly affecting platelet function[12]. Conversely, endothelial cells also secrete potent vasoconstrictors such as platelet activating factor (PAF), endothelin-1, thromboxane A_2

($TBXA_2$) and others. The regulation of vasoconstrictor and vasodilator secretion allows endothelial cells to precisely and exquisitely control vascular tone. The endothelium is also an ideal regulator of hemostasis, able to transform itself from a potent antithrombotic to a prothrombotic surface[12]. This hemostatic versatility permits rapid conformational change to accommodate mechanical damage or activation of endothelial cells by agents such as cytokines, endotoxin, hypoxia, or hemodynamic forces[11]. As barrier cells separating the intravascular and tissue compartments, endothelial cells possess surface adhesion molecules that interact with ligands and counterreceptors on other cells, most notably PMNs, to allow their passage through the vessel wall; a process termed transmigration.

1.2.3 Neutrophil/endothelial Interactions

1.2.3.1 PMN Rolling and Selectins

PMN passage to areas of injury is a key step to an adequate host immune response. Occurring mostly in post capillary venules, it involves a series of discrete interactions with endothelial cells. The first phase involves the *margination* of circulating neutrophils to the periphery of the vessel wall. This occurs when the PCV diameter is 50% larger than the diameter of the neutrophil and erythrocytes which are moving faster than leukocytes at the center of the vessel push leukocytes to the periphery[15]. The resultant proximity between neutrophils and endothelial cells allows for adhesive interactions to occur between their surface receptors. These weak initial interactions occurring primarily between surface selectins (L, E and P)[16] slow marginated neutrophils, and impart on them a rolling motion. PMN 'rolling' ability is also dependent on vascular rheologic forces, which are directly proportional to erythrocyte velocity and vascular shear stress[17].

Selectins are a family of glycoprotein adhesion molecules found on the surface of leukocytes (L-selectin), endothelial cells (E-selectin, P-selectin), and platelets (P-selectin). PMN L-selectin is inherently expressed on the surface of all quiescent leukocytes (except some populations of lymphocytes), and, depending on the degree of cellular activation, is either upregulated or shed by cleavage at the cell surface to its soluble form (sL-selectin)[18]. To induce neutrophil rolling, PMN L-selectin forms a weak bond with syalylated Lewis (SLe^x) carbohydrate determinants on the surface of the EC[16]. Endothelial P-selectin is also involved in neutrophil rolling by binding P-selectin glycoprotein ligand-1 (PSGL-1), a carbohydrate complex expressed by PMN, which also contains Sle^x determinants[19]. Blocking L or P-selectin in animals using a selectin binding carbohydrate (fucoidin) decreases in vivo neutrophil rolling following local insults[20]. Likewise, the use of specific monoclonal antibodies against selectins also reduce PMN rolling[21,22]. Furthermore, L-and P-selectin gene deficient mice demonstrate diminished rolling following activation[22,23].

1.2.3.2 PMN Adhesion, Integrins and the Immunoglobulin Superfamily

The weak selectin-mediated interactions causing neutrophil rolling on endothelium are believed to be a prerequisite for the stronger more permanent interactions that follow. In particular, neutrophil adherence to endothelium follows rolling and is largely mediated by PMN β_2-integrins (CD18/CD11) which elaborate high affinity bonds with endothelial surface receptors of the immunoglobulin (Ig) superfamily (intercellular adhesion molecule -1 [ICAM-1], ICAM-2). Integrins are a family of transmembrane heterodimeric proteins located on the cell surface which consist of α and β subunits. β_2 integrins are restricted to leukocytes and are essential to their normal trafficking. They are classified by their various α subunit isoforms (CD11a, CD11b,

CD11c), each of which can be upregulated depending upon the given conditions of neutrophil activation[24]. Enhanced CD11 expression has been demonstrated in neutrophils activated by various substances including N-formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, tumor necrosis factor-α (TNF-α), and other cytokines[25]. Endothelial ICAMs are also transmembrane polypeptides classified by five constant region C2 domains belonging to a given Ig superfamily[26]. Both endothelial ICAM-1 and ICAM-2 bind PMN surface β_2 integrins resulting in firm adhesion of the two cells. Several stimuli cause the upregulation of constitutively expressed surface ICAMs including endotoxin, IL-1 and TNF-α[27,28]. Monoclonal blockade against CD18/CD11 or ICAM-1 has demonstrated diminished neutrophil adherence to endothelium[27,29,30]. As well, CD18 and ICAM-1 gene knock-out animals also demonstrate diminished neutrophil adhesion to their endothelium[31-33].

Integrin mediated neutrophil adhesion to endothelium appears to be essential for host defence; a principle well illustrated in patients with Leukocyte Adhesion Deficiency-1 (LAD-1). Afflicted patients have a mutation of the CD18 integrin gene and are found to develop lethal bacterial infections early in life, as their neutrophils are unable to reach sites of infection[34]. On the other hand, numerous experiments using neutralizing monoclonal antibody blockade against β-integrins and/or ICAMs have demonstrated attenuated neutrophil mediated injury in models of allograft rejection, endotoxin challenge, hemorrhagic shock, ischemia/reperfusion and acute lung injury[35]. Thus, while neutrophil adhesion to endothelium is a key step for appropriate leukocyte trafficking, it is also an important mediator of tissue injury from systemic inflammation. Once the neutrophil is firmly adherent, it is able to transmigrate through the endothelial monolayer in order to reach the area of tissue injury or invasion.

1.2.3.3 PMN Transmigration and Directed Migration

PMN transmigration involves diapedesis, a process whereby neutrophils extend pseudopods through the narrow passages between endothelial cells, which carries the leukocyte through the endothelial monolayer[36]. Gaps between endothelial cells are poorly described but have been noted to widen following different stimuli. An in vitro model has demonstrated that adherence of neutrophils to endothelium results in disruptions of EC cell-cell interactions, augmenting endothelial cell permeability[37]. Electron microscopy suggests this process occurs at EC tri-cellular corners, where three adjacent endothelial cells are in contact[38]. Several leukocyte and endothelial adhesion molecules are involved in the process of diapedesis most notably platelet-endothelial cell adhesion molecule-1 (PECAM-1). PECAM-1 is concentrated in the lateral borders of endothelial cells as well as on the surface of leukocytes and platelets[39,40]. Blockade of PECAM-1 with monoclonal antibodies results in increased numbers of adherent neutrophils unable to undergo diapedesis[41,42]. Adhesion molecules responsible for other neutrophil interactions with endothelium also appear to govern neutrophil diapedesis and transmigration. In different animal inflammatory models, leukocyte emigration through the vessel wall was significantly reduced with monoclonal antibodies to CD18, CD11b, ICAM-1 and L-selectin[30,43]. Once transmigrated into the interstitial milieu PMNs migrate down chemotactic gradients to the given area of infection or injury in order to destroy unwanted substances and organisms.

1.2.3.4 PMN Degranulation, Phagocytosis and Cytotoxicity

Polymorphonuclear neutrophils are capable of destroying invading or discarded substances in two ways: phagocytosis or cytotoxic release of toxic products for

destruction in situ. Phagocytosis is triggered when unrecognized substances come in contact with neutrophils through antigenic or opsonin surface presentation. The principal opsonins are complement fragments and antibodies. The neutrophil ingests the presented substance by invaginating its cell membrane and engulfing it, incorporating it into a phagocytic vesicle or phagosome. The destruction of engulfed substances then occurs through degranulation or fusion of lysosomes containing enzymes and reactive oxygen species to the phagosome itself resulting in the formation of a phagolysosome. Degranulation may also occur when lysosomes fuse with the plasma membrane resulting in the release of contents to the outside environment. Cytotoxic degranulation to the extracellular milieu is thus the second manner by which neutrophils can destroy adjacent unwanted substances. However, as this process is not contained, it may inadvertently result in nearby host tissue injury.

The microbicidal substances released by degranulating PMNs are divided into two categories: proteolytic enzymes and reactive oxygen species (ROS)[44]. Enzymes including mostly proteinases such as elastase, myeloperoxidase (MPO), cathepsins, collagenases and esterases are found in intracellular granules and serve to destroy organic material through their digestion of polypeptides, nucleic acids and membranes. ROS, originate at the cell surface, where activation of the membrane associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme converts oxygen to potent oxidizing byproducts. This process termed the respiratory burst, partially reduces molecular oxygen (O_2) to superoxide radical (O_2^-) with subsequent conversion to equally reactive hydrogen peroxide (H_2O_2), singlet oxygen ($O\cdot$) and hydroxyl ($OH\cdot$) radicals. Furthermore, through the catalysis of neutrophil MPO, H_2O_2 converts Cl^- to the highly reactive hypochlorus acid (HOCl) likely the most potent of respiratory burst oxidizing byproducts. These oxygen free radicals released through degranulation destroy pathogens and unwanted organic substances by the rapid

disruption of lipid bilayers[45]. Thus, the microbicidal activity of neutrophils is achieved through the synergistic release of intracellular enzyme granules and membrane derived oxygen radicals, both capable of potent organic tissue destruction.

1.3 Hemorrhagic shock and resuscitation

Rapid blood loss resulting in hypovolemic shock can be a lethal event causing death. While blood loss can occur in different conditions (gastrointestinal bleeds, ruptured aneurysms), traumatic vessel injury, within or outside organs, is the main cause of hemorrhage in trauma. In the context of blunt or penetrating injuries, hemorrhagic shock accounts for more than half of all early trauma related deaths (<48 hours)[46]. More specifically, in the theatre of war, one in five injured combatants die in battle, with exsanguination being the single major, potentially salvageable cause of field deaths[5]. In the Vietnam conflict, for example, more than 50% of battlefield casualties died of hemorrhagic shock[47]. The importance of hemorrhagic shock is further underscored in ever-increasing modern urban trauma.

While most early trauma deaths are due to hemorrhage, few to none occurring beyond a week after injury are secondary to blood loss. The overwhelming majority of late trauma deaths are due to sepsis or organ dysfunction[48]. Nonetheless, an increasing body of evidence suggests that the manner in which the initial hemorrhage resuscitation is conducted, can profoundly affect the natural history of late deaths. Finding improved therapies for the resuscitation of hemorrhagic shock are thus of prime importance if reductions in both early and late mortality of trauma victims is to be envisaged.

1.3.1 Macrohemodynamic Dysfunction in Hemorrhagic shock

In 1895 John C. Warren described hemorrhagic shock as 'a momentary pause in the act of death'[49]. Currently, one of the better definitions, albeit less dramatic, can be

found in the American College of Surgeons Advanced Trauma Life Support (ATLS®) guidelines, which divide hemorrhagic shock into 4 classes (Table 1). Active hemorrhage deteriorates into shock when diminished effective circulating volume causes critical reductions in cardiac filling pressures. With hypovolemic reductions in cardiac output, tissue perfusion is proportionately decreased, triggering arterial baroreceptors (carotid sinus, aortic arch), volume (cardiac ventricles) and stretch (atria) receptors activating the sympathoadrenal axis. The ensuing result is a massive release of epinephrine, norepinephrine and subsequently, cortisol from the adrenal gland[50]. Other potent vasoconstrictors such as vasopressin from the posterior pituitary gland and angiotensin from the kidney are also concurrently secreted.

Table 1: ATLS® Classification of Hemorrhagic Shock

Class	Blood loss	Volume loss	HR	BP	RR	U/O	Mental status	Therapy
I	<15%	<800cc	ØΔ	ØΔ	ØΔ	ØΔ	ØΔ	+/- crystalloid
II	15-30%	0.8-1.5L	↑	ØΔ	↑	20-30cc/h	anxiety	crystalloid
III	30-40%	1.5-2L	↑	↓	↑	5-15cc/h	confusion	Blood + crystalloid
IV	>40%	>2L	↑↑	↓↓	↑↑	negligible	lethargy	Blood + crystalloid

ATLS: Advanced Trauma Life Support, HR: heart rate, BP: blood pressure, RR: respiratory rate, U/O: urine output
<: less than, >: greater than, ØΔ: no change, ↓:decrease, ↑:increase

While catecholamines immediately increase heart rate and minute ventilation, neuroendocrine reflexes, result in a slower, prolonged reabsorption of tissue fluids at the level of capillaries, thereby increasing effective circulating volume. Loss of up to 15% of total blood volume (Class I hemorrhagic shock) is well tolerated and usually does not affect hemodynamic parameters as compensatory mechanisms are capable to restore intravascular volume deficits rapidly. Blood volume deficits beyond 25% produce measurable deficits in blood pressure and rises in the sympathetic vascular tone of arterioles and capacitance vessels. Yet, vasoconstriction does not affect all capillary beds equally, favouring cardiac and cerebral tissue at the expense of splanchnic, renal and muscular beds[51]. Class II, III and IV hemorrhagic shock require prompt exogenous

fluid administration usually in the form of isotonic crystalloids such as lactated Ringer's (LR, RL) or normal saline (NS, 0.9% NaCl). Volume loss beyond 40% of total blood volume (Class IV hemorrhagic shock) is life threatening. Arterial pressure below 60 millimeters of mercury (mmHg) results in inadequate cerebral blood flow giving rise to extreme sympathetic activation with intense peripheral vasoconstriction[52].

Hemorrhagic shock decompensation arises from failure to maintain cardiac function despite maximal arterial and venous constriction. Failure to maintain sympathetic tone results in paradoxical vasodilatation and reductions in peripheral vascular resistance which further compromise cardiac filling pressures. The loss of vascular tone is a reliable indicator of impending irreversible hemorrhagic shock[53]. Two major factors appear responsible for this decompensation, both of which occur in the microcirculatory milieu: accumulation of blood in capacitance vessels and the development of leaky capillaries losing fluid into the interstitium (edema)[51].

1.3.2 Global tissue-level ischemia/reperfusion injury

The key end-organ event in shock is an inadequate oxygen delivery (DO_2) to meet increased tissue oxygen consumption (VO_2). Inadequate DO_2 resulting in persistently ischemic tissues is an early event in shock preceding hypotension and an important contributor to increased mortality[54,55]. The inability of the heart and vasculature to maintain physiologic function is believed to occur secondarily to the acidosis and toxemia arising from systemic circulation of metabolites released from ischemic tissues[56]. Prolonged tissue hypoxia results in the loss of membrane polarity and an inability to maintain trans-membrane ionic gradients with the subsequent inhibition of mitochondrial activity[51]. This further hampers cellular ability to generate high energy phosphate compounds which are vital for membrane integrity. Additionally, decompensating

hemorrhagic shock is accompanied by profound aberrations in coagulation processes, with dysfunctions in the reticuloendothelial system and alterations of immune function.

Irreversible hemorrhagic shock, therefore occurs when global ischemia/reperfusion injury can no longer be counterbalanced by compensatory mechanisms. Capillary beds become progressively occluded by swollen endothelium and sequestrated cells (particularly neutrophils) and debris preventing the restoration of downstream tissue perfusion and contributing further to cellular hypoxia. This process, termed the 'no-reflow phenomenon' is typically observed at the onset of irreversible hemorrhagic shock and can be detected by the lack of hemodynamic improvement despite adequate volume restoration[57]. As neutrophils circulate through sluggishly flowing capillaries they come in close proximity to activated endothelial cells, becoming themselves activated. The otherwise well ordered sequential EC/PMN interactions become chaotic and unbridled. Sequestered activated neutrophils may inadvertently release reactive oxygen species and proteolytic enzymes causing local tissue damage and worsening microvascular leakiness[58].

1.3.2.1 SIRS and MODS

There are numerous conditions where the host inflammatory response results in host tissue injury. Illnesses such as severe pancreatitis, the adult respiratory distress syndrome (ARDS), multiple trauma, and severe hemorrhagic shock all may result in persistent, unremitting over-stimulation of the host inflammatory response causing the systemic inflammatory response syndrome (SIRS)[59,60]. Multiple organ dysfunction syndrome (MODS) can be seen as the lethal complication of SIRS, often resulting in the patient's demise. Initially described as the cause of mortality following aortic aneurysm rupture, this condition is characterized by the sequential failure of different organ systems culminating in death[3]. While different, apparently heterogeneous conditions can

result in MODS they all share a setting of persistent stimulation of host immunity, precipitating an uncontrolled systemic inflammatory response causing remote organ injury[61]. Today, MODS is the leading cause of death in intensive care units carrying a mortality of 30-100%[62,63]. The recent apparent rise in incidence of this syndrome is likely due to key advances in the treatment of acutely ill patients, maintaining alive those who, in the past, would not have survived the initial illness[64,65]. Innovative health technology permitting the complete artificial support of failing organ systems is partly responsible for this outcome reversal.

The link between neutrophils, SIRS and MODS has been demonstrated by multiple lines of evidence. Neutrophil tissue sequestration and amount of neutrophil by-products have been correlated with the presence and severity of inflammation in different disease states. In hemorrhagic shock models, neutropenic animals developed diminished organ injury[66] and displayed a significant survival advantage compared to non neutropenics[57,58]. Other animal models involving local ischemia/reperfusion of muscle[67], lung[68] and brain[69] also revealed diminished tissue injury in neutropenic animals compared to controls. Reduced mortality and tissue injury has also been noted in neutropenic animals with acute pancreatitis[70], sepsis[71] and acute lung injury[72,73]. The specific event that initiates escalating neutrophil and inflammatory self-perpetuating activation remains elusive. The concept of gut bacterial translocation as this triggering event has been proposed by some[74,75]. The gut is one of the earliest tissues to lose its integrity following ischemia. Intestinal mucosa then loses its barrier function and luminal bacteria are allowed to translocate into the circulation. Systemic dissemination of bacterial proinflammatory substances (such as endotoxin) has been proposed as a key event in uncontrolled neutrophil activation contributing to the host's progression into systemic inflammation and multiorgan dysfunction[74,75]. Otherwise referred to as the 'gut-liver-lung axis', this attractive paradigm suggests that prolonged hypoperfusion from

hemorrhagic shock results in gut mucosal injury, local activation of neutrophils and the release of mediators and bacterial toxins into lymph and circulation. Once these factors reach the liver and lung, they induce further inflammatory changes, resulting in additional neutrophil activation and mediator release, leading to systemic inflammation and remote organ injury[51]. Hence, the neutrophil, with its considerable supply of toxic reactive oxygen species and proteolytic enzymes may be the key element in the tissue injury and organ damage occurring in systemic inflammatory conditions such as resuscitated hemorrhagic shock.

1.3.2.2 PMN Priming and Activation – The 'Two-Hit" Theory

Despite what would seem as an inherent tendency for neutrophils to be effortlessly activated, this process does not occur haphazardly. It is perhaps an evolutionary drive to prevent PMN oversensitivity to activation that has lead neutrophils to develop a step wise activation process to launch their microbicidal activity. Several investigators have observed that neutrophils can be *primed* by a given stimulus without degranulating or responding functionally. A subsequent stimulus then *activates* the PMN, for an augmented response with massive release of toxic products through degranulation[76]. Thus, priming is a process whereby the response of neutrophils to an activating stimulus is potentiated by prior exposure to a priming agent or stimulus which in itself does not elicit effector function[77]. In vitro, neutrophil priming agents such as lipopolysaccharide (LPS), phorbol myristate acetate (PMA), interleukin-1 (IL-1), certain leukotrienes, tumor necrosis factor (TNFα) and other mediators, have been shown, at small doses, not to elicit a functional response from neutrophils while inducing an augmented response with subsequent activation[78]. The distinctness of neutrophil priming and activation has been demonstrated *in vitro* in studies where activated but not

primed human neutrophils were capable of enhanced superoxide release and tissue sequestration as well as injury to endothelial cells[79]. While this would imply that neutrophils exist in three distinct states: resting, primed or activated, this is likely oversimplistic as PMNs more likely exist in a continuum of states spanning these three categories[78].

While this step-wise stimulation may have been originally intended to control over-exuberant PMN function, neutrophils continue to demonstrate dysfunctional exaggerated responses in conditions of systemic inflammation. Clearly, inappropriate levels of priming may also lead to unbridled and uncontrolled subsequent neutrophil activation leading to systemic inflammation and tissue injury[80]. Furthermore, as activation in the setting of prior priming is greatly potentiated, the step-wise activation process may result in augmented systemic inflammation.

The priming/activation concept is exemplified clinically by the two–hit or two-insult theory which maintains that in certain conditions, a given insult to the host (such as resuscitated hemorrhagic shock) may not immediately lead to the systemic inflammatory response. A second subsequent, even innocuous stimulus (aspiration, upper gastrointestinal bleed, minor surgery), leads to complete activation of the host inflammatory response with resultant tissue damage and organ injury[61]. I*n vivo* animal studies have demonstrated how two spaced insults resulted in microcirculatory compromise[81], and more recently how two-insult conditions lead to distinctly worse organ dysfunction. In a porcine model an intravenous pseudomonas infusion in the group submitted to antecedent resuscitated hemorrhage resulted in worse hemodynamic parameters and Acute Physiology And Chronic Health Evaluation (APACHE) scores than controls only receiving the infusion[82]. Similarly, in a murine model where a femoral fracture accompanied by resuscitated hemorrhagic shock constituted the first hit, only the group undergoing subsequent ceacal ligation and puncture (CLP) demonstrated

significantly worse post-mortem pulmonary and hepatic histologic injury in addition to an 85% reduction in survival[83].

1.3.2.3 Shock Resuscitation as a Systemic Reperfusion Injury

In either one or two hit conditions, the systemic hypoperfusion and global end-organ ischemia that accompany severe hemorrhagic shock may trigger the host inflammatory response with unintended neutrophil degranulation resulting in tissue injury. Intravascular replenishment with fluid must be urgently instituted to prevent end organ anoxia, loss of transmembrane ionic gradients and cellular death. Clearly, hemorrhagic shock must be reversed with adequate fluid resuscitation or the condition is uniformly lethal[84]. Nonetheless, while tissue ischemia in hemorrhagic shock activates the inflammatory response and can lead to neutrophil-mediated tissue injury, fluid resuscitation may, in fact, worsen these effects. Several studies report that despite adequate resuscitation, some hemorrhagic shock victims will develop systemic inflammation, organ dysfunction and subsequently die. Indeed, multiple organ dysfunction syndrome (MODS) is the most common cause of post-traumatic death occurring more than 48 hours after resuscitation[46,85,86]. Paradoxically, the restoration of nutritive tissue blood flow by resuscitation initiates a cascade of events at the microcirculatory level that may worsen the existing hypoxic cellular injury. Resuscitated hemorrhagic shock can be viewed as 'whole-body' ischemia/reperfusion (I/R) where different capillary beds first rendered ischemic by global hypoperfusion are subsequently reperfused by the administration of fluid. As with reperfusion in I/R injury, locally-released toxic ischemic by-products are no longer self-contained in the sluggish capillary beds where they are produced. Fluid resuscitation, recirculates these byproducts systemically causing further generalized inflammatory upregulation of cytokines, oxygen free radicals, archidonic acid metabolites and the other inflammatory mediators[51]. Thus,

resuscitated hemorrhagic shock is in itself a double insult to the host first through global hypoperfusion and second by systemic recirculation of mediators and toxic by-products of neutrophils and other inflammatory elements.

1.3.2.4 Inflammatory Mediators

The sustained presence of circulating inflammatory mediators following resuscitation of hemorrhagic shock results in global microcirculatory cellular activation. Arachidonic acid metabolism through the cyclo-oxygenase pathway (leading to eicosanoids: prostaglandins and thromboxanes) and through the lipoxygenase pathway (leading to leukotrienes) yields these key mediators that are produced and released in resuscitated hemorrhagic shock. Thromboxane A_2 (TBX A_2), a potent vasoconstrictor, is initially released in large quantities. TBX A_2 has a central role in resuscitated hemorrhagic shock and I/R injury promoting neutrophil margination and platelet aggregation into 'hemostatic plugs' and contributing to downstream tissue hypoxia[87]. Vasoconstricting leukotrienes are also released, further contributing to tissue hypoxia. Blockade of the lipooxygenase pathway has been shown to diminish metabolic injury and mortality in hemorrhagic shock[88]. Platelet activating factor (PAF) is another mediator that is secreted by neutrophils and endothelium in resuscitated hemorrhagic shock. PAF promotes neutrophil and platelet aggregation as well as vascular permeability, and itself activates both PMNs and ECs, making its effects self perpetuating[89]. While prostacyclin (PGI_2) and other vasodilators are subsequently released to restore perfusion, their actions are overwhelmed as hemorrhagic shock progresses to decompensation. The complement system is also activated early after hemorrhage and can induce rapid and fulminant injury to different organ systems by interacting with neutrophils and the humoral system[90].

Several cytokines circulate freely following resuscitated hemorrhagic shock. Unlike classic hormones, cytokines are active locally at concentrations not measurable systemically. They are produced at many different sites by a myriad of cell types and possess important autocrine, paracrine and endocrine functions on several tissues and cells including neutrophils and endothelial cells. The principal cytokines involved in resuscitated hemorrhagic shock are released mostly by activated macrophages. Tumor necrosis factor α (TNFα) a cytokine known to promote neutrophil respiratory burst and degranulation appears early in plasma and persists in tissue for long periods following resuscitation of hemorrhagic shock[91-93]. Interleukin 1 (IL-1) levels are not detected in plasma but are noted in tissue despite adequate resuscitation[91]. Mesenteric levels of interleukin 6 (IL-6) are also seen in hemorrhagic shock and are further increased with resuscitation[94]. Increased interleukin 8 (IL-8), which plays an important role in neutrophil chemoattraction, transmigration and adhesion to endothelium has also been demonstrated in severely injured trauma patients[95]. Several other cytokines are also released in large quantities following resuscitated hemorrhagic shock and their effects on neutrophil function and activation are becoming increasingly clear as research continues in these areas.

1.3.2.5 PMN function in resuscitated hemorrhagic shock

As in other SIRS conditions, PMNs from resuscitated hemorrhagic shock victims may become inappropriately activated by circulating mediators and byproducts of reperfused ischemic tissues. With greater degrees of blood loss, persistence of low flow conditions, and especially with resuscitation, the systemic release of these mediators may result in the inappropriate activation of neutrophils.

In particular, resuscitation of hemorrhagic shock promotes the indiscriminate release of reactive oxygen species not only by activated neutrophils but also by post-

ischemic endothelium[96]. Two mechanisms are responsible for this reperfusion-related response: First, molecular oxygen supplied to ischemic tissue with reperfusion enhances generation of large quantities of H_2O_2 and O_2^- by the xanthine/hypothanxine axis, serving as a cofactor and electron acceptor[97]. Second, protection by the physiologic antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase is greatly diminished. While in non-stressed physiologic conditions, SOD from red blood cells and other sources, is capable of destroying any freely existing superoxide radicals by dismutation, converting them to elemental oxygen and hydrogen peroxide[98], this process is easily overwhelmed following resuscitated hemorrhagic shock by reconversion of the resultant H_2O_2 back into hydroxyl radicals[97]. The unchecked levels of ROS circulating systemically produce tissue injury through lipid peroxidation. In bi-lipid membranes, this process is particularly damaging as it is self-perpetuating and continues until loss of membrane integrity is complete and destruction of the cell itself occurs[99]. The destructive effect of free radicals extends beyond lipid membranes, also interfering with nucleic acid integration in deoxyribonucleic acid (DNA), and protein assembly into vital enzymes and transport proteins[45,97,100,101]. In addition, free radicals further activate and injure endothelial cells resulting in microvascular loss of integrity and interstitial leakage of plasma, cells, and proteins promoting tissue edema. Activated endothelium further accentuates tissue destruction by ROS through a reduction in its release of nitric oxide (NO). NO's role as a potent vasodilator allowing nutritive blood flow to end capillaries is lost as is its highly effective ability to scavenge reactive oxygen radicals[102,103].

PMN elastase release is also inappropriately upregulated in resuscitated hemorrhagic shock. Rodent PMNs primed by trauma and hemorrhage demonstrate greater release of elastase when activated[104]. Conversely, administration a granulocyte elastase inhibitor reduces tissue injury in hemorrhagic shock and hepatic or pulmonary

ischemia/reperfusion studies[105-107]. Levels of other proteinases including cathepsins are also reported to be elevated in hemorrhagic shock[108].

Resuscitated hemorrhagic shock also alters cellular structure. Upregulated expression of adhesion molecules are seen in ECs and PMNs from resuscitated hemorrhagic shock victims. In rodent models of hemorrhagic shock, PMN CD18/CD11 expression is greatly augmented following standard resuscitation[109-111]. Similarly, PMN surface L-selectin levels were found to be increased in similar experiments by other authors[109,112,113]. Increased expression of endothelial adhesion molecules is also reported after hemorrhagic shock resuscitation with standard crystalloids. This has been noted with ICAM-1 and 2[114,115], vascular cell adhesion molecule 1 (VCAM-1)[115] and P-selectin[116,117].

The augmented expression of endothelial and PMN adhesion molecules is confirmed by enhanced PMN/EC interactions following standard resuscitation of hemorrhagic shock. In vivo evaluation with intravital microscopy has shown greatly increased neutrophil rolling and adhesion to endothelium after shock resuscitation in liver[118-121], intestinal mesentery[23,122-125], pancreas[126], skin[127,128] and cremaster which is the subject of the current work.

1.4 Isotonic fluid resuscitation

The optimal fluid for initial resuscitation of hemorrhage has been debated since the seventeenth century. In 1883, Sidney Ringer noted the importance of different electrolyte components of infusion solutions in heart reperfusion studies[129]. Half a century later Krebs created a fluid similar to plasma in ionic concentrations and tonicity[5]. At about the same time Hartman determined the critical role of sodium in physiologic fluid balance, and added sodium lactate to hydration solutions[130]. Lactated Ringer's was hence coined and the solution has since been used for hypovolemic states, essentially

unchanged. Nonetheless, in the years that followed World War I, intravascular deficits from hemorrhage were increasingly replaced by whole blood or plasma[131]. Following WWII, the notion that blood loss did not necessarily require replacement with blood products was emerging[132]. Yet, it was not before the combat experience in Vietnam that isotonic crystalloid was clearly established as the prime resuscitation fluid for hemorrhagic shock[5]. Wide usage of crystalloid resuscitation at that time rendered rare the overwhelming mortality and renal complications previously documented in hemorrhagic shock victims. Hence, the revolutionary concept that lost blood did not need to be replaced with blood, but with isotonic salt solutions had now become the norm. The key was to administer the fluid in sufficiently large volumes, three to four times the volume of shed blood, in order to restore cardiac filling pressures and output[133]. More recently, this ratio has been questioned and it has been argued that it should more likely be 7:1[52].

Nevertheless, even in Vietnam, while crystalloids permitted patients to survive otherwise lethal hemorrhage, it soon became evident that such large administered fluid volumes gave rise to other complications that could also result in death. Though first described in the First World War, severe pulmonary complications following massive crystalloid resuscitation were extensively documented in the Vietnam War[132]. It became clear that crystalloids ultimately fill the interstitial space, readily 'leaking' out of all capillary beds but most dramatically observed in the lung as pulmonary edema. 'Danang lung', 'shock lung' and 'traumatic wet lung' were initial names ascribed to the pulmonary dysfunction following trauma resuscitation which resulted in prolonged respirator usage[5]. Presently referred to as the 'acute respiratory distress syndrome' (ARDS), this entity is now known to also occur after other inflammatory conditions, often but not always, resuscitated with high crystalloid volumes. Concern over the increasing incidence of this

condition has encouraged research in alternative resuscitation fluids requiring more modest fluid doses to adequately restore effective circulating volume.

1.5 Colloid fluid resuscitation

One of the effects of the massive isotonic crystalloid volumes required to resuscitate hemorrhagic shock is to reduce vascular colloid osmotic pressure resulting in disseminated tissue plasma leakage and edema. The administration of exogenous colloids in such conditions has been proposed by some to maintain effective circulating volume while reducing intravascular fluid losses and preventing the accumulation of tissue edema. Colloids are fluid solutions containing large macromolecules unable to cross physiologic vascular pores and channels. These macromolecules generate a colloid oncotic intravascular pressure (COP) which prevents osmotic leakage of fluid out of the vasculature. As long ago as WWI, colloid fluid use was reported for resuscitation as it could maintain hemodynamic effects for longer periods than crystalloids[131].

Colloids are divided into natural and synthetic forms. Red blood cells, platelets and plasma are typical natural colloids which can generate powerful oncotic pressure by their large sized particles (red cells, proteins). WWI, Korean War and most WWII casualties were resuscitated with human plasma and whole blood [5]. Unfortunately the limited supply and risk of contamination has limited resuscitation with blood products in more recent times. Another natural colloid is albumin, the main circulating protein in the plasma of mammals. Albumin solutions (5%, 25% and others) are available commercially and are excellent to maintain colloid oncotic pressure. The use of albumin for shock resuscitation is widespread and has been reported in various conditions including post-operative ARDS[134], burns[135], cirrhosis[135], cardiac bypass[136] and hypovolemic shock[137]. Yet, large reviews of randomized control trials involving the administration of albumin in critically ill patients have revealed increases in mortality

rates and have led to emphatic recommendations that it not be used outside rigorously controlled clinical trials[138-140]. While some strong disagreement and contradicting reports followed[141,142], the use of albumin in critically ill patients has remained cautious.

The main synthetic colloids are starches and gelatins. Starches are subdivided into dextrans and hydroxyethyl (HES) varieties. Dextran use in North America is limited, particularly due to its high risk of coagulopathic complications. HES solutions have been associated with fewer such side effects and less renal complications, particularly if of lower molecular weight and degree of hydroxyl substitution[143,144]. The use of HES in critically ill patients is increasing in Canada and certain parts of Europe, greatly surpassing that of albumin, in part due to its lower cost, safety and efficacy[145-148]. While its use following injury remains guarded, studies in trauma patients found diminished fluid requirements and few side effects[149,150]. Gelatins are extensively used worldwide but minimally in North America and Europe. In hemorrhagic shock, they are effective in increasing circulating plasma volume, capillary wedge pressure, cardiac index and oxygen delivery with few adverse effects on pulmonary function and hemostasis[151,152]. Anaphylactic responses secondary to gelatin use appear to be more frequent than with other colloids[153,154].

Consideration of available data in major meta-analyses of several prospective randomized trials have suggested that colloids offer no advantages to injured patients, and may possibly increase their mortality[155-158]. Current evidence would thus suggest that crystalloids should remain the resuscitation fluid of choice in hemorrhagic shock trauma victims and that colloids be used only in exceptional cases.

1.6 Hypertonic saline

1.6.1 Improved Macrohemodynamic Pathophysiology

Hypertonic saline is particular in that, as a crystalloid, it is a simple salt solution without macromolecules, yet similar to colloids it is administered in small quantities which remain intravascular, drawing fluid from the cellular and interstitial space. Table 2 shows the compositions of some infusion solutions as well as their relative intravascular expansion. Increased serum osmolality from small doses (4cc/kg) of 7.5% sodium chloride (HTS) instantly expand effective circulating volume by creating a potent transcapillary osmotic gradient which mobilizes intracellular (from endothelial cells, red blood cells) and interstitial fluid, into the vascular compartment[159,160]. This fluid exchange occurs in the microcirculatory milieu, in capillary and post-capillary beds; the precise location where ischemia-reperfusion injury is initiated during resuscitation of hemorrhagic shock. Hemorrhagic shock first renders capillary endothelial cells hypoxic, while resuscitation then makes them edematous resulting in narrowing and occlusion of capillaries contributing to downstream tissue hypoxia. On the other hand, hypertonic saline mobilizes intracellular fluid from erythrocytes and endothelial cells preventing such cellular swelling during resuscitation. The resultant reduction in capillary hydraulic resistance, improves microcirculatory perfusion and permits better post-ischemic organ recovery[161,162]. Concurrently, large intravascular fluid mobilization yields rapid restoration of mean arterial pressure (MAP) giving rise to an elevated cardiac output (CO), oxygen delivery and extraction[160,163-165]. In addition to increases in cardiac output through higher filling pressures, HTS may have direct cardiac effects improving ventricular contractile force, dP/dt_{max} and stroke work at equal or lower atrial filling pressures than with isotonic fluids[166,167]. This effect is believed to occur through osmolar reductions in cardiac edema, restoring cardiac membrane potential[168,169]. Nonetheless, the most important benefits of hypertonic saline resuscitation may not reside with its early

macrohemodynamic effects but instead on its ability to modulate the inflammatory response at the microcirculatory level.

Table 2: FLUID THERAPY

Fluid	Intravascular volume change (amount infused: amount remaining intravascular)	Osmolality (mOsm/L)	Na+ (mEq/L)	Cl- (mEq/L)	K+ (mEq/L)	Ca++ (mEq/L)	Lactate (mmol/L)	Dextrose (g/100ml)	pH	COP (mm Hg)	Other
D5W	10:1	252	-	-	-	-	-	50	4.0	-	
D5½NS	7:1	280	77	77	-	-	-	50	4.2	-	
NS	4:1	308	154	154	-	-	-	-	5.0	-	
RL	4:1	273	130	109	4	5	28	-	6.5	-	
7.0% HTS	1:4	2396	1197	1197	-	-	-	-		-	
PTS	1:1	326	154	154	-	-	-	-	5.0	40	HES 10g/100ml
25% albumin	1:3.5	-	140	-	-	-	-	-	-	100	albumin 25g/100ml
Plasma		**290**	**144**	**107**	**5**	**5**	**-**	**0.8 (glc)**	**7.4**		

D5W: 5% dextrose solution, NS: normal saline, RL: Ringer's lactate, HTS: hypertonic saline, PTS: pentastarch solution, HES: hydroxyethyl starch, glc: glucose

1.7.2 **Manuscript #1**

Hypertonic Saline and the Microcirculation

José L. Pascual MD, Kosar A. Khwaja MD, Prosanto Chaudhury MD
and Nicolas V. Christou MD, PhD, FRCS(C), FACS, FCCM

Presented in part at the Fluid Resuscitation in Combat meeting of the Canadian Defence and Civil Institute of Environmental Medicine (DCIEM), Toronto, Canada, October 25, 2001.
Published in the Journal of Trauma Vol 54: S135-S140 (2003)

Keywords: hypertonic saline, HTS, Ringer's lactate, immunity, host defence, resuscitation, shock, endothelial cells, leukocytes, microcirculation, vessel permeability, leukocyte-endothelial interactions, leukocyte adhesion, L-selectin, CD11b.

Abstract

The systemic inflammation that occurs in shock states is believed to promote over-exuberant microcirculatory activation, the release of toxic proteases and oxygen radicals causing microvascular damage, and subsequent tissue and organ injury. Although shock-associated microvascular failure is often unresolved following standard resuscitation, hypertonic saline (HTS) appears to reduce microvascular collapse, restoring vital nutritional blood flow. In addition, hypertonic fluids tend to blunt the upregulation of leukocyte and endothelial adhesion molecules that occurs with isotonic resuscitation of shock. Recently, direct evaluation by intravital microscopy has shown that HTS resuscitation dampens the interactions between leukocytes, platelets and endothelium found with Ringer's lactate (RL)-resuscitation. Furthermore, fewer cellular interactions have been correlated with attenuations in microvascular wall permeability after resuscitation with HTS. Better characterization of microcirculatory effects by hypertonic saline may provide mechanisms for improved morbidity and mortality associated with hypertonic resuscitation.

For several decades hypertonic saline (HTS) solutions have been known to restore physiological hemodynamics in hypotensive conditions such as hemorrhagic shock. Resuscitation with isotonic fluid (Ringer's lactate [RL], normal saline [0.9% NaCl, NS]) is the current standard of care and often involves the administration of several liters of fluid to restore microvascular perfusion. The advantages of small volume resuscitation[168] with hypertonic saline (4cc/kg 7.5% NaCl) are its ease of transport, speed of administration and rapid correction of perturbed hemodynamics. Restoration of normal intravascular physiology by HTS is achieved through a potent transcapillary osmotic gradient that causes a net shift of fluid from the interstitium, endothelial cells (ECs), and red blood cells (RBCs) to the intravascular space. Animal studies have demonstrated that HTS-resuscitation rapidly restores mean arterial pressure, peripheral tissue perfusion, cardiac contractility and urinary output, primarily by increasing cardiac preload.[160,163] Although large, multicenter, randomized studies evaluating HTS and HTS-starch mixtures in traumatic shock have demonstrated safety and efficacy, they were unable to show a clear survival advantage over standard isotonic therapy.[170-173] Nonetheless, some reports did suggest important advantages in HTS-resuscitation, namely reductions in post-resuscitation complications such as renal failure, coagulopathies and acute respiratory distress syndrome (ARDS).[173] Despite some recommendations to use HTS as the first line therapy for resuscitation of shock[5], to this day, the routine use of HTS across North American and European hospitals is limited.

In the last 15 years, a revived interest in hypertonic saline has occurred after certain reports demonstrated HTS-mediated immune protection, possibly improving trauma outcomes.[174] Systemic inflammatory injury primarily occurs at the microcirculatory milieu in conditions such as sepsis, burns, pancreatitis and severe hemorrhagic shock. It is this microcirculatory environment where hypertonic saline appears to exert its beneficial immune effects.

Intravital videomicroscopy (IVM), a recent technical advance in the study of living tissue, has provided an insight into the pivotal events that occur in microcirculatory beds of animals with systemic inflammation. IVM permits the direct observation of animal (and even human) microcirculation by placing a thin, translucent, live tissue over the objective lens of a microscope attached to a video camera. Real-time flow is visualized in microvessels (arterioles, venules and capillaries), distinguishing the traffic of different circulating elements such as red blood cells (RBC), leukocytes (LEU), platelets and fibrin clots. Tagging different cells with fluorescent markers further allows the precise identification of visualized elements. Moreover, with similar labeling of non-cellular circulating macromolecules (albumin, dextran), appropriate ultraviolet filters allow live quantification of local macromolecular leakage from a given vessel or vessels. Simple image analysis software can compare the fluorescent signal within the vessel with that of the neighboring interstitial space, yielding a light intensity ratio (permeability index, PI) corresponding to the degree of macromolecular leakage and proportional to the vessel's permeability.

The following review will discuss three different components of the effects of HTS on the microcirculation. The first section examines the known alterations by HTS of microvascular rheology, flow and vessel constriction/dilation. The subsequent segment explores the activation, receptor expression and interactions of microcirculation leukocytes, endothelial cells and platelets. Finally, hypertonic saline modifications of vessel wall integrity, permeability and the appearance of surrounding tissue and organ edema are discussed.

Unfortunately, a lack of uniformity in methods evaluating hypertonic fluid administration makes comparisons across studies difficult. Some evaluations compare isotonic fluids (RL, NS) to HTS alone, and others compare them to hypertonic saline-dextran (HSD) solutions or HTS-hydroxyethyl starch (HTS/HES) solutions. In addition,

while volumes of HTS or hypertonic saline-starch solutions studied are usually 4cc/kg, volumes of isotonic fluids range from 4cc/kg to 3-4 times the shed blood volumes (over 40cc/kg).

Microvascular hemodynamics

HTS promotes microcirculatory flow by preventing the capillary collapse seen in shock that is only partially improved by standard isotonic resuscitation. Laser Doppler flow analysis cannot discriminate between types of vessel (capillary, venules or arterioles), or between vessel calibers, but it does, nonetheless, provide a general assessment of *in vivo* regional blood flow. This technique was used in hemorrhagic shock models resuscitated with either RL (3-4 times the shed blood volume) or HTS/HSD regimens (4cc/kg or equivalent). Two hours following resuscitation, Doppler signals from intestinal mucosa and renal cortex were 40-50% lower with RL resuscitation.[175,176] Comparison of mucosal blood flow following administration of either HSD or isotonic dextran was also performed in a model simulating sepsis with lipopolysaccharide (LPS) infusion. HSD-treated animals had twice the Doppler signal of isotonic controls.[177] Interestingly, HSD animals had a survival rate three fold greater than controls. With traumatic injury, a spinal compression model found that subsequent HTS administration increased blood flow, better preserved spinal cord function acutely and accelerated recovery from chronic injury.[178]

The effects of HTS on microcirculatory physiology can be further characterized with intravital microscopy models. IVM on pia mater windows of rabbits subjected to brain percussion injury followed by HSD showed an absence of arteriolar *dilatation* otherwise seen with brain percussion alone.[179] No isotonic controls were evaluated, and the authors were unable to correlate these findings with differences in intracranial

pressure (ICP) between groups. Using IVM of rabbit tenissimus muscle, Mazzoni *et al* demonstrated that HTS and HSD-resuscitation of hemorrhagic shock completely restored capillary patency to pre-shock levels. In contrast, RL administration resulted in persistent capillary narrowing, unchanged from non-resuscitated shock.[162] They subsequently corroborated this finding with increased Doppler flow signals from the muscle surface of HSD-resuscitated animals using a similar model.[180]

Hepatic intravital microscopy models of hemorrhagic shock have also evaluated the rheologic effects of HTS. Although initial studies revealed equal sinusoidal narrowing with RL or HSD resuscitation[181], subsequent comparisons between hypertonic-hydroxyethyl starch, HSD regimens and RL have demonstrated that both hypertonic regimens reduced the number of non-perfused sinusoids (18% and 14% respectively) found in RL (24%) controls.[119,161] Additionally, morphometric analysis demonstrated smaller EC thickness in HSD than RL-resuscitated animals. The authors proposed that HSD resuscitation reduced endothelial cell swelling, which prevented the congestion of narrow sinusoidal passages seen with isotonic resuscitation.

More precise intravital microscopy experiments have quantified microvascular rheological alterations mediated by HTS. In one study, ileal mucosa microscopy in rat hemorrhagic shock demonstrated that resuscitation with HSD or at least twice the shed blood volume of RL equally restored arteriolar diameters to pre-shock levels, but a 75% diameter reduction persisted if blood or dextran alone was used.[182,183] Still, most other IVM studies quantifying microvascular diameters and flow have determined that HTS may increase general microvascular flow by constricting capacitance vessels. Comparing equal volumes of 7.5% and 0.9% saline in the resuscitation of hemorrhagic shock, cheek pouch IVM showed twice the arteriolar flow with HTS but no differences in arteriolar diameters.[184] Nevertheless, there was considerable venular constriction found only in HTS-treated animals. Furthermore, post-resuscitation venular flow took 1 hour to

normalize with HTS but only 10 minutes in NS-treated controls. These results highlight how hypertonicity mobilizes fluid from high capacitance sources (i.e.: venules) to increase intravascular volume. In a more recent report, Torres Filho *et al* used a hemorrhage/resuscitation rat model of exteriorized mesentery IVM and similarly demonstrated that HTS increased post-resuscitation arteriolar flow without changing arteriolar and venular diameters.[185] In addition, *vasomotion,* described as the continuous rhythmic contraction and relaxation of a vessel, was found to be 80% greater in HTS-treated animals than RL controls. This increased vasomotion was offered as a possible mechanism for microhemodynamic improvement by HTS. Unfortunately both latter studies compared equal volumes of NS and HTS, arguably under-resuscitating RL animals. An interesting additional arm would have been animals resuscitated with 3 to 4 times the shed blood volume of isotonic crystalloid.

Thus, microvascular rheological alterations by HTS-resuscitation lead to increases in arteriolar flow at the expense of temporary venoconstriction but resulting in generalized improvements in microcirculatory patency.

Cellular receptor expression and interactions in the microcirculation

The microcirculatory milieu is the primary site where HTS exerts its effects on circulating elements and their interactions.

Polymorphonuclear neutrophils (PMNs) follow an orderly passage from the vasculature to appropriate sites of action where an injury or infection has occurred (Figure 1). With such insults, PMNs marginate to the vessel periphery and roll along the surface of the vessel wall, interacting with endothelial cells through surface selectins (L, E and P).[16] Theses initial weak interactions permit strong adhesion of surface β_2-integrins (CD18/CD11) to endothelial receptors of the Ig superfamily (ICAM-1, ICAM-2), completely arresting the PMN on the vessel wall.[186] Through diapedesis the PMN then

passes to the interstitial space migrating down chemotactic gradients to reach the source of tissue injury, infection or inflammation.[62]

Disruption of this orderly PMN passage out of the microcirculation has been implicated in the pathophysiology of organ dysfunction promoting the progression of sepsis, burns, multiple trauma and resuscitated hemorrhagic shock to the systemic

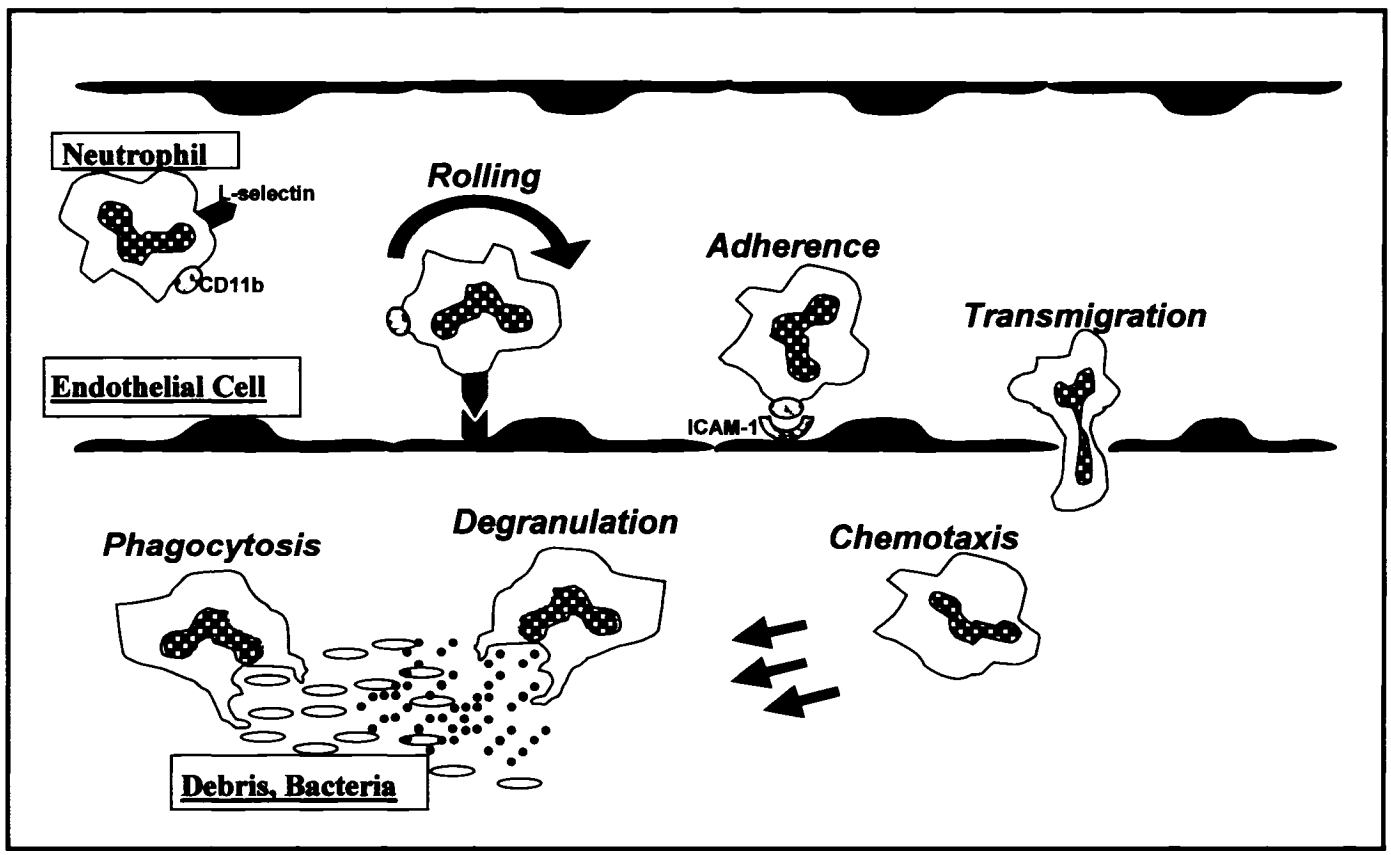

FIGURE 1: Schematic diagram of the passage of neutrophils from the vasculature to the area of injury or infection: The activated neutrophil rolls on the vessel wall, slowed from central blood flow by weak interactions between surface L-selectin and Sialyl Lewis X (Sial-Le^x) bases on the surface of the endothelial cell (EC). PMN rolling permits surface CD11b adhesion molecules to strongly interact with EC receptors of the immunoglobulin superfamily (ICAMs). The PMN then transmigrates through the vessel wall, migrating down a chemotactic gradient to perform physiologic functions (phagocytosis, degranulation) in the area of injury.

inflammatory response syndrome (SIRS), multiple organ dysfunction (MODS) and sometimes death. Inappropriate neutrophil-endothelial (PMN/EC) interactions are believed to promote the release of proteases and oxygen free radicals contributing to severe microvascular injury and tissue edema. (Figure 2)

Increasing evidence demonstrates that HTS may improve morbidity and mortality in different forms of localized or systemic inflammation. In particular, hemorrhagic shock models resuscitated with HTS have shown a reduction in lung injury, with fewer PMNs in bronchioalveolar lavage fluid as well as reductions in liver injury. Furthermore, some of these animal models have shown increased survival with HTS-resuscitation.[109,187-189] Key alterations in the function and interactions of PMNs and ECs have been related to

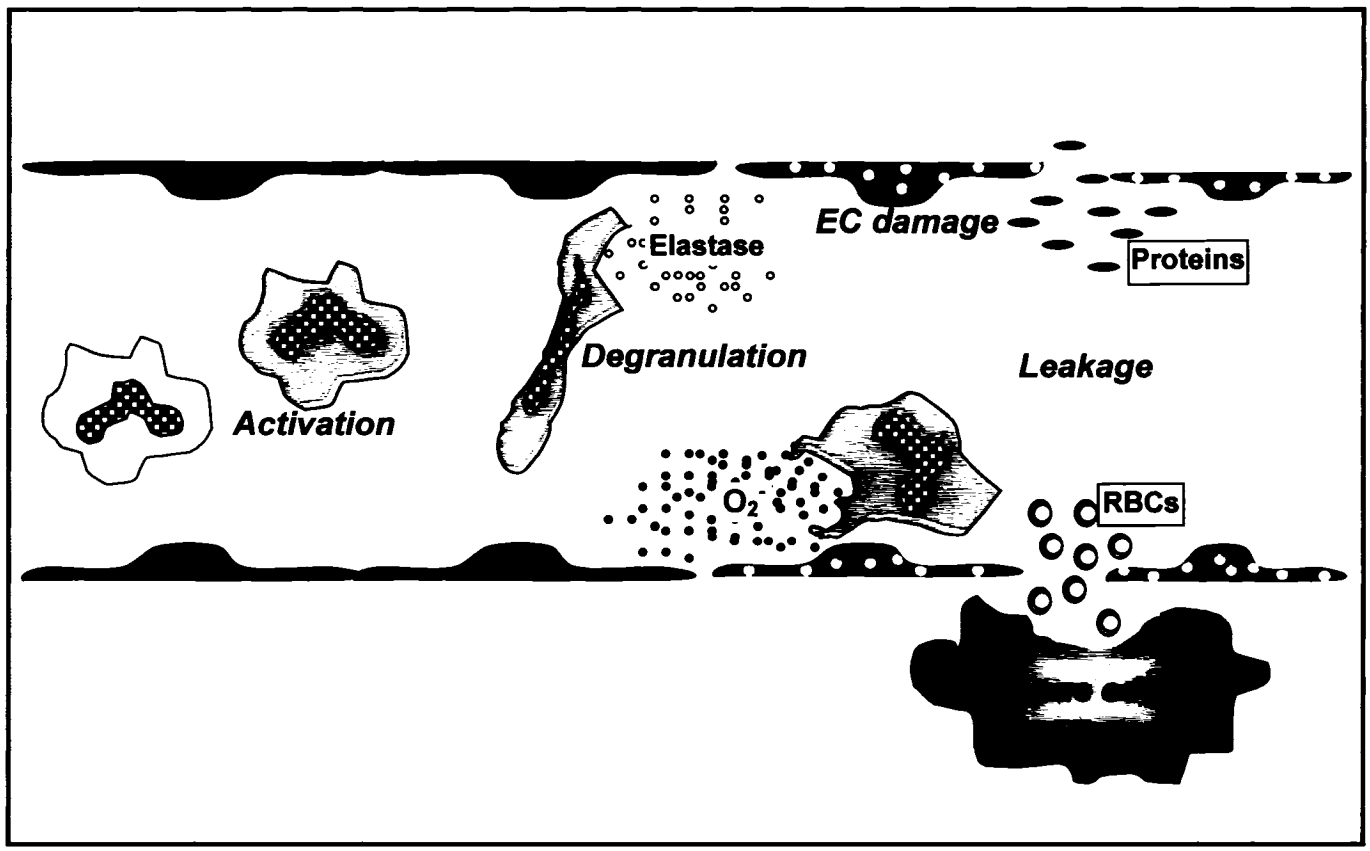

FIGURE 2: Schematic diagram of microvascular disturbances in systemic inflammation: The neutrophil becomes inappropriately activated, prematurely degranulating and releasing toxic elastases, and oxygen free radicals inside or immediately outside the vasculature. These substances injure the microcirculatory endothelium, increasing vascular permeability and allowing fluids, cells and macromolecules to leak out and cause tissue edema.

HTS administration. *In vitro* exposure to hypertonicity attenuates PMN activation, cytotoxicity, and release of superoxide and elastase.[190-193] Incubation with hypertonic media reduces human PMN surface L-selectin and CD11b.[109,190,193] Furthermore, when human volunteers receive hypertonic saline infusions, their PMN CD11b expression is

reduced in comparison to those receiving RL.[194] Yet, although HTS-resuscitation in animals appears to reduce L-selectin, *in vivo*, CD11b alterations remain unclear.[109,112,187,189]

Endothelial cell expression of adhesion molecules is also modified by HTS. Diminished *in vitro* endothelial ICAM-1 has been reported when LPS activation was followed by incubation in HTS[195]. *In vivo* studies have similarly found diminished pulmonary and hepatic ICAM-1 levels in HTS-resuscitated animals as compared with RL controls[109,195]. Additionally, Sun *et al* report less ICAM-1 protein and gene expression in rat spleen and lung resuscitated with HTS instead of RL.[115] While in some reports E (endothelium) and P selectin (platelets and endothelium) were unchanged by differential

FIGURE 3: Representative photomicrograph from mouse cremaster intravital videomicroscopy 90 minutes after RL-resuscitation of hemorrhagic shock (original magnification X 1920). Note the rolling (arrowhead) and adherent leukocytes (arrow). (Adapted from Pascual JL, Ferri LE, Chaudhury P, et al. Hemorrhagic shock resuscitation with a low molecular weight starch reduces neutrophil-endothelial interactions and vessel leakage in vivo. *Surg Infect.* 2001;2:275-288, with permission.)

resuscitation[112], Alam *et al* report elevations in both receptors in spleen and lung tissue following RL but not HTS-resuscitation of hemorrhagic shock.[196]

IVM studies have contributed further credibility to the idea that blunting of adhesion molecule expression by HTS reduces aberrant EC/PMN interactions, resulting in less organ and tissue injury. Inflammatory insults followed by HTS administration consistently demonstrate fewer leukocyte interactions in different microvascular beds. In burns, for example, HTS-resuscitated rats demonstrated diminished leukocyte stasis (adhesion) in pial windows, in comparison with those receiving standard Parkland formula using RL.[197] In local trauma and ischemia/reperfusion models, prior HTS infusion resulted in fewer rolling and adherent leukocytes than in isotonic or untreated controls.[198,199]

EC-PMN interactions in hemorrhage-resuscitation have been well characterized by hepatic videomicroscopy. Comparable to reports with other models, they show attenuation of leukocyte stasis in sinusoids and post-sinusoidal venules if HTS/starch replaces resuscitation with RL.[118,119,181]. According to some authors, swollen and stiff PMNs may become passively 'trapped' in narrowed sinusoidal passages making these findings not necessarily applicable to other microcirculatory beds.[200-202].

To circumvent the possible uniqueness of hepatic microcirculation, our group developed a resuscitated hemorrhagic shock model evaluated with cremaster intravital microscopy. In a moderate hemorrhagic shock model (45 mmHg for 45 minutes) followed by resuscitation with RL (twice the shed blood volume) or HTS (4cc/kg), we evaluated leukocyte rolling and adherence on endothelium for up to 90 minutes. As seen in figure 3[203] several leukocytes can be noted rolling or adherent to the endothelium of a post capillary venule in murine cremaster 90 minutes following resuscitation with RL. Unlike HTS or SHAM animals, persistent elevations in PMN adherence with RL were found beyond 15 minutes after resuscitation (Figure 4[204]). Similar differences between

groups were also found in leukocyte rolling and rolling velocity. With respect to HTS effect on platelet interaction with neutrophils, few studies can be found in the literature. With skin IVM in hamsters, Saeltzer and colleagues describe finding 25 to 35% fewer leukocyte platelet aggregates when HTS was used instead of RL to resuscitate hemorrhagic shock.[205] The authors further report fewer rolling and adhering leukocytes 60 minutes after resuscitation with HTS but greater numbers in RL controls.[205]

Clearly, HTS has profound effects on adhesion receptors and interactions

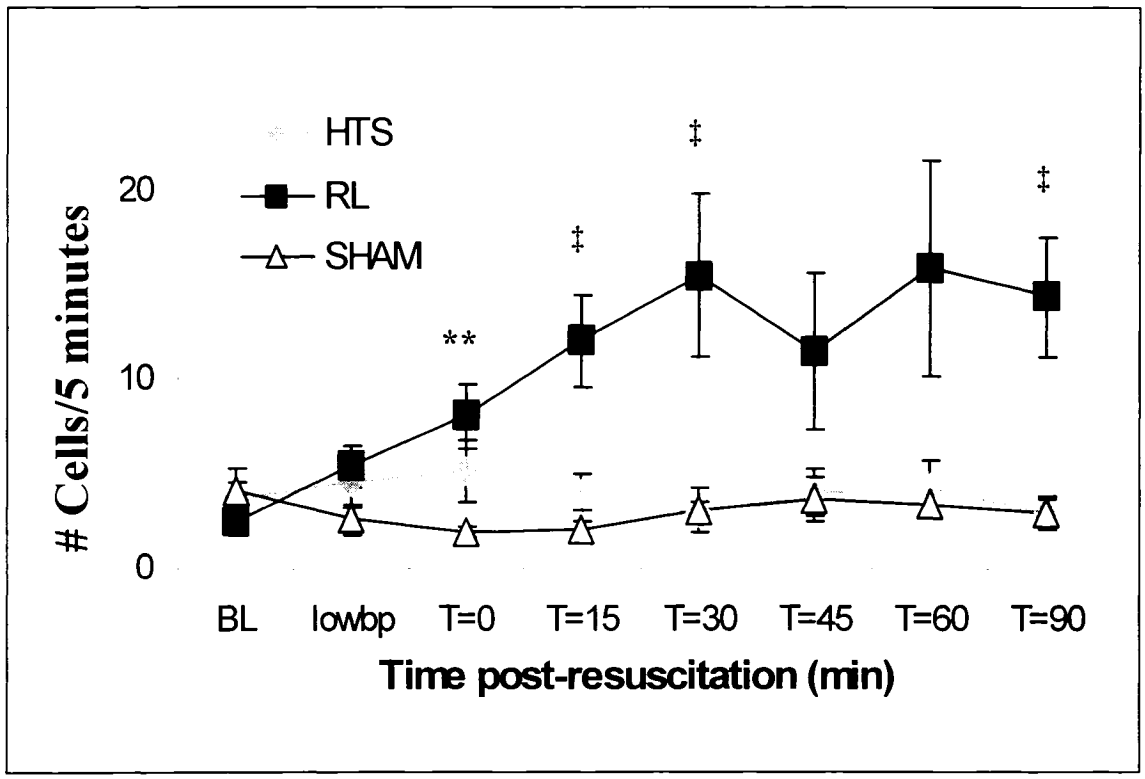

FIGURE 4: Leukocyte adhesion with differential fluid resuscitation of hemorrhagic shock. Adherent leukocytes in post-capillary venules of mouse cremaster at baseline (BL) hypotension (lowbp) and at different times after resuscitation (T=0-90 minutes). Compared with HTS and SHAM, RL animals displayed greater leukocytes adhering to endothelial cells. Values expressed are mean number of stationary PMN initially and during 5 minutes on a given 100μm vessel length ± SEM. **RL vs SHAM only: $p<0.05$, ‡RL vs either HTS or SHAM: $p<0.05$. There were no significant differences between HTS and SHAM at any time point. (Adapted from Pascual JL, Ferri LE, Seely AJ, et al. Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces in vivo vascular leakage. *Ann Surg.* 2002;236:634-642, with permission.)

between different cellular elements of the microcirculation. How this effect alters the microvascular injury and leakage seen with standard resuscitation will be discussed in the subsequent section.

Microvascular integrity and permeability

The systemic inflammation that sometimes accompanies severe hemorrhagic shock, sepsis, trauma and burns has long been associated to increased tissue edema and the concept of 'leaky capillaries'. The theory first proposed 25 years ago maintains that systemic inflammation causes inappropriate unleashing of host defense products (proteases, oxygen radicals) creating microvascular injury and leakage of plasma and proteins into surrounding interstitium.[206,207] More recently, authors have postulated that inappropriate cellular activation (neutrophils, macrophages, endothelium) and their over-exuberant interactions may be responsible for the systemic release of toxic products that cause vessel injury.(Figure 2)

Different experimental methods can estimate microvascular permeability changes. Dyess *et al* measured hind paw lymph flow and capillary filtration coefficients 6 hours after burn injury in differently resuscitated dogs.[208] Although only HTS administration increased lymph flow initially, by the end of experiments, HTS, HSD and dextran alone displayed flows equal to untreated burn controls. Concurrently, capillary filtration coefficients were greatest with HTS-treatment and lowest in dextran-treated animals throughout the experiment. Although this study would suggest a greater loss of vascular integrity with HTS treatment, most current studies demonstrate HTS-mediated decreases in microvascular permeability.

The wet to dry (W/D) ratio is a simple method for estimating tissue water content by comparing the tissue's 'wet' weight (weighed immediately after death of the animal) to the 'dry' weight (weighed after 14 days of drying at 90°C). This assessment of tissue edema was used in a sheep model of 75% burns, demonstrating a diminished W/D ratio in colon, liver, pancreas, skeletal muscle and *non-burned* skin when the animals received HSD instead of RL.[209] This underscores the notion that systemic inflammation causes increased tissue edema or microvascular leakiness *systemically*, remote from the area of direct injury. HTS in pancreatitis demonstrated half the lung W/D ratio and one tenth the alveolar protein content compared with an equal volume of NS.[210] Using histologic assessment of tissue edema, Jerome and colleagues demonstrated less tissue water if 4-hour muscle ischemia was reperfused with blood supplemented with HSD.[211] Also, using histology, a rat model of hemorrhagic shock followed by intratracheal infection demonstrated HTS-associated reduction in lung water.[109] The authors further correlated these results with *ex vivo* reductions of radio-labeled albumin leakage in the alveoli of HTS animals.

Perivascular macromolecular leakage can be further evaluated directly using epifluorescent intravital microscopy. Filtered ultraviolet light (480 nm) exposure allows IVM to quantify fluorescent macromolecules (FITC-labeled dextran, albumin) leaking into the interstitium. In a model simulating local infection by treating hamster cheek pouches with topical LPS, subsequent epifluorescent analysis demonstrated 40% less leakage of fluorescent (FITC)-labeled dextran if animals had been pre-treated with HTS.[212] Similarly, in models of local ischemia reperfusion, or severe burns, various intravital microscopy windows have shown that FITC-labeled macromolecule leakage *in vivo* was much greater if RL had been administered instead of HTS. [198,213] Both studies correlated fewer adherent leukocytes with diminished vascular leakage in HTS-resuscitated animals than in RL controls.[197] Likewise, our own hemorrhagic shock model using murine

cremaster videomicroscopy found 45% less *in vivo* vascular leakage of FITC-labeled albumin simultaneously to fewer rolling and adherent leukocytes with HTS compared to RL

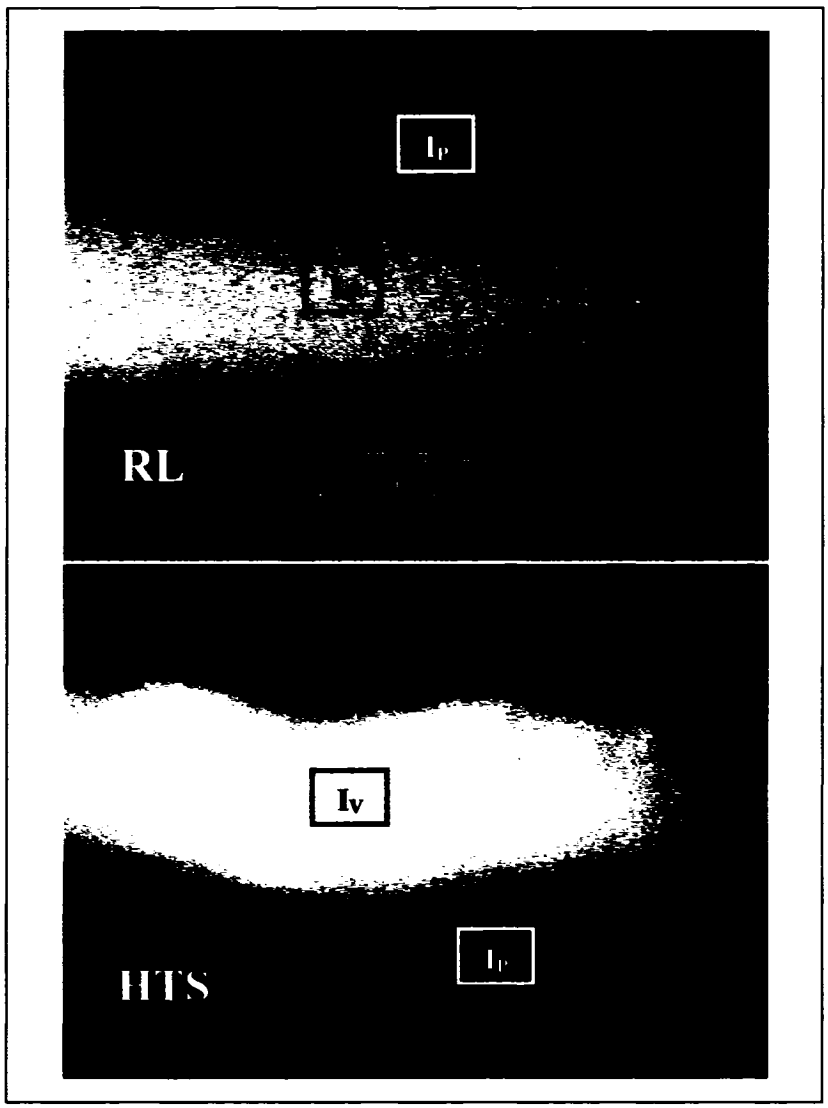

FIGURE 5: Representative examples of fluorescent intravital microscopic images of mouse cremaster contrasting the greater interstitial leakage of fluorescent-labeled (FITC) albumin from postcapillary venules after RL compared to HTS-resuscitation. Ip (perivenular fluorescent intensity) and Iv (venular fluorescent intensity) represent regions evaluated for light intensity. (Adapted from Pascual JL, Ferri LE, Seely AJ, et al. Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces in vivo vascular leakage. *Ann Surg.* 2002;236:634-642, with permission.)

resuscitation[204]. Figure 5 shows representative epifluorescent cremaster

photomicrographs from HTS and RL-resuscitated animals captured 2 hours after resuscitation. Extravasation of fluorescent albumin, as seen by the ratio of fluorescence (whiteness) outside (I_p) to than inside (I_v) the venule, is significantly greater in a vessel of a RL-resuscitated animal than in the HTS counterpart.

Although human clinical evidence finds hypertonic saline resuscitation safe and efficacious in restoring macro-vascular hemodynamics (e.g., arterial pressure, cardiac output etc), studies to date have been unable to clearly establish clinical superiority over isotonic crystalloid. In contrast, mounting data suggests that HTS imparts many advantages at the microcirculatory level. Improved capillary patency and arteriolar flow have been demonstrated directly and indirectly in different models simulating local and systemic insults. Several reports indicate that hypertonicity reduces expression of surface leukocyte and endothelial adhesion molecules. Furthermore, direct evidence now demonstrates that HTS blunts the considerable augmentation in leukocyte-endothelium interactions seen with standard isotonic resuscitation of shock. Different shock animal models have shown that a reduction in leukocyte-endothelial interactions by HTS administration can be correlated both spatially and chronologically with a diminution in microcirculatory vascular leakage. Further research is needed to determine whether these findings are clinically beneficial, and able, in the long run, to improve outcome in syndromes of systemic inflammation.

End of Manuscript #1

(Please see end notes incorporated as part of introduction's end notes, p58)

1.7.3 Hypertonic Saline and Neutrophils

Thus, prior exposure of activated neutrophils to hypertonicity blunts expression of their adhesion receptors and their ability to interact with and injure endothelium. Furthermore, important effects of hypertonic saline on the cytotoxic function of activated neutrophils have also been reported. In vitro studies have demonstrated blunting of N-formyl-methionyl-leucyl-phenylalanine (fMLP)-activated neutrophil superoxide release with prior incubation in hypertonic media[189,193,214,215]. Other experiments used mesenteric lymph from hemorrhaged rodents resuscitated with either HTS or RL as a PMN priming agent. Ex vivo neutrophils primed with lymph from HTS animals demonstrated significantly less superoxide release compared to that from RL counterparts[111,216,217]. In vivo, PMNs from HTS-resuscitated hemorrhagic swine also demonstrate reduced intracellular superoxide burst as compared to those from RL counterparts[114].

As with reactive oxygen species, the release of proteolytic enzymes during neutrophil degranulation is also altered by hypertonicity. While some studies indicate a potentiating effect of hypertonicity for elastase release upon neutrophil activation[218], more recent evaluations find that hypertonicity, at clinically relevant levels, blunts the enzyme's release, providing hypertonic exposure precedes both neutrophil priming and activation[193,219].

While hypertonicity blunts PMN cytotoxicity, its effect on other PMN functions such as phagocytosis is equivocal. Some studies report ehanced phagocytic function whilst others find hypertonicity attenuates phagocytosis[191,214,189,194].

The molecular biology behind the effects of hypertonic saline on neutrophils has been investigated by several groups. Neutrophil shrinking by cytoskeletal shape changes that occur upon exposure to hypertonic environments may be at the basis of altered PMN function. PMN cytosol, relatively hypotonic in physiologic conditions, loses water to equalize osmolality with the hypertonic interstitial milieu. Cellular shrinkage and

loss of intracellular water causes a reorganization of the neutrophil's cytoskeleton inducing actin fiber polymerization to accommodate the new cell shape[215]. Interestingly, actin polymerization and cytoskeletal rearrangement are known to also be critical events necessary for converting a rolling neutrophil into one firmly adherent to endothelium[220]. Indeed, the differential expression of PMN and endothelial surface adhesion molecules responsible for conformational change (rolling to adherence) is partly controlled by cytoskeletal structure. Hence, the cellular cytoskeleton is not a passive matrix but rather a complex frame, anchoring membrane bound receptors and signaling kinases and providing channels for vesicular and protein trafficking[221-223]. Adequate cellular signal transduction is impaired without a functioning cytoskeleton. HTS mediated changes in cytoskeletal organization may thus modify normal signaling component relationships and result in dysfunctional signal transduction. In fact, HTS alters the phosphorylation of specific signal transduction kinases, those of the mitogen activated protein kinase (MAPK) family. In particular, (MAPK) p38, which appears to function as an osmoreceptor in human neutrophils, has been shown to become weakly phosphorylated upon exposure to hypertonicity[224]. MAPK p38 is known to significantly contribute to cellular activation, participating in the intracellular cascade regulating stress activated signal transduction[225]. Interestingly, in vitro experiments demonstrate that while HTS weakly activates MAPK p38, it also completely prevents subsequent MAPK p38 activation by standard activating agents such as lipopolysacharide (LPS) or fMLP[225,226]. Furthermore, phosphorylation of MAPK p38 has been consistently associated with upregulation of neutrophil adhesion molecules. While LPS or PAF cause considerable kinase activation and PMN CD11b expression, kinase phosphorylation and integrin expression with these agents is reduced to less than half with prior exposure to HTS[225,227]. As with kinase activation, HTS alone also upregulates PMN integrin expression, but to a very mild degree. These studies thus suggest that the weak

MAPKp38 activation by hypertonicity renders the neutrophil refractory to other stimuli without inducing a functional cellular response nor upregulating its CD11b expression.

In parallel to these studies, another set of investigations evaluating the cytotoxic response of PMNs has shown that while fMLP and PAF neutrophil activation results in a considerable release of superoxide and elastase, prior exposure to mildly hypertonic media (10-20 mM) results in significant inhibition of fMLP/PAF-mediated cytotoxicity[226,227]. In the same experimental conditions, western blot analysis reveals that PAF activation of MAPK p38 is reduced by half if PMNs are similarly preincubated with clinically relevant hypertonicity levels[227]. Complementary experiments demonstrating that HTS blunts fMLP but not PMA-mediated PMN superoxide release further suggest that HTS affects the signal transduction cascade distal to the receptor responsible for ROS production (NADPH), as PMA activation is receptor-independent[227,228]. These findings relating PMN cytotoxicity and adhesion molecule expression to alterations of MAPKp38 by HTS are further complemented by studies on PMN actin polymerization. Ciesla and colleagues report that clinically relevant levels of HTS cause a 30% increase in PMN F-actin polymerization while fMLP stimulation causes a 100% increase. Exposure to HTS prior to fMLP stimulation completely abrogates fMLP increases[215]. Again, this data suggests that while HTS itself weakly polymerizes neutrophil cytoskeletal actin, it renders the cytoskeleton completely unresponsive to full polymerization by activating agents.

Hence, taken together this data suggests that HTS reorganizes the actin cytoskeleton of neutrophils to a state unfavorable for MAPK p38 activation which is otherwise necessary for CD11b upregulation and ROS/proteolytic enzyme release. This attractive paradigm as the molecular mechanism of HTS immune benefits may be an oversimplification, as recent experiments have demonstrated independent alterations in cellular exocytosis following HTS-related changes in neutrophil cytoskeletal shape[229].

Whether HTS-mediated cytoskeletal modifications alter PMN structure and function primarily through dysfunctional signal transduction or peptide trafficking remains to be seen, and will likely be clarified in future studies.

1.7.4 Hypertonic Saline and Other Cells

Immune modulation by hypertonic saline has also been evaluated on cells other than neutrophils. T lymphocytes were the first leukocytes to demonstrate functional alterations by hypertonic saline. T-cell suppression by certain stimuli was reversed by exposure to hypertonic saline, causing augmented lymphocyte proliferation and enhanced cell mediated immunity[230-232]. In vivo hemorrhagic shock studies later confirmed proliferation of CD4+ cells and natural killer cells if HTS had been used for resuscitation[233]. HTS may also affect platelet function by reducing their adhesiveness and ability to form a hemostatic plug[234,235]. Endothelial cells exposed to hypertonic saline demonstrate reductions in adhesion molecule expression as discussed above. HTS may additionally cause increased EC PGI_2 production through increased cyclo-oxygenase enzyme activity[236,237]. Little is known about the effect of hypertonicity on macrophages but in vitro studies demonstrate that with prior hypertonic incubation macrophage activation results in increased IL-10 release and suppressed TNFα production[238,239].

1.7.5 Protective Effects of Hypertonic Saline on End Organs

While hypertonic saline exposure clearly alters the structure and function of neutrophils, endothelial cells and several other microcirculatory components, the key question is whether these effects translate into changes in inflammation-mediated tissue injury in end organs. Several animal models have demonstrated reductions in neutrophil tissue sequestration by HTS resuscitation. Pancreatitis rat models have shown reduced lung myeloperoxidase (MPO) activity, a marker of total neutrophil tissue content if HTS

was used instead of NS for resuscitation[210]. In the same study, HTS animals also demonstrated reduced protein leakage in broncoalveolar lavage (BAL) fluid, diminished lung edema, and attenuated histologic lung injury compared to NS counterparts. An animal model using intermittent infrarenal aortic cross-clamping to cause lower torso ischemia/reperfusion also demonstrated diminished pulmonary MPO, edema and histologic injury, with HTS administration prior to reperfusion[240]. In the same study, measurement of liver transaminases revealed better hepatic function in HTS animals compared to isotonic controls. In a hepatic ischemia/reperfusion study, Oreopoulos *et al* also showed hypertonic saline-mediated reductions in liver transaminase levels but additionally found diminished histologic injury in the livers of the same animals[195].

End organ benefits from hypertonic resuscitation are also seen following resuscitation of hemorrhagic shock. Moreover, improved survival of hypertonic saline resuscitated animals has been shown in certain hemorrhagic shock studies[188,241]. In hemorrhaged rodent models, numerous authors report diminished BAL neutrophil sequestration, lung permeability and lung MPO, following HTS resuscitation. Concurrently they demonstrate diminished lung histologic injury from hours[115,241] to days after resuscitation in these animals[187,242,243]. Furthermore, PMNs taken from these same HTS resuscitated animals, were found to produce 60% less superoxide than those from RL counterparts[187]. In similar studies, HTS resuscitation also resulted in improved small bowel histologic morphology compared to that in isotonic resuscitated controls[244,245]. Two-hit models, where hemorrhagic shock is subsequently followed by an infectious second insult, further underscore persistent end-organ benefits by HTS resuscitation. Rizoli and colleagues describe a model where hemorrhaged rats were resuscitated with either RL or HTS and subsequently received intra-tracheal LPS as a second hit[109]. In this elegant study they demonstrated how, compared to RL-resuscitation, HTS diminished lung MPO, BAL protein leakage and PMN sequestration, diminished PMN

and endothelial adhesion molecule expression and reduced histologic lung injury[109]. These results from a two-hit model were confirmed by subsequent studies from the same authors[110] and others using caecal ligation and puncture (CLP) as an alternate second hit[188]. In this and other models, gut mucosal bacterial translocation and the resultant bacteremia occurring with isotonic hemorrhagic shock resuscitation was concomitantly reduced with HTS resuscitation[188,233,242,245,246].

Clear evidence exists that resuscitated hemorrhagic shock can result in disregulated activation of the host inflammatory response, in part through tissue injury caused by the inappropriate activation of PMNs resulting in their indiscriminate release of toxic metabolites. Small volumes of hypertonic saline instead of standard isotonic crystalloid resuscitation not only restore hemodynamic parameters efficiently, but also appear to blunt the exuberant inflammatory response, specifically altering the structure and function of activated neutrophils. Both in vitro and ex-vivo studies exist supporting the immune modulating effects of hypertonic saline resuscitation, but few analyses have linked this data to in vivo investigations.

General Hypothesis

Compared to lactated Ringer's, hypertonic saline resuscitation diminishes in vivo the activation of neutrophils and endothelial cells resulting in their reduced interactions and in the attenuation of tissue injury following hemorrhagic shock.

Specific Objectives #1

1. To establish a murine model of resuscitated hemorrhagic shock allowing in vivo evaluation of cremaster microcirculation by intravital microscopy.
2. To compare short-term leukocyte rolling and adhesion to endothelium before, during and up to 90 minutes after either hypertonic saline or lactated Ringer's resuscitation of hemorrhagic shock.
3. To compare venular permeability in the same animals' microcirculatory bed after either hypertonic saline or lactated Ringer's resuscitation of hemorrhagic shock

Specific Objectives #2

1. To establish a two-insult murine model where severe resuscitated hemorrhagic shock (1^{st} insult) is followed by a mimicked pulmonary infection (2^{nd} insult) one hour later.
2. To compare in vivo neutrophil adhesion to endothelium hours after resuscitation with either lactated Ringer's or hypertonic saline in a two-insult hemorrhagic shock model.
3. In the same animals, to concurrently compare lung sequestration of neutrophils one day after resuscitation with either lactated Ringer's or hypertonic saline in two-insult conditions.
4. In the same animals, to concurrently evaluate with histologic analysis the degree of neutrophil margination and transmigration occurring in lung 24 hours after resuscitation with either lactated Ringer's or hypertonic saline in two-insult conditions

Specific Objectives #3

1. To determine if attenuation of in vivo neutrophil-endothelial interactions persist a day after resuscitation with hypertonic saline in a two-insult hemorrhagic shock model.
2. To determine if in vivo reduction of microvascular permeability persist 22 hours after resuscitation with hypertonic saline in a two-insult hemorrhagic shock model.

3. To evaluate differences in soluble ICAM-1 plasma levels one day after resuscitation with either lactated Ringer's or hypertonic saline in two-insult conditions.
4. In the same animals, to concurrently compare lung and cremaster parenchymal tissue injury one day after resuscitation with either lactated Ringer's or hypertonic saline in two-insult conditions.

Specific Objectives #4

1. To establish if attenuation of neutrophil adherence to endothelium by hypertonic saline is the principal mechanism by which it improves pulmonary injury after hemorrhagic shock.
    a. To compare resuscitation with hypertonic saline alone and with RL supplemented with anti PMN adhesion blockade (by monoclonal antibodies against PMN CD11b and endothelial ICAM-1).
    b. In these conditions, to evaluate if RL supplemeted with anti-PMN adhesion blockade is able to reproduce the immune effects of HTS with respect to:
        i. Early neutrophil rolling and adhesion to endothelium in vivo
        ii. Early microvascular permeability in vivo
        iii. One day functional behavior of live animals
        iv. One day neutrophil lung sequestration
        v. One day lung and cremaster histologic injury

1.8 References

1. Baue, A.E. Multiple, progressive, or sequential systems failure. A syndrome of the 1970s. *Arch Surg* **110**, 779-81 (1975).
2. Eiseman, B., Beart, R. & Norton, L. Multiple organ failure. *Surg Gynecol Obstet* **144**, 323-6 (1977).
3. Bone, R.C. Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS). *Ann Intern Med* **125**, 680-7 (1996).
4. Salazar, C.F. *The treatment of war wounds in Graeco-Roman antiquity*, xxvii, 299 p. (Brill, Leiden ; Boston, 2000).
5. Pope, A.F.G., Longnecker. D. E. Editors. *Fluid Resuscitation: State of the Science for Treating Combat Casualties and Civilian Injuries*, 196 (National Academy Press, 1999).
6. Malech, H.L. & Gallin, J.I. Current concepts: immunology. Neutrophils in human diseases. *N Engl J Med* **317**, 687-94 (1987).
7. Hoffman, R. *Hematology : basic principles and practice*, xxxi, 2584 , [24] of plates (Churchill Livingstone, New York, 2000).
8. Cannistra, S.A. & Griffin, J.D. Regulation of the production and function of granulocytes and monocytes. *Semin Hematol* **25**, 173-88 (1988).
9. Boggs, D.R. The kinetics of neutrophilic leukocytes in health and in disease. *Semin Hematol* **4**, 359-86 (1967).
10. Lieschke, G.J. & Burgess, A.W. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (1). *N Engl J Med* **327**, 28-35 (1992).
11. Braunwald, E. *Harrison's principles of internal medicine*, (McGraw-Hill Medical Publishing Division, New York Montreal, 2001).
12. Braunwald, E., Zipes, D.P. & Libby, P. *Heart disease : a textbook of cardiovascular medicine*, (Saunders, Philadelphia, 2001).
13. Guo, J.P., Murohara, T., Buerke, M., Scalia, R. & Lefer, A.M. Direct measurement of nitric oxide release from vascular endothelial cells. *J Appl Physiol* **81**, 774-9. (1996).
14. Lefer, A.M. & Lefer, D.J. The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. *Cardiovasc Res* **32**, 743-51. (1996).
15. Schmid-Schonbein, G.W., Usami, S., Skalak, R. & Chien, S. The interaction of leukocytes and erythrocytes in capillary and postcapillary vessels. *Microvasc Res* **19**, 45-70 (1980).
16. Bevilacqua, M.P. & Nelson, R.M. Selectins. *J Clin Invest* **91**, 379-87 (1993).
17. Perry, M.A. & Granger, D.N. Role of CD11/CD18 in shear rate-dependent leukocyte-endothelial cell interactions in cat mesenteric venules. *J Clin Invest* **87**, 1798-804 (1991).
18. Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-14 (1994).
19. McEver, R.P. & Cummings, R.D. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest* **100**, S97-103 (1997).
20. Kubes, P., Jutila, M. & Payne, D. Therapeutic potential of inhibiting leukocyte rolling in ischemia/reperfusion. *J Clin Invest* **95**, 2510-9 (1995).
21. Mansson, P., Zhang, X.W., Jeppsson, B., Johnell, O. & Thorlacius, H. Critical role of P-selectin-dependent rolling in tumor necrosis factor- alpha-induced leukocyte adhesion and extravascular recruitment in vivo. *Naunyn Schmiedebergs Arch Pharmacol* **362**, 190-6. (2000).
22. Ley, K. et al. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J Exp Med* **181**, 669-75. (1995).
23. Scalia, R., Armstead, V.E., Minchenko, A.G. & Lefer, A.M. Essential role of P-selectin in the initiation of the inflammatory response induced by hemorrhage and reinfusion. *J Exp Med* **189**, 931-8. (1999).
24. Granger, D.N. & Kubes, P. The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. *J Leukoc Biol* **55**, 662-75 (1994).
25. Carlos, T.M. & Harlan, J.M. Membrane proteins involved in phagocyte adherence to endothelium. *Immunol Rev* **114**, 5-28 (1990).
26. Springer, T.A. Adhesion receptors of the immune system. *Nature* **346**, 425-34 (1990).
27. Salas, A. et al. Heparin attenuates TNF-alpha induced inflammatory response through a CD11b dependent mechanism. *Gut* **47**, 88-96 (2000).
28. Yan, H.C. et al. Human/severe combined immunodeficient mouse chimeras. An experimental in vivo model system to study the regulation of human endothelial cell-leukocyte adhesion molecules. *J Clin Invest* **91**, 986-96 (1993).
29. Horie, Y., Wolf, R., Miyasaka, M., Anderson, D.C. & Granger, D.N. Leukocyte adhesion and hepatic microvascular responses to intestinal ischemia/reperfusion in rats. *Gastroenterology* **111**, 666-73 (1996).

30. Kurose, I. et al. Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. *Circ Res* **74**, 336-43 (1994).
31. Yadav, S.S. et al. L-selectin and ICAM-1 mediate reperfusion injury and neutrophil adhesion in the warm ischemic mouse liver. *Am J Physiol* **275**, G1341-52. (1998).
32. von Andrian, U.H. et al. In vivo behavior of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes. *J Clin Invest* **91**, 2893-7 (1993).
33. Henderson, R.B. et al. The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and alpha4 integrin in the inflammatory response of neutrophils. *J Exp Med* **194**, 219-26. (2001).
34. Anderson, D.C. & Springer, T.A. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu Rev Med* **38**, 175-94 (1987).
35. Smith, C.W. Endothelial adhesion molecules and their role in inflammation. *Can J Physiol Pharmacol* **71**, 76-87 (1993).
36. Armstrong, M., Jr., Needham, D., Hatchell, D.L. & Nunn, R.S. Effect of pentoxifylline on the flow of polymorphonuclear leukocytes through a model capillary. *Angiology* **41**, 253-62 (1990).
37. Del Maschio, A. et al. Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. *J Cell Biol* **135**, 497-510 (1996).
38. Burns, A.R. et al. Neutrophil transendothelial migration is independent of tight junctions and occurs preferentially at tricellular corners. *J Immunol* **159**, 2893-903 (1997).
39. Dejana, E., Zanetti, A. & Del Maschio, A. Adhesive proteins at endothelial cell-to-cell junctions and leukocyte extravasation. *Haemostasis* **26 Suppl 4**, 210-9 (1996).
40. Luscinskas, F.W., Ma, S., Nusrat, A., Parkos, C.A. & Shaw, S.K. Leukocyte transendothelial migration: a junctional affair. *Semin Immunol* **14**, 105-13 (2002).
41. Muller, W.A., Weigl, S.A., Deng, X. & Phillips, D.M. PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med* **178**, 449-60 (1993).
42. Kuwabara, H., Tanaka, S., Sakamoto, H., Oryu, M. & Uda, H. Antibody mediated ligation of platelet/endothelial cell adhesion molecule-1 (PECAM-1) on neutrophils enhances adhesion to cultured human dermal microvascular endothelial cells. *Kobe J Med Sci* **42**, 233-41 (1996).
43. Zimmerman, B.J. et al. Molecular determinants of lipid mediator-induced leukocyte adherence and emigration in rat mesenteric venules. *Am J Physiol* **266**, H847-53 (1994).
44. Weiss, S.J. Tissue destruction by neutrophils [see comments]. *N Engl J Med* **320**, 365-76 (1989).
45. Halliwell, B., Clement, M.V. & Long, L.H. Hydrogen peroxide in the human body. *FEBS Lett* **486**, 10-3. (2000).
46. Sauaia, A. et al. Epidemiology of trauma deaths: a reassessment. *J Trauma* **38**, 185-93. (1995).
47. Maughon, J.S. An inquiry into the nature of wounds resulting in killed in action in Vietnam. *Mil Med* **135**, 8-13. (1970).
48. Rotstein, O.D. Novel strategies for immunomodulation after trauma: revisiting hypertonic saline as a resuscitation strategy for hemorrhagic shock [In Process Citation]. *J Trauma* **49**, 580-3 (2000).
49. Warren, J.C. *To work in the vineyard of surgery ; the reminiscences of J. Collins Warren, 1842-1927*, viii, 288 (Harvard University Press, Cambridge,, 1958).
50. Greenfield, L.J. & Mulholland, M.W. *Surgery : scientific principles and practice*, xxvi, 2381 (Lippincott Williams & Wilkins, Philadelphia, 2001).
51. Peitzman, A.B. et al. Hemorrhagic shock. *Curr Probl Surg* **32**, 925-1002. (1995).
52. Orlinsky, M., Shoemaker, W., Reis, E.D. & Kerstein, M.D. Current controversies in shock and resuscitation. *Surg Clin North Am* **81**, 1217-62, xi-xii (2001).
53. Bond, R.F. & Johnson, G., 3rd. Cardiovascular adrenoreceptor function during compensatory and decompensatory hemorrhagic shock. *Circ Shock* **12**, 9-24 (1984).
54. Shoemaker, W.C., Appel, P.L., Kram, H.B., Waxman, K. & Lee, T.S. Prospective trial of supranormal values of survivors as therapeutic goals in high-risk surgical patients. *Chest* **94**, 1176-86. (1988).
55. Shoemaker, W.C., Appel, P.L. & Kram, H.B. Role of oxygen debt in the development of organ failure sepsis, and death in high-risk surgical patients. *Chest* **102**, 208-15. (1992).
56. Lefer, A.M. Eicosanoids as mediators of ischemia and shock. *Fed Proc* **44**, 275-80 (1985).
57. Barroso-Aranda, J., Schmid-Schonbein, G.W., Zweifach, B.W. & Engler, R.L. Granulocytes and no-reflow phenomenon in irreversible hemorrhagic shock. *Circ Res* **63**, 437-47 (1988).
58. Barroso-Aranda, J. & Schmid-Schonbein, G.W. Transformation of neutrophils as indicator of irreversibility in hemorrhagic shock. *Am J Physiol* **257**, H846-52 (1989).
59. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis [see comments]. *Crit Care Med* **20**, 864-74 (1992).
60. Levy, M.M. et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med* **31**, 1250-6 (2003).

61. Moore, F.A. & Moore, E.E. Evolving concepts in the pathogenesis of postinjury multiple organ failure. *Surg Clin North Am* **75**, 257-77. (1995).
62. Ahmed, N. & Christou, N. Systemic inflammatory response syndrome: interactions between immune cells and the endothelium. *Shock* **6**, S39-42 (1996).
63. Baker, C.C., Oppenheimer, L., Stephens, B., Lewis, F.R. & Trunkey, D.D. Epidemiology of trauma deaths. *Am J Surg* **140**, 144-50 (1980).
64. Carrico, C.J., Meakins, J.L., Marshall, J.C., Fry, D. & Maier, R.V. Multiple-organ-failure syndrome. *Arch Surg* **121**, 196-208 (1986).
65. Deitch, E.A. Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg* **216**, 117-34 (1992).
66. Tsukada, K. et al. Neutrophil accumulation and damage to the gastric mucosa in resuscitated hemorrhagic shock is independent of inducible nitric oxide synthase. *Shock* **11**, 319-24 (1999).
67. Hirose, J., Yamaga, M., Kato, T., Ikebe, K. & Takagi, K. Effects of a hydroxyl radical scavenger, EPC-K1, and neutrophil depletion on reperfusion injury in rat skeletal muscle. *Acta Orthop Scand* **72**, 404-10 (2001).
68. Eppinger, M.J., Jones, M.L., Deeb, G.M., Bolling, S.F. & Ward, P.A. Pattern of injury and the role of neutrophils in reperfusion injury of rat lung. *J Surg Res* **58**, 713-8 (1995).
69. Atochin, D.N., Fisher, D., Demchenko, I.T. & Thom, S.R. Neutrophil sequestration and the effect of hyperbaric oxygen in a rat model of temporary middle cerebral artery occlusion. *Undersea Hyperb Med* **27**, 185-90 (2000).
70. Inoue, S. et al. Anti-neutrophil antibody attenuates the severity of acute lung injury in rats with experimental acute pancreatitis. *Arch Surg* **130**, 93-8. (1995).
71. Terashima, T. et al. Neutrophil-induced lung protection and injury are dependent on the amount of Pseudomonas aeruginosa administered via airways in guinea pigs. *Am J Respir Crit Care Med* **152**, 2150-6 (1995).
72. McClintock, S.D., Till, G.O., Smith, M.G. & Ward, P.A. Protection from half-mustard-gas-induced acute lung injury in the rat. *J Appl Toxicol* **22**, 257-62 (2002).
73. Abraham, E. Neutrophils and acute lung injury. *Crit Care Med* **31**, S195-9 (2003).
74. Marshall, J.C., Christou, N.V. & Meakins, J.L. The gastrointestinal tract. The "undrained abscess" of multiple organ failure. *Ann Surg* **218**, 111-9 (1993).
75. Marshall, J.C. The ecology and immunology of the gastrointestinal tract in health and critical illness. *J Hosp Infect* **19 Suppl C**, 7-17 (1991).
76. McCall, C.E., DeChatelet, L.R., Cooper, M.R. & Shannon, C. Human toxic neutrophils. 3. Metabolic characteristics. *J Infect Dis* **127**, 26-33 (1973).
77. Condliffe, A.M., Kitchen, E. & Chilvers, E.R. Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci (Lond)* **94**, 461-71 (1998).
78. Swain, S.D., Rohn, T.T. & Quinn, M.T. Neutrophil priming in host defense: role of oxidants as priming agents. *Antioxid Redox Signal* **4**, 69-83 (2002).
79. Patrick, D.A., Moore, F.A., Moore, E.E., Barnett, C.B. & Silliman, C.C. Neutrophil priming and activation in the pathogenesis of postinjury multiple organ failure. *New Horiz* **4**, 194-210 (1996).
80. Matzner, Y. Acquired neutrophil dysfunction and diseases with an inflammatory component. *Semin Hematol* **34**, 291-302 (1997).
81. Spain, D.A. et al. Decreased alpha-adrenergic response in the intestinal microcirculation after "two-hit" hemorrhage/resuscitation and bacteremia. *J Surg Res* **84**, 180-5. (1999).
82. Eissner, B., Matz, K., Smorodchenko, A., Roschmann, A. & v Specht, B.U. Chronic porcine two-hit model with hemorrhagic shock and Pseudomonas aeruginosa sepsis. *Eur Surg Res* **34**, 61-7. (2002).
83. van Griensven, M. et al. Polymicrobial sepsis induces organ changes due to granulocyte adhesion in a murine two hit model of trauma. *Exp Toxicol Pathol* **54**, 203-9 (2002).
84. Guyton, A.C. & Hall, J.E. *Circulatory shock and physiology of its treatment. In Textbook of medical physiology*, xliii, 1148 (W.B. Saunders, Philadelphia, 1996).
85. Smail, N., Wang, P., Cioffi, W.G., Bland, K.I. & Chaudry, I.H. Resuscitation after uncontrolled venous hemorrhage: Does increased resuscitation volume improve regional perfusion? *J Trauma* **44**, 701-8. (1998).
86. Cryer, H.G. Therapeutic approaches for clinical ischemia and reperfusion injury. *Shock* **8**, 26-32. (1997).
87. Paterson, I.S. et al. Thromboxane mediates the ischemia-induced neutrophil oxidative burst. *Surgery* **106**, 224-9. (1989).
88. Bitterman, H., Smith, B.A. & Lefer, A.M. Beneficial actions of antagonism of peptide leukotrienes in hemorrhagic shock. *Circ Shock* **24**, 159-68 (1988).
89. Stahl, G.L., Craft, D.V., Lento, P.H. & Lefer, A.M. Detection of platelet-activating factor during traumatic shock. *Circ Shock* **26**, 237-44 (1988).

90. Fosse, E., Mollnes, T.E., Aasen, A.O., Trumpy, J.H. & Stokke, T. Complement activation following multiple injuries. *Acta Chir Scand* **153**, 325-30 (1987).
91. Molina, P.E. et al. Early organ-specific hemorrhage-induced increases in tissue cytokine content: associated neurohormonal and opioid alterations. *Neuroimmunomodulation* **4**, 28-36 (1997).
92. Jiang, J. et al. Expression of TNF alpha, IL-1 beta, IL-6 mRNA, release of TNF alpha in vital organs and their relationship with endotoxin translocation following hemorrhagic shock. *Chin Med Sci J* **12**, 41-6 (1997).
93. Meldrum, D.R. et al. Hemorrhage activates myocardial NFkappaB and increases TNF-alpha in the heart. *J Mol Cell Cardiol* **29**, 2849-54 (1997).
94. Tamion, F. et al. Gut ischemia and mesenteric synthesis of inflammatory cytokines after hemorrhagic or endotoxic shock. *Am J Physiol* **273**, G314-21 (1997).
95. Donnelly, T.J. et al. Cytokine, complement, and endotoxin profiles associated with the development of the adult respiratory distress syndrome after severe injury. *Crit Care Med* **22**, 768-76 (1994).
96. Wink, D.A. & Mitchell, J.B. Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med* **25**, 434-56. (1998).
97. Halliwell, B., Gutteridge, J.M. & Cross, C.E. Free radicals, antioxidants, and human disease: where are we now? *J Lab Clin Med* **119**, 598-620. (1992).
98. Granger, D.N. Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *Am J Physiol* **255**, H1269-75. (1988).
99. Cheeseman, K.H. & Slater, T.F. An introduction to free radical biochemistry. *Br Med Bull* **49**, 481-93. (1993).
100. Halliwell, B. & Gutteridge, J.M. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett* **307**, 108-12. (1992).
101. Halliwell, B., Clement, M.V., Ramalingam, J. & Long, L.H. Hydrogen peroxide. Ubiquitous in cell culture and in vivo? *IUBMB Life* **50**, 251-7. (2000).
102. Ochoa, J.B. et al. Nitrogen oxide levels in patients after trauma and during sepsis. *Ann Surg* **214**, 621-6 (1991).
103. Jacob, T.D. et al. Nitric oxide production is inhibited in trauma patients. *J Trauma* **35**, 590-6; discussion 596-7 (1993).
104. Toth, B. et al. Gender dimorphism in neutrophil priming and activation following trauma-hemorrhagic shock. *Int J Mol Med* **11**, 357-64 (2003).
105. Kushimoto, S., Okajima, K., Okabe, H. & Binder, B.R. Role of granulocyte elastase in the formation of hemorrhagic shock-induced gastric mucosal lesions in the rat. *Crit Care Med* **24**, 1041-6 (1996).
106. Kushimoto, S. et al. Role of granulocyte elastase in ischemia/reperfusion injury of rat liver. *Crit Care Med* **24**, 1908-12 (1996).
107. Ishikawa, N., Oda, M., Kawaguchi, M., Tsunezuka, Y. & Watanabe, G. The effects of a specific neutrophil elastase inhibitor (ONO-5046) in pulmonary ischemia-reperfusion injury. *Transpl Int* **16**, 341-6 (2003).
108. Dzienis-Koronkiewicz, E., Debek, W. & Chyczewski, L. Hemorrhagic shock-induced changes in the cathepsin D activity in the intestinal wall and blood serum in rats. *Eur J Pediatr Surg* **8**, 288-94 (1998).
109. Rizoli, S.B. et al. Immunomodulatory effects of hypertonic resuscitation on the development of lung inflammation following hemorrhagic shock. *J Immunol* **161**, 6288-96 (1998).
110. Rizoli, S.B., Kapus, A., Parodo, J., Fan, J. & Rotstein, O.D. Hypertonic immunomodulation is reversible and accompanied by changes in CD11b expression. *J Surg Res* **83**, 130-5 (1999).
111. Deitch, E.A., Shi, H.P., Feketeova, E., Hauser, C.J. & Xu, D.Z. Hypertonic saline resuscitation limits neutrophil activation after trauma-hemorrhagic shock. *Shock* **19**, 328-33 (2003).
112. Angle, N. et al. Hypertonic saline resuscitation reduces neutrophil margination by suppressing neutrophil L selectin expression. *J Trauma* **45**, 7-12; discussion 12-3 (1998).
113. Pan, J., Fu, G., Lin, X. & et al. The expression of L-selectin at transcription and translation level in hemorrhagic shock rat. *Zhonghua Nei Ke Za Zhi* **41**, 472-5 (2002).
114. Rhee, P. et al. Lactated Ringer's solution resuscitation causes neutrophil activation after hemorrhagic shock. *J Trauma* **44**, 313-9 (1998).
115. Sun, L.L. et al. Early up-regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in rats with hemorrhagic shock and resuscitation. *Shock* **11**, 416-22 (1999).
116. Olinde, J.G. et al. Persantine attenuates hemorrhagic shock-induced P-selectin expression. *Am Surg* **66**, 1093-7; discussion 1097-8. (2000).
117. Akgur, F.M. et al. Role of superoxide in hemorrhagic shock-induced P-selectin expression. *Am J Physiol Heart Circ Physiol* **279**, H791-7. (2000).
118. Corso, C.O., Okamoto, S., Ruttinger, D. & Messmer, K. Hypertonic saline dextran attenuates leukocyte accumulation in the liver after hemorrhagic shock and resuscitation. *J Trauma* **46**, 417-23 (1999).

119. Vollmar, B., Lang, G., Menger, M.D. & Messmer, K. Hypertonic hydroxyethyl starch restores hepatic microvascular perfusion in hemorrhagic shock. *Am J Physiol* **266**, H1927-34 (1994).
120. Bauer, C., Marzi, I., Bauer, M., Fellger, H. & Larsen, R. Interleukin-1 receptor antagonist attenuates leukocyte-endothelial interactions in the liver after hemorrhagic shock in the rat. *Crit Care Med* **23**, 1099-105. (1995).
121. Maier, M., Strobele, H., Voges, J., Bauer, C. & Marzi, I. Attenuation of leukocyte adhesion by recombinant TNF-binding protein after hemorrhagic shock in the rat. *Shock* **19**, 457-61 (2003).
122. Moncure, M. et al. Heme-oxygenase-1 mRNA expression affects hemorrhagic shock-induced leukocyte adherence. *J Trauma* **55**, 118-25 (2003).
123. Balogh, Z. et al. Dalteparin sodium treatment during resuscitation inhibits hemorrhagic shock-induced leukocyte rolling and adhesion in the mesenteric microcirculation. *J Trauma* **52**, 1062-9; discussion 1070 (2002).
124. Miyabe, M. et al. Sodium nitroprusside decreases leukocyte adhesion and emigration after hemorrhagic shock. *Anesth Analg* **94**, 296-301, table of contents (2002).
125. Childs, E.W., Smalley, D.M., Moncure, M., Miller, J.L. & Cheung, L.Y. Effect of WEB 2086 on Leukocyte Adherence in Response to Hemorrhagic Shock in Rats. *J Trauma* **49**, 1102-1107. (2000).
126. von Dobschuetz, E., Hoffmann, T. & Messmer, K. Diaspirin cross-linked hemoglobin effectively restores pancreatic microcirculatory failure in hemorrhagic shock. *Anesthesiology* **91**, 1754-62 (1999).
127. Botzlar, A., Nolte, D. & Messmer, K. Effects of ultra-purified polymerized bovine hemoglobin on the microcirculation of striated skin muscle in the hamster. *Eur J Med Res* **1**, 471-8 (1996).
128. Nolte, D., Botzlar, A., Pickelmann, S., Bouskela, E. & Messmer, K. Effects of diaspirin-cross-linked hemoglobin (DCLHb) on the microcirculation of striated skin muscle in the hamster: a study on safety and toxicity. *J Lab Clin Med* **130**, 314-27 (1997).
129. Ringer, S. A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *Journal of Physiology* **4**, 29-42 (1883).
130. Hartmann, A.F. Theory and practice of parenteral fluid administration. *Journal of the American Medical Association* **103**, 1349-54 (1934).
131. Cannon, W.B. *Traumatic shock*, 201 (Appleton, New York,, 1923).
132. Jenkins, M., Jones, RF, Wilson B, Moyer,CA. Congestive atelectasis: a complication of the intravenousinfusion of fluids. *Ann Surg* **132**, 327-47 (1950).
133. Canizaro, P.C., Prager, M.D. & Shires, G.T. The infusion of Ringer's lactate solution during shock. Changes in lactate, excess lactate, and pH. *Am J Surg* **122**, 494-501. (1971).
134. Skillman, J.J., Restall, D.S. & Salzman, E.W. Randomized trial of albumin vs. electrolyte solutions during abdominal aortic operations. *Surgery* **78**, 291-303. (1975).
135. Goodwin, C.W., Dorethy, J., Lam, V. & Pruitt, B.A., Jr. Randomized trial of efficacy of crystalloid and colloid resuscitation on hemodynamic response and lung water following thermal injury. *Ann Surg* **197**, 520-31. (1983).
136. Marelli, D. et al. Does the addition of albumin to the prime solution in cardiopulmonary bypass affect clinical outcome? A prospective randomized study. *J Thorac Cardiovasc Surg* **98**, 751-6. (1989).
137. Erstad, B.L., Gales, B.J. & Rappaport, W.D. The use of albumin in clinical practice. *Arch Intern Med* **151**, 901-11. (1991).
138. Human albumin administration in critically ill patients: systematic review of randomised controlled trials. Cochrane Injuries Group Albumin Reviewers. *Bmj* **317**, 235-40. (1998).
139. Bunn, F. et al. Human albumin solution for resuscitation and volume expansion in critically ill patients. The Albumin Reviewers. *Cochrane Database Syst Rev*, CD001208 (2000).
140. Alderson, P. et al. Human albumin solution for resuscitation and volume expansion in critically ill patients. *Cochrane Database Syst Rev*, CD001208 (2002).
141. Webb, A.R. The appropriate role of colloids in managing fluid imbalance: a critical review of recent meta-analytic findings. *Crit Care* **4 Suppl 2**, S26-32 (2000).
142. Wilkes, M.M. & Navickis, R.J. Patient survival after human albumin administration. A meta-analysis of randomized, controlled trials. *Ann Intern Med* **135**, 149-64 (2001).
143. Treib, J. et al. All medium starches are not the same: influence of the degree of hydroxyethyl substitution of hydroxyethyl starch on plasma volume, hemorrheologic conditions, and coagulation. *Transfusion* **36**, 450-5. (1996).
144. Schortgen, F. et al. Effects of hydroxyethylstarch and gelatin on renal function in severe sepsis: a multicentre randomised study. *Lancet* **357**, 911-6. (2001).
145. Waxman, K., Holness, R., Tominaga, G., Chela, P. & Grimes, J. Hemodynamic and oxygen transport effects of pentastarch in burn resuscitation. *Ann Surg* **209**, 341-5. (1989).

146. London, M.J. et al. A randomized clinical trial of 10% pentastarch (low molecular weight hydroxyethyl starch) versus 5% albumin for plasma volume expansion after cardiac operations. *J Thorac Cardiovasc Surg* **97**, 785-97. (1989).
147. Rackow, E.C. et al. Effects of pentastarch and albumin infusion on cardiorespiratory function and coagulation in patients with severe sepsis and systemic hypoperfusion. *Crit Care Med* **17**, 394-8. (1989).
148. Robblee, J. et al. *Principles of fluid therapy management: the role of colloid plasma volume expanders*, 41 (DuPont Pharma Educational Tools, 1999).
149. Younes, R.N. et al. Use of pentastarch solution in the treatment of patients with hemorrhagic hypovolemia: randomized phase II study in the emergency room. *World J Surg* **22**, 2-5. (1998).
150. Mauritz, W., Schimetta, W., Oberreither, S. & Polz, W. Are hypertonic hyperoncotic solutions safe for prehospital small-volume resuscitation? Results of a prospective observational study. *Eur J Emerg Med* **9**, 315-9 (2002).
151. Wu, J.J. et al. Hemodynamic response of modified fluid gelatin compared with lactated ringer's solution for volume expansion in emergency resuscitation of hypovolemic shock patients: preliminary report of a prospective, randomized trial. *World J Surg* **25**, 598-602. (2001).
152. Eremenko, A.A. & Kuslieva, E.V. [Clinical experience with the use of gelofusin (gelatin) in anesthesiology, resuscitation and intensive care]. *Anesteziol Reanimatol*, 58-61. (2001).
153. Ford, S.A., Kam, P.C., Baldo, B.A. & Fisher, M.M. Anaphylactic or anaphylactoid reactions in patients undergoing cardiac surgery. *J Cardiothorac Vasc Anesth* **15**, 684-8. (2001).
154. Wahl, R. & Kleinhans, D. IgE-mediated allergic reactions to fruit gums and investigation of cross-reactivity between gelatine and modified gelatine-containing products. *Clin Exp Allergy* **19**, 77-80. (1989).
155. Velanovich, V. Crystalloid versus colloid fluid resuscitation: a meta-analysis of mortality. *Surgery* **105**, 65-71 (1989).
156. Schierhout, G. & Roberts, I. Fluid resuscitation with colloid or crystalloid solutions in critically ill patients: a systematic review of randomised trials. *Bmj* **316**, 961-4. (1998).
157. Alderson, P., Schierhout, G., Roberts, I. & Bunn, F. Colloids versus crystalloids for fluid resuscitation in critically ill patients. *Cochrane Database Syst Rev* **2**(2000).
158. Rizoli, S.B. Crystalloids and colloids in trauma resuscitation: a brief overview of the current debate. *J Trauma* **54**, S82-8 (2003).
159. Velasco, I.T., Rocha e Silva, M., Oliveira, M.A. & Silva, R.I. Hypertonic and hyperoncotic resuscitation from severe hemorrhagic shock in dogs: a comparative study. *Crit Care Med* **17**, 261-4. (1989).
160. Velasco, I.T., Pontieri, V., Rocha e Silva, M., Jr. & Lopes, O.U. Hyperosmotic NaCl and severe hemorrhagic shock. *Am J Physiol* **239**, H664-73 (1980).
161. Corso, C.O., Okamoto, S., Leiderer, R. & Messmer, K. Resuscitation with hypertonic saline dextran reduces endothelial cell swelling and improves hepatic microvascular perfusion and function after hemorrhagic shock. *J Surg Res* **80**, 210-20 (1998).
162. Mazzoni, M.C., Borgstrom, P., Intaglietta, M. & Arfors, K.E. Capillary narrowing in hemorrhagic shock is rectified by hyperosmotic saline-dextran reinfusion. *Circ Shock* **31**, 407-18 (1990).
163. Schmall, L.M., Muir, W.W. & Robertson, J.T. Haemodynamic effects of small volume hypertonic saline in experimentally induced haemorrhagic shock. *Equine Vet J* **22**, 273-7. (1990).
164. Chiara, O. et al. Resuscitation from hemorrhagic shock: experimental model comparing normal saline, dextran, and hypertonic saline solutions. *Crit Care Med* **31**, 1915-22 (2003).
165. Matsuoka, T. & Wisner, D.H. Resuscitation of uncontrolled liver hemorrhage: effects on bleeding, oxygen delivery, and oxygen consumption. *J Trauma* **41**, 439-45 (1996).
166. Kien, N.D., Reitan, J.A., White, D.A., Wu, C.H. & Eisele, J.H. Cardiac contractility and blood flow distribution following resuscitation with 7.5% hypertonic saline in anesthetized dogs. *Circ Shock* **35**, 109-16. (1991).
167. Traverso, L.W., Bellamy, R.F., Hollenbach, S.J. & Witcher, L.D. Hypertonic sodium chloride solutions: effect on hemodynamics and survival after hemorrhage in swine. *J Trauma* **27**, 32-9. (1987).
168. Nakayama, S., Sibley, L., Gunther, R.A., Holcroft, J.W. & Kramer, G.C. Small-volume resuscitation with hypertonic saline (2,400 mOsm/liter) during hemorrhagic shock. *Circ Shock* **13**, 149-59 (1984).
169. Mouren, S. et al. Mechanisms of increased myocardial contractility with hypertonic saline solutions in isolated blood-perfused rabbit hearts. *Anesth Analg* **81**, 777-82. (1995).
170. Wade, C.E., Kramer, G.C., Grady, J.J., Fabian, T.C. & Younes, R.N. Efficacy of hypertonic 7.5% saline and 6% dextran-70 in treating trauma: a meta-analysis of controlled clinical studies. *Surgery* **122**, 609-16 (1997).
171. Vassar, M.J., Perry, C.A., Gannaway, W.L. & Holcroft, J.W. 7.5% sodium chloride/dextran for resuscitation of trauma patients undergoing helicopter transport. *Arch Surg* **126**, 1065-72. (1991).

172. Shackford, S.R. et al. Hypertonic saline resuscitation of patients with head injury: a prospective, randomized clinical trial. *J Trauma* **44**, 50-8 (1998).
173. Mattox, K.L. et al. Prehospital hypertonic saline/dextran infusion for post-traumatic hypotension. The U.S.A. Multicenter Trial. *Ann Surg* **213**, 482-91 (1991).
174. Junger, W.G. et al. Hypertonic saline resuscitation: a tool to modulate immune function in trauma patients? *Shock* **8**, 235-41 (1997).
175. Diebel, L.N., Robinson, S.L., Wilson, R.F. & Dulchavsky, S.A. Splanchnic mucosal perfusion effects of hypertonic versus isotonic resuscitation of hemorrhagic shock. *Am Surg* **59**, 495-9. (1993).
176. Behrman, S.W., Fabian, T.C., Kudsk, K.A. & Proctor, K.G. Microcirculatory flow changes after initial resuscitation of hemorrhagic shock with 7.5% hypertonic saline/6% dextran 70. *J Trauma* **31**, 589-98; discussion 599-600. (1991).
177. Oi, Y. et al. Hypertonic saline-dextran improves intestinal perfusion and survival in porcine endotoxin shock. *Crit Care Med* **28**, 2843-50. (2000).
178. Tuma, R.F., Vasthare, U.S., Arfors, K.E. & Young, W.F. Hypertonic saline administration attenuates spinal cord injury. *J Trauma* **42**, S54-60 (1997).
179. Hartl, R. et al. Hypertonic/hyperoncotic saline attenuates microcirculatory disturbances after traumatic brain injury. *J Trauma* **42**, S41-7 (1997).
180. Mazzoni, M.C., Warnke, K.C., Arfors, K.E. & Skalak, T.C. Capillary hemodynamics in hemorrhagic shock and reperfusion: in vivo and model analysis. *Am J Physiol* **267**, H1928-35 (1994).
181. Bauer, M. et al. Comparative effects of crystalloid and small volume hypertonic hyperoncotic fluid resuscitation on hepatic microcirculation after hemorrhagic shock. *Circ Shock* **40**, 187-93 (1993).
182. Scalia, S.V. et al. Mesenteric microcirculatory changes in nonlethal hemorrhagic shock: the role of resuscitation with balanced electrolyte or hypertonic saline/dextran. *J Trauma* **33**, 321-5. (1992).
183. Scalia, S. et al. Persistent arteriolar constriction in microcirculation of the terminal ileum following moderate hemorrhagic hypovolemia and volume restoration. *J Trauma* **30**, 713-8. (1990).
184. Bouskela, E., Grampp, W. & Mellander, S. Effects of hypertonic NaCl solution on microvascular haemodynamics in normo- and hypovolaemia. *Acta Physiol Scand* **140**, 85-94. (1990).
185. Torres Filho, I.P., Contaifer Junior, D., Garcia, S. & Torres, L.N. Effects of hypertonic saline solution on mesenteric microcirculation. *Shock* **15**, 353-9. (2001).
186. Etzioni, A. Integrins--the glue of life. *Lancet* **353**, 341-3 (1999).
187. Angle, N. et al. Hypertonic saline resuscitation diminishes lung injury by suppressing neutrophil activation after hemorrhagic shock. *Shock* **9**, 164-70 (1998).
188. Coimbra, R. et al. Hypertonic saline resuscitation decreases susceptibility to sepsis after hemorrhagic shock. *J Trauma* **42**, 602-6; discussion 606-7 (1997).
189. Murao, Y. et al. Does the timing of hypertonic saline resuscitation affect its potential to prevent lung damage? *Shock* **14**, 18-23. (2000).
190. Rhee, P. et al. Human neutrophil activation and increased adhesion by various resuscitation fluids. *Crit Care Med* **28**, 74-8. (2000).
191. Hampton, M.B., Chambers, S.T., Vissers, M.C. & Winterbourn, C.C. Bacterial killing by neutrophils in hypertonic environments. *J Infect Dis* **169**, 839-46 (1994).
192. Ciesla DJ, M.E., Zallen G, Biffl WL, Elzi DJ, Silliman CC. Hypertonic saline attenuation of neutrophil cytotoxic function is reversed upon return to normotonicity. *Surgical Forum* **L**, 189-91 (1999).
193. Ciesla, D.J., Moore, E.E., Zallen, G., Biffl, W.L. & Silliman, C.C. Hypertonic saline attenuation of polymorphonuclear neutrophil cytotoxicity: timing is everything. *J Trauma* **48**, 388-95 (2000).
194. Angle, N. et al. Hypertonic saline infusion: can it regulate human neutrophil function? *Shock* **14**, 503-8. (2000).
195. Oreopoulos, G.D. et al. In vivo and in vitro modulation of intercellular adhesion molecule (ICAM)-1 expression by hypertonicity. *Shock* **14**, 409-14; discussion 414-5. (2000).
196. Alam, H.B. et al. E- and P-selectin expression depends on the resuscitation fluid used in hemorrhaged rats. *J Surg Res* **94**, 145-52. (2000).
197. Barone, M., Jimenez, F., Huxley, V.H. & Yang, X.F. Morphologic analysis of the cerebral microcirculation after thermal injury and the response to fluid resuscitation. *Acta Neurochir Suppl* **70**, 267-8 (1997).
198. Nolte, D. et al. Attenuation of postischemic microvascular disturbances in striated muscle by hyperosmolar saline dextran. *Am J Physiol* **263**, H1411-6 (1992).
199. Spera, P.A., Arfors, K.E., Vasthare, U.S., Tuma, R.F. & Young, W.F. Effect of hypertonic saline on leukocyte activity after spinal cord injury. *Spine* **23**, 2444-8; discussion 2448-9 (1998).
200. Bauer, M., Zhang, J.X., Bauer, I. & Clemens, M.G. ET-1 induced alterations of hepatic microcirculation: sinusoidal and extrasinusoidal sites of action. *Am J Physiol* **267**, G143-9 (1994).
201. McCuskey, R.S. & Reilly, F.D. Hepatic microvasculature: dynamic structure and its regulation. *Semin Liver Dis* **13**, 1-12 (1993).

202. Nishida, J., McCuskey, R.S., McDonnell, D. & Fox, E.S. Protective role of NO in hepatic microcirculatory dysfunction during endotoxemia. *Am J Physiol* **267**, G1135-41 (1994).
203. Pascual, J.L. et al. Hemorrhagic shock resuscitation with a low molecular weight starch reduces neutrophil-endothelial interactions and vessel leakage in vivo. *Surg Infect (Larchmt)* **2**, 275-87; discussion 287-8 (2001).
204. Pascual, J.L. et al. Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces in vivo vascular leakage. *Ann Surg* **236**, 634-42. (2002).
205. Saetzler RK, B.M., Buckman Jr RF, Eynon AC, Tuma RF. Hypertonic saline attenuates leukocyte/endothelium and leukocyte/platelet interactions following hemorrhagic shock. *Surgical Forum* **47**, 41-3 (1996).
206. Webb, W.R. & Wax, S.D. Post-traumatic respiratory distress syndrome. *Compr Ther* **5**, 19-23. (1979).
207. Barrios, R., Inoue, S. & Hogg, J.C. Intercellular junctions in "shock lung". A freeze-fracture study. *Lab Invest* **36**, 628-35. (1977).
208. Dyess, D.L., Ardell, J.L., Townsley, M.I., Taylor, A.E. & Ferrara, J.J. Effects of hypertonic saline and dextran 70 resuscitation on microvascular permeability after burn. *Am J Physiol* **262**, H1832-7. (1992).
209. Kinsky, M.P., Milner, S.M., Button, B., Dubick, M.A. & Kramer, G.C. Resuscitation of severe thermal injury with hypertonic saline dextran: effects on peripheral and visceral edema in sheep. *J Trauma* **49**, 844-53. (2000).
210. Shields, C.J. et al. Hypertonic saline attenuates end-organ damage in an experimental model of acute pancreatitis. *Br J Surg* **87**, 1336-40. (2000).
211. Jerome, S.N., Akimitsu, T. & Korthuis, R.J. Leukocyte adhesion, edema, and development of postischemic capillary no- reflow. *Am J Physiol* **267**, H1329-36 (1994).
212. de Carvalho, H., Matos, J.A., Bouskela, E. & Svensjo, E. Vascular permeability increase and plasma volume loss induced by endotoxin was attenuated by hypertonic saline with or without dextran. *Shock* **12**, 75-80 (1999).
213. Barone, M., Jimenez, D.F., Huxley, V.H. & Yang, X.F. Cerebral vascular response to hypertonic fluid resuscitation in thermal injury. *Acta Neurochir Suppl* **70**, 265-6 (1997).
214. Matsumoto, T. et al. Neutrophil function in hyperosmotic NaCl is preserved by phosphoenol pyruvate. *Urol Res* **19**, 223-7 (1991).
215. Ciesla, D.J., Moore, E.E., Musters, R.J., Biffl, W.L. & Silliman, C.A. Hypertonic saline alteration of the PMN cytoskeleton: implications for signal transduction and the cytotoxic response. *J Trauma* **50**, 206-12. (2001).
216. Gonzalez, R.J. et al. Hyperosmolarity abrogates neutrophil cytotoxicity provoked by post- shock mesenteric lymph. *Shock* **18**, 29-32. (2002).
217. Zallen, G. et al. Hypertonic saline resuscitation abrogates neutrophil priming by mesenteric lymph. *J Trauma* **48**, 45-8. (2000).
218. Patrick, D.A. et al. Hypertonic saline activates lipid-primed human neutrophils for enhanced elastase release. *J Trauma* **44**, 592-7; discussion 598 (1998).
219. Ciesla, D.J., Moore, E.E., Biffl, W.L., Gonzalez, R.J. & Silliman, C.C. Hypertonic saline attenuation of the neutrophil cytotoxic response is reversed upon restoration of normotonicity and reestablished by repeated hypertonic challenge. *Surgery* **129**, 567-75. (2001).
220. Sheikh, S., Gratzer, W.B., Pinder, J.C. & Nash, G.B. Actin polymerisation regulates integrin-mediated adhesion as well as rigidity of neutrophils. *Biochem Biophys Res Commun* **238**, 910-5 (1997).
221. Ingber, D.E. Tensegrity: the architectural basis of cellular mechanotransduction. *Annu Rev Physiol* **59**, 575-99 (1997).
222. Niggli, V., Djafarzadeh, S. & Keller, H. Stimulus-induced selective association of actin-associated proteins (alpha-actinin) and protein kinase C isoforms with the cytoskeleton of human neutrophils. *Exp Cell Res* **250**, 558-68 (1999).
223. Li, S. et al. Distinct roles for the small GTPases Cdc42 and Rho in endothelial responses to shear stress. *J Clin Invest* **103**, 1141-50 (1999).
224. Junger, W.G. et al. Hypertonic saline activates protein tyrosine kinases and mitogen- activated protein kinase p38 in T-cells. *J Trauma* **42**, 437-43; discussion 443-5 (1997).
225. Rizoli, S.B., Kapus, A., Parodo, J. & Rotstein, O.D. Hypertonicity prevents lipopolysaccharide-stimulated CD11b/CD18 expression in human neutrophils in vitro: role for p38 inhibition. *J Trauma* **46**, 794-8; discussion 798-9 (1999).
226. Junger, W.G. et al. Hypertonicity regulates the function of human neutrophils by modulating chemoattractant receptor signaling and activating mitogen-activated protein kinase p38. *J Clin Invest* **101**, 2768-79 (1998).

227. Ciesla, D.J., Moore, E.E., Gonzalez, R.J., Biffl, W.L. & Silliman, C.C. Hypertonic saline inhibits neutrophil (PMN) priming via attenuation of p38 MAPK signaling. *Shock* **14**, 265-9; discussion 269-70. (2000).
228. Orlic, T., Loomis, W.H., Shreve, A., Namiki, S. & Junger, W.G. Hypertonicity increases cAMP in PMN and blocks oxidative burst by PKA-dependent and -independent mechanisms. *Am J Physiol Cell Physiol* **282**, C1261-9 (2002).
229. Rizoli, S.B., Rotstein, O.D., Parodo, J., Phillips, M.J. & Kapus, A. Hypertonic inhibition of exocytosis in neutrophils: central role for osmotic actin skeleton remodeling. *Am J Physiol Cell Physiol* **279**, C619-33. (2000).
230. Junger, W.G., Liu, F.C., Loomis, W.H. & Hoyt, D.B. Hypertonic saline enhances cellular immune function. *Circ Shock* **42**, 190-6 (1994).
231. Coimbra, R., Junger, W.G., Liu, F.C., Loomis, W.H. & Hoyt, D.B. Hypertonic/hyperoncotic fluids reverse prostaglandin E2 (PGE2)-induced T-cell suppression. *Shock* **4**, 45-9 (1995).
232. Loomis, W.H., Namiki, S., Hoyt, D.B. & Junger, W.G. Hypertonicity rescues T cells from suppression by trauma-induced anti-inflammatory mediators. *Am J Physiol Cell Physiol* **281**, C840-8 (2001).
233. Hirsh, M., Dyugovskaya, L., Bashenko, Y. & Krausz, M.M. Reduced rate of bacterial translocation and improved variables of natural killer cell and T-cell activity in rats surviving controlled hemorrhagic shock and treated with hypertonic saline. *Crit Care Med* **30**, 861-7. (2002).
234. Wilder, D.M., Reid, T.J. & Bakaltcheva, I.B. Hypertonic resuscitation and blood coagulation. In vitro comparison of several hypertonic solutions for their action on platelets and plasma coagulation. *Thromb Res* **107**, 255-61 (2002).
235. Scherer, R., Giebler, R., Kampe, S. & Kox, W.J. Effects of hypertonic saline hydroxyethyl starch solution on collagen-induced platelet aggregation and ATP secretion. *Infusionsther Transfusionsmed* **21**, 310-4 (1994).
236. Arbabi, S., Rosengart, M.R., Garcia, I. & Maier, R.V. Hypertonic saline solution induces prostacyclin production by increasing cyclooxygenase-2 expression. *Surgery* **128**, 198-205. (2000).
237. Arbabi, S., Garcia, I., Bauer, G. & Maier, R.V. Hypertonic saline induces prostacyclin production via extracellular signal-regulated kinase (ERK) activation. *J Surg Res* **83**, 141-6. (1999).
238. Cuschieri, J., Gourlay, D., Garcia, I., Jelacic, S. & Maier, R.V. Hypertonic preconditioning inhibits macrophage responsiveness to endotoxin. *J Immunol* **168**, 1389-96 (2002).
239. Oreopoulos, G.D. et al. Synergistic induction of IL-10 by hypertonic saline solution and lipopolysaccharides in murine peritoneal macrophages. *Surgery* **130**, 157-65 (2001).
240. Shields, C.J. et al. Hypertonic saline infusion for pulmonary injury due to ischemia- reperfusion. *Arch Surg* **138**, 9-14. (2003).
241. Gurfinkel, V., Poggetti, R.S., Fontes, B., da Costa Ferreira Novo, F. & Birolini, D. Hypertonic saline improves tissue oxygenation and reduces systemic and pulmonary inflammatory response caused by hemorrhagic shock. *J Trauma* **54**, 1137-45 (2003).
242. Yada-Langui, M.M. et al. Hypertonic saline and pentoxifylline prevent lung injury and bacterial translocation after hemorrhagic shock. *Shock* **14**, 594-8 (2000).
243. Murao, Y., Loomis, W., Wolf, P., Hoyt, D.B. & Junger, W.G. Effect of dose of hypertonic saline on its potential to prevent lung tissue damage in a mouse model of hemorrhagic shock. *Shock* **20**, 29-34 (2003).
244. Murao, Y. et al. Hypertonic saline resuscitation reduces apoptosis and tissue damage of the small intestine in a mouse model of hemorrhagic shock. *Shock* **20**, 23-8 (2003).
245. Shi, H.P., Deitch, E.A., Da Xu, Z., Lu, Q. & Hauser, C.J. Hypertonic saline improves intestinal mucosa barrier function and lung injury after trauma-hemorrhagic shock. *Shock* **17**, 496-501. (2002).
246. Reed, L.L. et al. The effect of hypertonic saline resuscitation on bacterial translocation after hemorrhagic shock in rats. *Surgery* **110**, 685-8; discussion 688-90. (1991).

Preface to Manuscript #2

Despite several in vitro and ex vivo reports of reduced adhesion molecule expression by neutrophils and endothelial cells exposed to hypertonic saline, few hemorrhagic shock models have evaluated live neutrophil interactions with endothelium immediately after resuscitation. Furthermore, no previous investigation comparing HTS to standard resuscitation regimens has assessed whether these altered EC/PMN interactions result in differences in the live permeability of the microvasculature. In the 2nd manuscript we demonstrate how, immediately after hemorrhagic shock resuscitation with hypertonic saline, in vivo neutrophil rolling and adhesion to endothelium as well as concurrent microvascular permeability are attenuated in comparison to that observed in RL-resuscitated counterparts.

Manuscript #2

Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces *in vivo* vascular leakage

José L. Pascual MD, Lorenzo E. Ferri MD, Andrew J.E. Seely MD, Giuseppina Campisi MSc, Prosanto Chaudhury MD, Betty Giannias BSc, David C. Evans MD, FRCS(C), Tarek Razek MD, FRCS(C), René P. Michel† MD, FRCP(C) and Nicolas V. Christou MD, PhD, FRCS(C), FACS, FCCM

Presented in part at the 30th International Educational & Scientific Symposium of the Society of Critical Care Medicine, San Francisco, California, February 10 to 14, 2001.
Published in the *Annals of Surgery*, Vol. 236, No. 5, 634-642 (2002).

Keywords: Hemorrhagic shock, resuscitation, fluids, trauma, adhesion receptors, neutrophil/endothelial interactions, ICAM-1, intravital microscopy, vessel permeability, hypertonic saline, Ringer's lactate

Mini-abstract

7.5% hypertonic saline was compared to Ringer's lactate and sham resuscitation in a murine model of hemorrhagic shock studying cremaster muscle with intravital microscopy. Compared to Ringer's lactate and sham, hypertonic saline resuscitation diminished *in vivo* vascular leakage and was associated with diminished neutrophil rolling and adhesion to endothelium.

Structured Abstract

***Background*:** The polymorphonuclear neutrophil (PMN) has been implicated in the pathogenesis of endothelial cell (EC) damage and organ injury following hemorrhagic shock. Compared to Ringer's lactate (RL), hypertonic saline (HTS) resuscitation diminishes PMN and EC adhesion molecule expression and organ sequestration of PMNs.

Objective: To evaluate the *in vivo* effects of HTS-resuscitation on EC-PMN interactions and vascular permeability after hemorrhagic shock.

Methods: In a murine model of hemorrhagic shock (50mmHg for 45minutes followed by resuscitation) using intravital microscopy on cremaster muscle, we studied PMN/EC interactions and vascular leakage (epifluorescence after 50mg/kg IV fluorescent albumin) in three resuscitation groups: **HTS** (shed blood + 4cc/kg 7.5%HTS, n=12), **RL** (shed blood + RL [2X shed blood volume], n=12) and **SHAM** (No hemorrhage or resuscitation, n=9). EC ICAM-1 expression was evaluated by immunohistochemistry. Data, presented as mean ± SEM, were evaluated by ANOVA with Bonferroni correction.

***Results*:** There were no differences between groups in flow mechanics. Compared to RL, HTS animals (T=90minutes) displayed diminished PMN rolling (95.3±19.8 vs 34.2±13.3 PMN/2min, p=0.03) and PMN adhesion to EC (14.3±3.1 vs. 2.9±0.8

PMN/5min, p=0.002) at time intervals beyond T=0. There were no differences between the SHAM and HTS groups. Vascular leakage was 45% lower in HTS than in RL-resuscitated animals ($p<0.01$). Cremaster EC ICAM-1 expression was similar in the two groups.

***Conclusion*:** Using HTS instead of RL to resuscitate hemorrhagic shock diminishes vascular permeability *in vivo* by altering PMN/EC interactions. HTS could serve as a novel means of immunomodulation in hemorrhagic shock victims, potentially reducing PMN-mediated tissue injury.

Over thirty years ago, the Vietnam War established the current standard use of isotonic crystalloid fluids (normal saline [NS] and lactated Ringer's [RL]) for the resuscitation of hemorrhagic shock. Subsequent studies have demonstrated that crystalloids represent an effective and inexpensive means to restore intravascular volume and additionally offer a survival advantage over colloids in the resuscitation of traumatic hemorrhagic shock (1).

More recently, "small volume resuscitation"(2) with 4ml of 7.5% NaCl per kg body weight of hypertonic saline (HTS) has been proposed in the treatment of hemorrhagic shock. Whereas isotonic fluid administration requires large volumes, hypertonic resuscitation offers the advantages of ease of transport, speed of administration and almost instantaneous hemodynamic effect. The intravascular administration of 7.5% NaCl rapidly creates a potent transcapillary osmotic gradient causing intravascular movement of water from the interstitium, endothelial cells (ECs), and red blood cells, a process that could potentially reduce third space fluid sequestration in the lungs of patients with traumatic pulmonary contusions or in the brain following head trauma(3). Early animal studies showed that HTS-resuscitation rapidly restored mean arterial pressure (MAP), peripheral tissue perfusion, cardiac contractility and oxygen consumption, mainly through vasodilatation of precapillary resistance vessels and increases in cardiac preload (5, 6). Although large multicentric randomized human clinical trials subsequently confirmed that HTS-resuscitation was safe and efficacious, they failed to demonstrate a clear survival advantage over standard isotonic resuscitation (4, 33). Nonetheless, one multicentric randomized control trial has demonstrated fewer post-resuscitation complications such as ARDS, renal failure and coagulopathies, with the use of HTS (4).

Many of the complications following the resuscitation of hemorrhagic shock may be related to alterations in host immunity. In particular, the polymorphonuclear

neutrophil (PMN), one of the principal host immune effector cells, has been implicated in the development of organ dysfunction and death following sepsis, burns, multiple trauma as well as hemorrhagic shock resuscitation (8-10). Although the PMN is essential in protecting the host from traumatic and infectious insults, it may also turn its potent defenses inappropriately against the host, activated by, and contributing to the severity of injury.

The sequential events in the passage of PMNs from the vasculature to their sites of action have been well characterized. First marginating to the periphery of the vessel, the PMN rolls on the vessel wall, interacting with ECs through surface selectins (L, E and P) (11). These weak interactions allow PMN β_2-integrins (CD18/CD11) to strongly interact with endothelial receptors of the Ig superfamily (ICAM-1, ICAM-2) (12), resulting in firm adhesion of the two cell types, and permitting subsequent PMN diapedesis between endothelial cells to reach the injured site (13).

The inappropriate upregulation of these PMN/EC interactions in the ischemia/reperfusion injury of hemorrhagic shock-resuscitation is believed to be an important step in the host's progression to systemic inflammation and subsequent remote organ injury. There are data suggesting that activated PMNs are then sequestered in end organs where they unleash a cytotoxic arsenal of proteases and oxygen radicals causing injury to endothelium resulting in vascular leakage, tissue edema, and eventually organ damage (8, 10, 14).

Much evidence now exists demonstrating that HTS-resuscitation of hemorrhagic shock alters PMN activity. When human PMNs are incubated in hypertonic media, they display decreased phagocytosis, cytotoxicity, cellular activation and superoxide production (16-19). *In vivo* studies have shown that both neutrophil and endothelial adhesion molecule expression is reduced in HTS-resuscitated animals as compared to those receiving RL (15, 20, 22). These alterations in adhesion molecules suggest that

HTS may impart functional changes in PMNs and ECs. More importantly, animal models of hemorrhagic shock resuscitated with HTS have shown reductions in lung and liver injury, diminished bronchioalveolar lavage PMN and diminished pulmonary myeloperoxidase (MPO - total PMN content) as well as decreases in mortality (15, 21, 23).

Models of hemorrhagic shock-resuscitation studied with intravital microscopy suggest that the beneficial effects of HTS may be due to altered EC-PMN interactions (24-26). However, the consequences of such altered EC-PMN interactions following HTS-resuscitation of hemorrhagic shock remain elusive and the link to altered adhesion molecule expression and to subsequent tissue edema is unclear. We thus hypothesized that through diminished endothelial ICAM-1 expression, HTS-resuscitation of hemorrhagic shock lessens venular EC-PMN interactions, leading to decreased *in vivo* vascular leakage in remote organs. We tested this hypothesis in a murine model of resuscitated hemorrhagic shock using intravital microscopy of cremaster muscle.

Methods

Materials and solutions

Sodium chloride (0.9% NaCl) and RL solutions were purchased from Baxter Corporation (Toronto, Canada), 2-methylbutane (isopentane) and frozen tissue embedding medium (Histo Prep™) from Fisher Scientific Ltd. (Montreal, Canada), 5% NMS mouse serum from Dako Diagnostics Canada Inc. (Mississauga, Canada), heparin (10 000 USP units/ml) from Organon Teknika (Toronto, Canada) and bovine fluorescein isothiocyanate (FITC)-labeled albumin and 0.5% BSA from Sigma Chemicals (St-Louis, MO). The anesthetics used were ketamine from Wyeth-Ayerst (Guelph, Canada) and

xylazine from Bayer (Etobicoke, Canada). The monoclonal antibodies used were hamster anti-mouse CD54 3-E2 (primary, 1:50 dilution) and biotin conjugated mouse anti-hamster IgG cocktail (secondary, 1:50 dilution) from BD Pharmigen Inc. (Mississauga, Canada). Tris buffer [50mmol/L Tris/HCl, 150 mmol/L NaCl, pH 7.6], 0.03% H_2O_2 solution, buffered bicarbonate solution (BBS) [NaCl 132 mM, KCl 4.7 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1.2 mM, NaOH 18mM, pH 7.4] and HTS [7.5% NaCl in dH_2O] were prepared in our laboratory.

Intravital microscopy model

Approval for these studies was obtained from the McGill University Animal Care Committee. CD1 male mice (Charles River: St-Constant, Canada), 25-30gm, were fed *ad libitum* and housed in standard care facilities for 3-5 days prior to study. Acclimated mice were anaesthetized by intra-peritoneal injection (xylazine = 6.7mg/kg, ketamine = 13.4mg/kg) and the right internal jugular vein and carotid artery were cannulated with polyethylene (PE)-10 tubing. The internal jugular vein catheter was used for administration of resuscitation fluids, FITC-labeled albumin, and intermittent 50µl boluses of xylazine/ketamine solution as required to maintain anaesthesia. Blood pressure was monitored continuously through a pressure transducer (Living Systems Instrumentation: Burlington, VT) connected to the carotid artery catheter.

After closing the cervical incision, the mouse cremaster was prepared for intravital microscopy as described previously (27). Briefly, a longitudinal incision was made in the scrotal tissue, and the cremaster muscle exteriorized and dissected free of the testicle and epididymis. The cremaster was fixed to a plexiglass stage at five points with 4-0 silk and continuously perfused with thermostat-controlled (37° C) BBS for the remainder of the experiment. The stage was placed on a Nikon TE 300 inverted

microscope (Nikon Canada: Montreal, Canada), and the tissue was imaged at 2120X magnification.

Non-branching post-capillary venules (PCVs) of 20-40μm diameter were evaluated for the velocity of blood flow at their center (V_{RBC}) using an optical Doppler velocimeter (Microcirculation Research Institute: College Station, TX), and were considered suitable only if V_{RBC} was 2.0-4.0 mm/sec. Live microscopic images were captured with a high definition black and white video camera (CCD High Performance Camera, COHU, San Diego, CA), transferred to a monitor (Trinitron Color monitor – SSM-14NE, Sony: Toronto, Canada), and recorded on videotape with a video recorder (VR564, RCA: Toronto, Canada) for subsequent analysis without knowledge of treatment groups. A video Time-Date Generator (model WJ-810, Panasonic: Toronto, Canada) projected the time, date, and stopwatch function onto the monitor at all times. A temperature probe (Bio Medic Data Systems, Seaford, DE) introduced subcutaneously after cannulation of vessels was used to measure temperature intermittently. Body temperature was maintained at 37° C with a radiant heat lamp throughout the study period. All animals were sacrificed by xylazine/ketamine overdose followed by cervical dislocation at the completion of experiments.

Experimental protocol and randomization of study groups

Hemorrhagic shock was induced by withdrawing blood from the carotid artery catheter over 10 to 20 min with a tuberculin syringe previously flushed with 25 U heparin until MAP reached 50 mmHg. Hypotension was maintained for 45 minutes, by further blood withdrawals if MAP rose above 50 mmHg, or by re-infusions of 0.05 ml of withdrawn blood if MAP fell below 40 mmHg. The syringe containing the withdrawn blood was placed in a warmed (37° C) shaker (Roto Mix, Barnstead/Thermolyne:

Dubuque, IA) until reinfusion. After 45 minutes of hypotension, the animals were resuscitated using the regimen randomly assigned prior to anaesthesia.

The 33 mice were randomized to one of three resuscitation regimens. In the **HTS** group, 12 mice were resuscitated with 4cc/kg of 7.5% hypertonic saline immediately followed by the entire volume of withdrawn blood. In the **RL** group, 12 mice were resuscitated with lactated Ringer's, infusing twice the volume of withdrawn blood, immediately followed by the withdrawn blood itself. Both fluid regimens have been demonstrated to adequately resuscitate mice after such hemorrhagic shock (23, 28). The **SHAM** group, composed of 9 mice, underwent vascular cannulation and cremaster preparation but was not subjected to hemorrhage or resuscitation. Once resuscitated, both RL and HTS mice were studied for sequential EC-PMN interactions using video recordings and epiluminescence. *Post-mortem,* the cremaster was prepared for immunohistochemistry as outlined below.

Quantification of EC-PMN interactions

Seven-minute video recordings of each animal's microvasculature were captured at the following time intervals: baseline (BL) [prior to hemorrhage and 30 minutes after a stabilization period following the completion of surgery], during hypotension (lowbp), immediately after resuscitation (T=0), every 15 minutes during the subsequent hour (T=15, 30, 45, 60) and 90 minutes after resuscitation (T=90). These recordings were subsequently played back off line and EC-PMN interactions assessed without prior knowledge of treatment.

We measured central vessel flow velocity (V_{RBC}) using Doppler velocimetry, and calculated mean velocity (V_{MEAN}) from V_{RBC} using the formula: $V_{MEAN} = V_{RBC}/1.6$. Shear rate (γ), in sec^{-1}, was calculated using the formula: $\gamma = 8 \times (V_{RBC}/D_V)$, where D_V is the venular diameter measured directly off line using calipers. Shear stress was calculated

using the formula: shear stress = 0.25 X shear rate. PMNs were not labeled with fluorescent markers that could affect their adhesive properties and, as available evidence suggests, all leukocytes visualized were considered to be PMNs (29). Neutrophil rolling was defined as the number of neutrophils crossing a line perpendicular to the long axis of the vessel, that were moving at a rate slower than erythrocytes over a period of two minutes. Rolling velocity was calculated as the mean transit time of ten neutrophils over a given 100 μm length of post-capillary venule and was expressed in μm/second. Neutrophil adherence was defined as the number of cells stationary for a minimum of 30 seconds in a 100 μm length of venule during a five-minute period and pre-adherence was defined as the number of immobile neutrophils in the same 100 μm vessel section at the initiation of counting. Total neutrophil adherence was the sum of neutrophil adherence and pre-adherence for a given sample.

Fluorescent quantification of vascular permeability

Ninety minutes after resuscitation and following the completion of all video recordings, 50 mg/kg of fluorescein isothiocyanate (FITC)–labeled bovine serum albumin were injected intravenously through the jugular catheter. Fifteen minutes later, epiluminescence microscopy was performed using a fluorescence source powered by a high-pressure mercury lamp (HB 10103AF, Nikon Canada: Toronto, Canada). Using a high definition black and white video camera (CCD High Performance Camera, COHU: San Diego, CA) moving images were transferred to a computer (Dimension XPS B866r, Dell Canada: Toronto, Canada) and captured by a frame grabber from Scion Image processing software (Scion Image for Windows, Scion Corporation: Frederick, MD). Using a frozen digital image of the fluorescent post-capillary venule, gray levels [0 (black) to 256 (white)] were measured in three equal areas within the venule (venular

intensity, I_v), as well as three separate equal areas in the perivenular space (perivenular intensity, I_p) (Figure 6). Using the means of I_p and I_v measurements, vascular leakage (permeability index, PI) was calculated with the formula $I_p / I_v = PI$. Thus, the degree of luminescence 'leaking' out of the vessel created by leakage of a fluorescent macromolecule (albumin) was use as a marker of the loss of integrity (permeability) of the given vessel wall.

Cremaster endothelial cell expression of ICAM-1

Following all recordings and fluorescent evaluations and prior to sacrifice, the cremaster was delicately resected, placed in embedding medium, snap frozen in isopentane submerged in liquid nitrogen and stored at -80°C. Five μm-thick frozen sections were cut on a cryostat, stained with hamster anti-mouse ICAM-1 mAb 3E2, washed and stained with biotinylated mouse anti-hamster IgG cocktail. After further rinses with Tris-buffer, the slides were developed using a standard DAB detection kit (Ventana Basic DAB Detection Kit, Ventana Medical Systems Inc.: Tucson, AZ), and counterstained with haematoxylin for 4 minutes. After mounting, the slides were examined at a magnification of 250X and graded semi-quantitatively for intensity of vascular endothelial cell ICAM-1 expression by one of us (RPM) without prior knowledge of treatment group, using the following scale: no staining [0], weak staining [1], moderate staining [2] and strong staining [3]; ten vessels per slide were graded and a mean grade calculated for each animal (30).

Systemic total white blood cell and PMN counts:

Four hundred μl of blood removed by cardiac puncture prior to animal sacrifice were used for hemoglobin, white blood cell count and differential determination. The

Royal Victoria Hospital hematology laboratories performed these analyses using an Advia Hematology System (model 120, Bayer Systems: Hialeah, FL).

Statistical Analysis:

All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Differences between groups were compared using analysis of variance with Bonferroni correction with Systat 8 data analysis software (SPSS, Chicago, IL). Probabilities < 0.05 were deemed statistically significant.

Results

Vascular and hemodynamic mechanics

Measurements of V_{RBC}, V_{MEAN}, shear rates and shear velocities in the three groups and at all time intervals (except during hypotension) were not statistically different ($p>0.05$) (Table 1). All groups displayed similar PMN rolling, PMN rolling velocity and PMN adherence at baseline (BL) as well as during hypotension (lowbp) (Figures 2, 3 and 5).

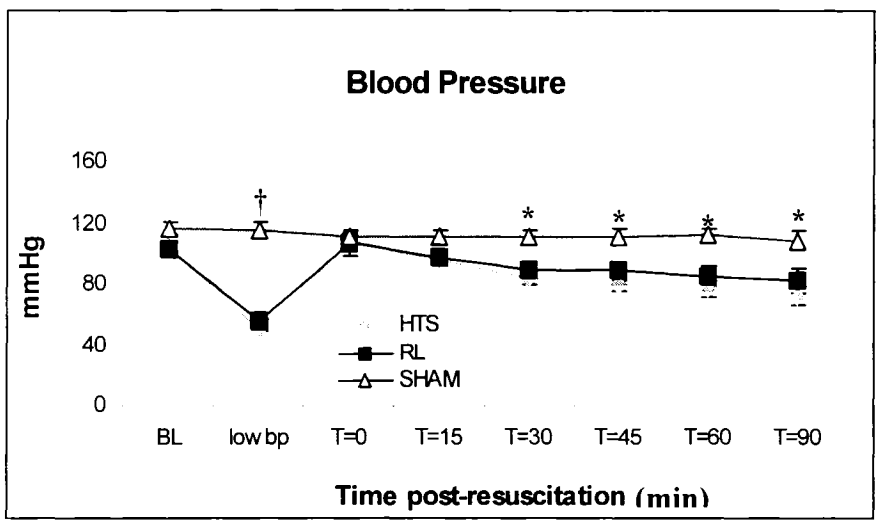

FIGURE 1: Compared to the two resuscitation groups, SHAM animals had a significantly higher blood pressure 30 min after resuscitation and thereafter. Values are expressed as mean (mmHg) ± SEM. *SHAM vs either HTS or RL: $p<0.05$, †SHAM vs either HTS or RL: $p<0.001$. There were no significant differences between HTS and RL at any time point.

Post-capillary venular diameter (range 20.0 – 39.0 μm, mean 27.11 ± 0.23 μm), duration of hypotension, (mean 45.1 ± 3.2 minutes), withdrawn blood volume (mean 485.3 ± 81.56 μl) and *post mortem*

Table 1. MICROVASCULAR MECHANICS

	Baseline	Hypotension	T=0 minutes	T=15 minutes	T=30 minutes	T=45 minutes	T=60 minutes	T=90 minutes
V_{RBC}								
SHAM	3.01±0.70	2.90±0.55	2.98±0.70	3.09±0.61	3.05±0.64	2.92±0.56	2.77±0.57	2.65±0.47
RL	3.58±1.24	1.82±0.65**	3.21±1.16	2.97±0.71	2.48±0.63	2.57±0.55	2.47±0.86	2.31±0.80
HTS	3.18±1.05	1.75±0.25**	3.63±0.93	3.36±1.02	3.08±1.04	2.75±0.94	2.67±0.94	2.36±0.69
V_{MEAN}								
SHAM	1.88±0.44	1.81±0.34	1.86±0.44	1.93±0.38	1.90±0.40	1.82±0.35	1.72±0.35	1.66±0.29
RL	2.23±0.68	1.13±0.37**	2.00±0.49	1.86±0.44	1.55±0.37	1.60±0.28	1.54±0.48	1.44±0.32
HTS	1.99±0.51	1.09±0.15**	2.27±0.48	2.10±0.56	1.92±0.53	1.72±0.43	1.67±0.25	1.47±0.14
Shear rate								
SHAM	0.59±0.15	0.57±0.11	0.59±0.14	0.61±0.11	0.60±0.12	0.57±0.11	0.54±0.12	0.53±0.11
RL	0.65±0.24	0.35±0.13**	0.60±0.22	0.55±0.16	0.47±0.15	0.48±0.12	0.46±0.16	0.42±0.12
HTS	0.58±0.19	0.33±0.09**	0.67±0.21	0.61±0.20	0.55±0.15	0.49±0.13	0.47±0.13	0.39±0.10
Shear stress								
SHAM	0.14±0.04	0.14±0.03	0.15±0.04	0.15±0.03	0.15±0.03	0.14±0.03	0.14±0.03	0.13±0.03
RL	0.16±0.06	0.08±0.03**	0.15±0.05	0.14±0.04	0.12±0.04	0.12±0.03	0.11±0.04	0.10±0.03
HTS	0.14±0.05	0.08±0.02**	0.17±0.05	0.15±0.05	0.14±0.04	0.12±0.03	0.12±0.03	0.10±0.03

Except for the hypotensive period, central flow velocity (V_{RBC}), mean flow velocity (V_{MEAN}), vessel shear rates and shear stress showed no statistical differences between groups. All results are shown as mean ± SD, and unless otherwise stated, $p>0.05$ for all comparisons between groups.
**SHAM vs either RL or HTS: $p<0.01$.

hemoglobin values (mean 111.6 ± 34.1 g/L) were similar in all groups ($p>0.05$ for all comparisons). RL-resuscitated animals took longer to resuscitate than HTS animals (20.3 ± 7.5 vs. 12.6 ± 4.2 minutes respectively, P=0.01), but no differences between groups were found in the time it took to hemorrhage animals (mean 7.93 ± 3.9 minutes). HTS and RL groups displayed similar blood pressures ($p>0.05$) at all time points, but SHAM mice had significantly higher blood pressures than both HTS and RL animals during the hypotension time point and onwards from 30 minutes following resuscitation (see figure 1).

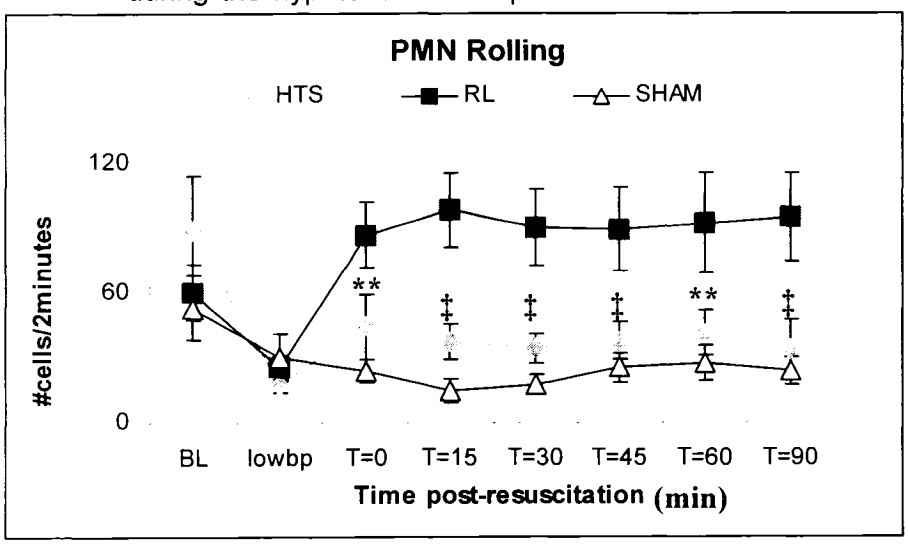

FIGURE 2: RL animals displayed more rolling neutrophils than either HTS or SHAM groups. Values are expressed as mean number of rolling neutrophils per 2-minute period ± SEM. **RL vs SHAM only: $p<0.05$, ‡RL vs either HTS or SHAM: $p<0.05$. There were no significant differences between HTS and SHAM at any time point.

Effect of hypertonic saline on PMN rolling and adhesion to EC

HTS-resuscitated animals displayed less

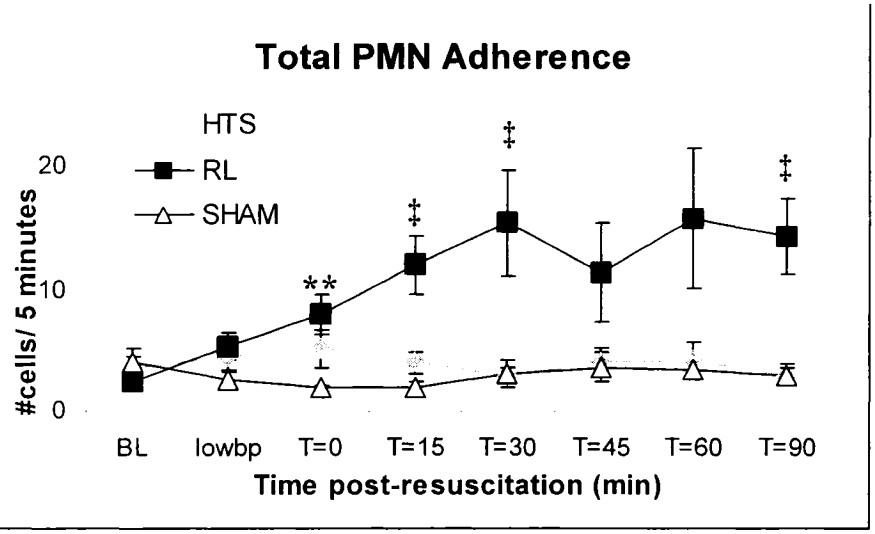

FIGURE 3: As compared to HTS and SHAM, RL animals had higher numbers of neutrophils adhering to endothelial cells. Values are expressed as mean number of stationary PMN on a given 100μm vessel length ± SEM. **RL vs SHAM only: $p<0.05$, ‡RL vs either HTS or SHAM: $p<0.05$. There were no significant differences between HTS and SHAM at any time point.

than half the number of rolling neutrophils seen in RL-resuscitated animals at all time points following resuscitation. RL-resuscitated animals had a three-fold greater number of rolling neutrophils than SHAM animals. (Figures 2 and 4) PMN rolling was similar in the SHAM and HTS groups ($p>0.05$ at all time points). Total neutrophil adherence to endothelium was similar in HTS and SHAM groups throughout the time points, but after fifteen minutes, adherence levels in RL-animals increased significantly, to almost four times those observed in SHAM and HTS at T=60 (Figures 3 and 4). PMN rolling velocity tended to be greater in both HTS and SHAM as compared to RL at all time points following resuscitation (Figure 5). Although during hypotension, both resuscitated groups had neutrophil rolling velocities that were half those of SHAM animals, these did not differ significantly between HTS and SHAM animals in the remaining time points.

Macromolecular leakage in HTS-resuscitated animals

Vascular permeability (PI) was slightly higher in HTS than in SHAM mice (36% ± 0.02 and 27% ± 0.02, respectively, $p=0.03$) but both groups displayed significantly less leakage than RL mice (66% ± 0.2, $p<0.01$ vs either HTS or SHAM) (Figures 6 and 7).

Cremaster ICAM-1 expression in resuscitation groups

Cremasteric muscle vascular EC expression of ICAM-1 was similar in both HTS and RL animals (mean grade 1.69 ± 0.3 and 1.82 ± 0.2, respectively, $p>0.05$).

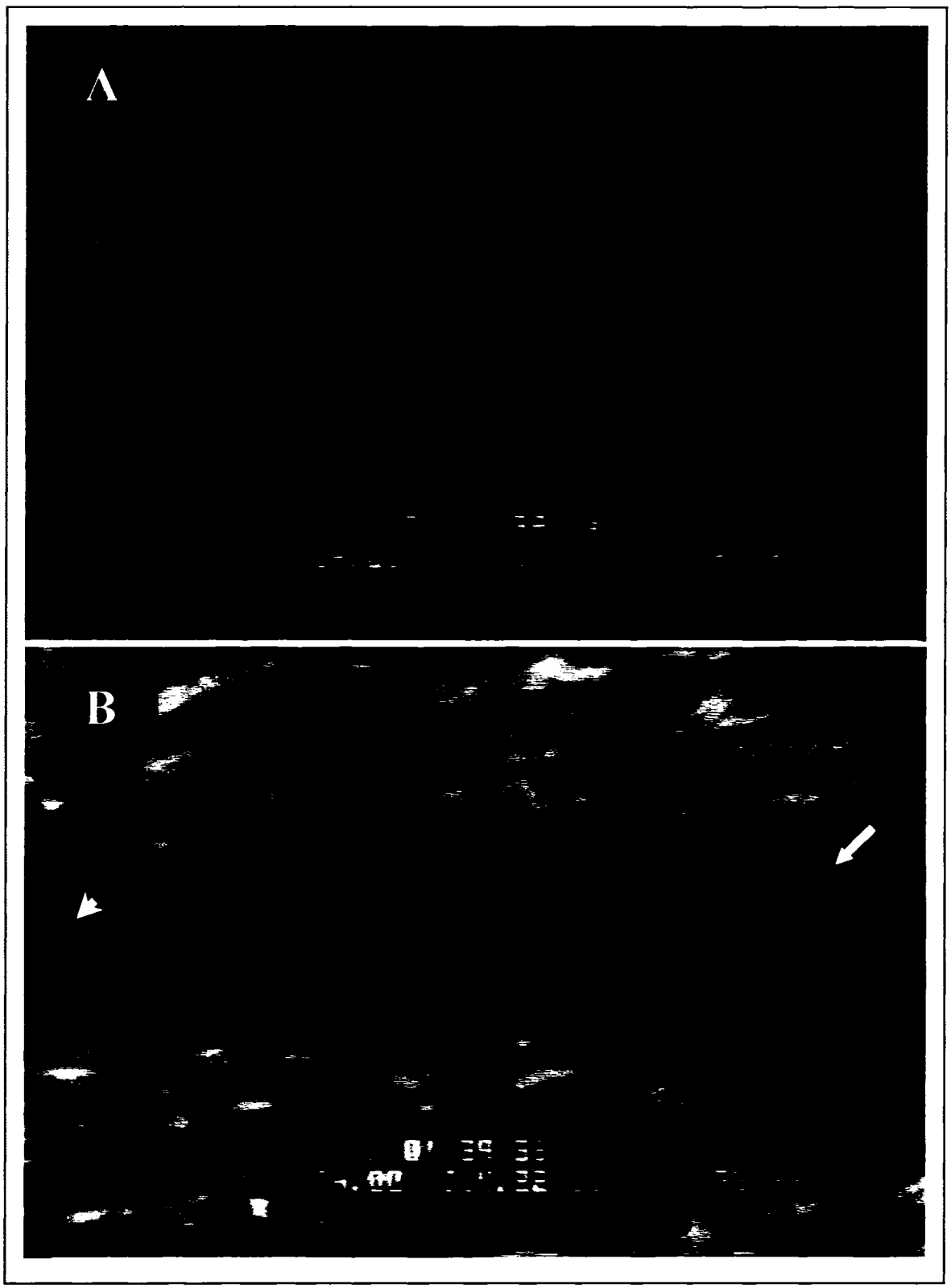

Figure 4: Representative examples of intravital microscopic images contrasting the lack of PMN rolling (arrowhead) and PMN adherence (arrow) in HTS (A) as compared to RL (B) vessels at T=90 minutes after resuscitation.

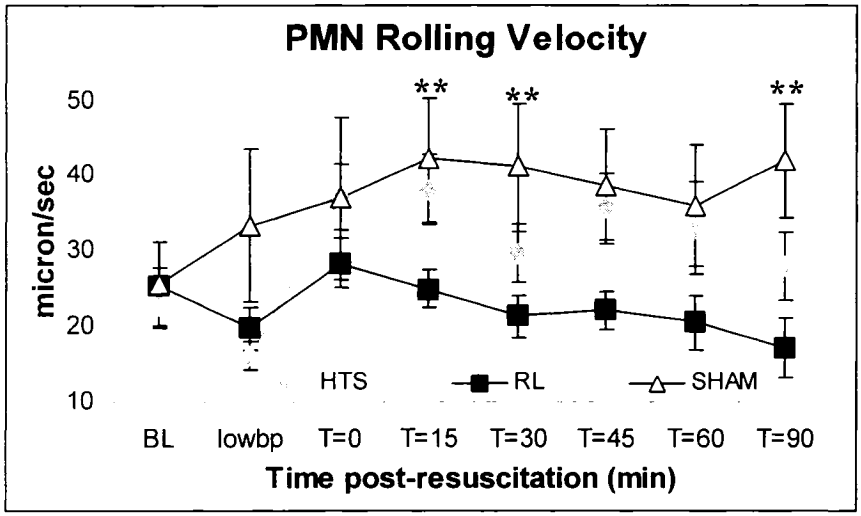

FIGURE 5: HTS animals tended to have higher PMN rolling velocities than RL animals. Values are expressed as the mean velocity (μm/s) of 10 PMNs crossing a given vessel section ± SEM. **RL vs SHAM only: $p<0.05$. There were no significant differences between HTS and SHAM at any time point.

Total white blood cell and PMN counts

Both circulating leukocyte ($p<0.05$) and neutrophil counts ($p=0.06$) tended to be higher in HTS animals (72.1 ± 31.4 and 1.98 ± 0.86, respectively) than in both SHAM (3.16 ± 0.36 and 0.14 ± 0.08, respectively) and RL animals (14.9 ± 10.14 and 0.28 ± 0.12, respectively). The proportion of circulating leukocytes that were PMNs was similar in all groups (data not shown).

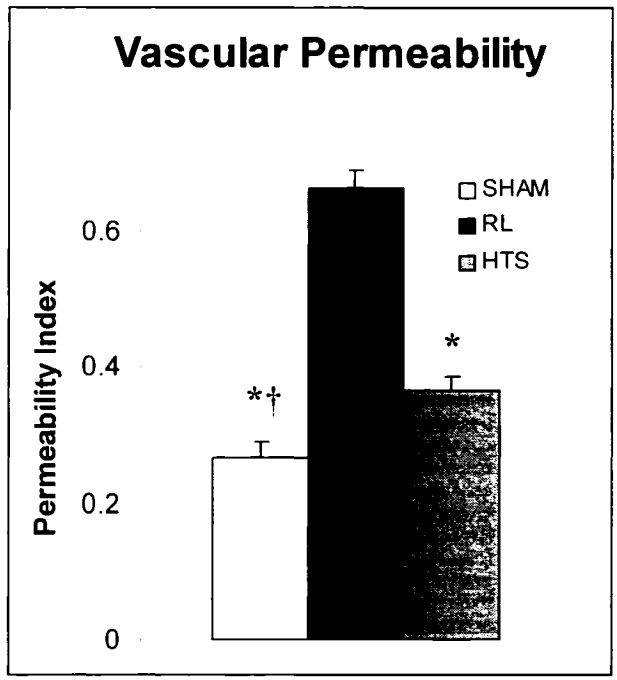

FIGURE 6: Vascular permeability index (PI) (where I_P/I_V= PI) is an estimation of vessel leakage and was higher in HTS as compared to SHAM animals, while RL animals had the highest PI of all. Values are expressed as % permeability index ± SEM. †vs HTS: $p<0.05$, *vs RL: $p<0.01$.

Discussion

For the last two decades hypertonic saline has become an increasingly popular resuscitation fluid, showing a trend to increased posttraumatic survival both in the early phase (< 12 hrs post trauma) and, in the late phase (days to weeks after trauma) (4, 6, 7, 31-33).

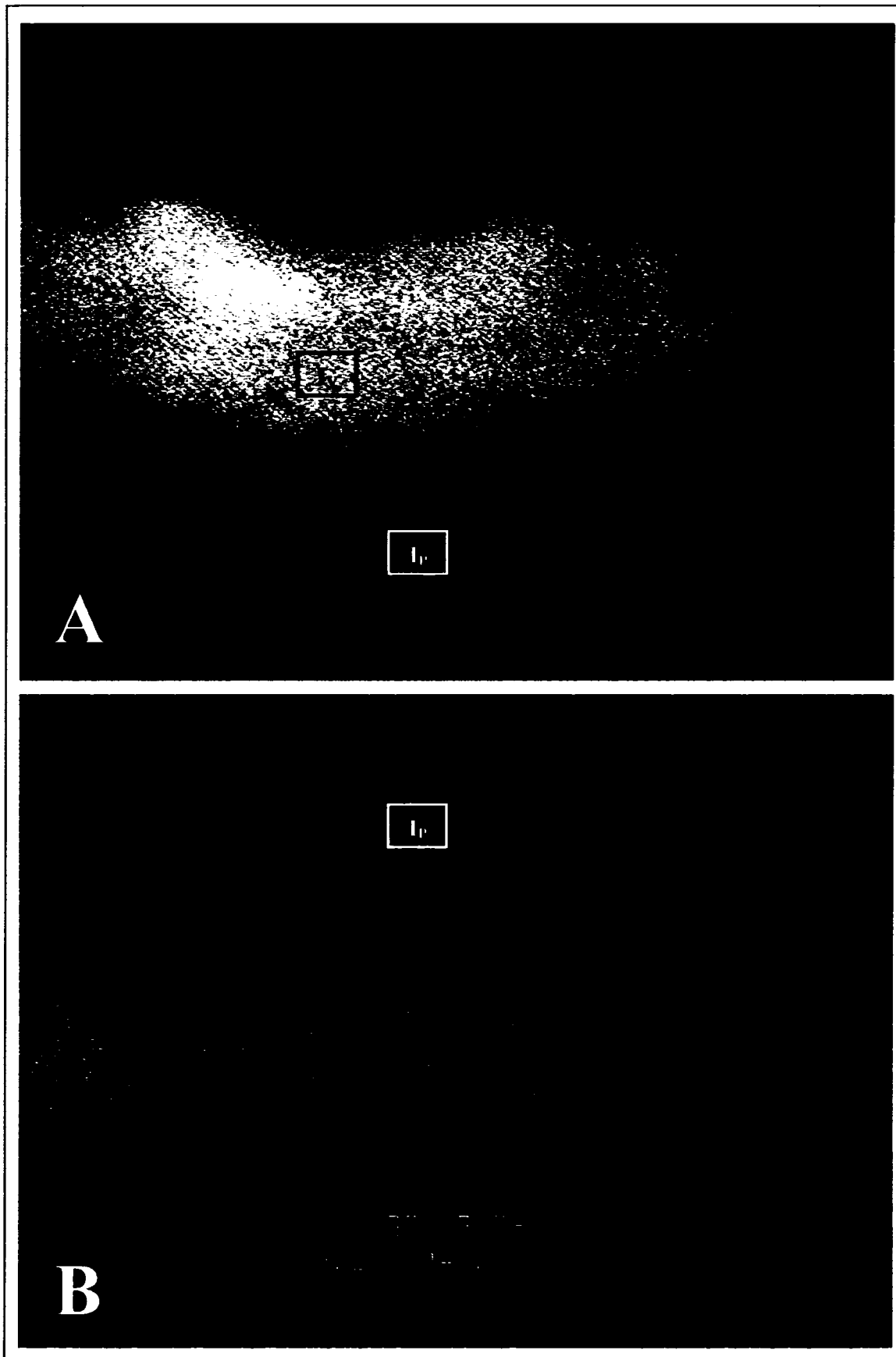

FIGURE 7: Representative examples of fluorescent intravital microscopic images contrasting the low leakage of FITC-labeled albumin from post-capillary venules in HTS (A) compared with RL-resuscitated (B) animals. I_P (perivenular light intensity) and I_V (venular light intensity) represent regions evaluated for fluorescence.

The early phase effect is believed to be due to a functional increase in cardiac preload, primarily by inducing an osmotic fluid shift from the cells and interstitium into the vasculature and leading to increases in systemic blood pressure and cardiac output as well as decreases in small vessel capacitance (4, 31). In contrast, the late beneficial effects of HTS-resuscitation are unlikely due to simple physiological alterations. The late morbidity and mortality of traumatic hemorrhage has been attributed to the "second hit"; that is, a relatively less important inflammatory event (pneumonia, aspiration, minor surgery) that occurs days to weeks after the traumatic injury and initiates progression to the systemic inflammatory response syndrome (SIRS), organ failure and eventual death (34). Clinical and animal studies conducted in the last decade have provided strong evidence suggesting that this late effect of HTS-resuscitation is due to an immune modulation of leukocytes, in particular neutrophils (21, 35-37).

We believe that the present study provides novel evidence demonstrating that HTS alters interactions between neutrophils and endothelial cells. In the present study using a "single-hit" murine model of hemorrhagic shock, resuscitation with hypertonic saline specifically diminished the number of rolling and adherent PMN while tending to increase PMN rolling velocities; there were no differences in these parameters between HTS-treated mice and the SHAM controls.

The effect of hypertonic saline on PMN/EC interactions has been studied previously. *In vitro* studies of cultured endothelial monolayers demonstrate diminished PMN adherence (15), whereas *in vivo* models of reperfused local pressure-induced ischemia display fewer rolling and 'sticking' venular PMNs after pretreatment with HTS (38). Using intravital microscopy of pial windows, HTS administration after cerebral percussion injury or systemic thermal injury also reduced EC-PMN interactions (39, 40). Liver intravital microscopy has probably yielded the most exciting findings to date about EC-PMN interactions following the differential fluid resuscitation of hemorrhagic shock.

Indeed, several studies report decreases in capillary and sinusoidal luminal narrowing and improved flow parameters (25, 41-43), as well as diminished EC-PMN interactions (sinusoids and post-sinusoidal venules) in animals resuscitated with hypertonic fluids (24-26). Because none of these studies evaluated the effects of HTS alone, but rather used hypertonic saline-sugar resuscitation regimens, it is difficult to reach a conclusion on the individual effect of HTS. Dextrans and other sugars have been shown to exert by themselves powerful anti-adhesive influences on PMNs (44-46). Furthermore, these studies in hepatic tissue employ fluorescent labeling of PMNs (rhodamine, acridine orange) even though such fluorescent markers are also known to affect the adhesive capacities of PMNs (47). Moreover, as liver sinusoids are not lined by typical endothelium it may be inappropriate to apply findings from the hepatic microvasculature to the rest of the systemic vasculature. An additional difference between the hepatic and systemic microvasculatures is that in the former, the possible 'trapping' of swollen and stiff PMNs may occur due to the narrowing of sinusoidal passages following crystalloid resuscitation (48-50). Thus, while the lung capillary beds may also demonstrate similar PMN 'trapping', the liver microvascular architecture may not be representative of other systemic capillary beds where presumably PMN arrest and become sequestered by standard receptor mediated transmigration.

Although knowledge about PMN/EC interactions is extensive, there is less evidence demonstrating an associated HTS-mediated decrease in vascular permeability following hemorrhagic shock resuscitation. HTS given after local pressure-induced ischemia reduced *in vivo* fluorescent dextran leakage, which was correlated with diminished EC-PMN interactions (38). A rodent cheek pouch intravital microscopic study evaluating vascular leakage in systemic endotoxic shock reported diminished EC-PMN interactions and leakage after HTS-resuscitation (51). Following the resuscitation of hemorrhagic shock, *post mortem* studies revealed less radioactive albumin leakage into

pulmonary tissue if HTS was used for resuscitation instead of RL (15, 52). Thus several studies evaluating multiple ways of creating systemic and local tissue injury have demonstrated concurrent increases in PMN interactions with enmdothelium and loss of vascular integrity resulting in more tissue edema. The present study represents the first attempt at characterizing *in vivo* post-capillary venous permeability following resuscitation of systemic hemorrhagic shock. Leakage of labeled albumin was evaluated with epifluorescence microscopy and revealed almost 50% less leakage if HTS was used for resuscitation instead of RL. This enhanced vascular leakage in RL animals rapidly reached a plateau after 15-30 minutes, whereas HTS animals never showed a significant increase in vascular permeability. Although we cannot establish a direct causal relationship between HTS-mediated decreases in EC-PMN interactions and vascular leakage, a strong association both chronologically and spacially is evident.

Much evidence exists supporting HTS-mediated modulation of PMN and EC adhesion molecule expression. The *in vitro* activation of PMNs from HTS-resuscitated animals and humans results in diminished L-selectin (15, 20) and CD11b (15, 18, 19, 37, 53) expression on the PMN surface. Diminished *in vitro* endothelial ICAM-1 protein and mRNA expression has been reported when LPS activation was followed by incubation in HTS (54). *In vivo* studies evaluating pulmonary and hepatic ICAM-1 mRNA expression in HTS-resuscitated animals have also demonstrated diminished receptor levels as compared to RL controls (15, 54). Moreover, in another *in vivo* study, Sun *et al* (22) found diminished splenic ICAM-1 protein expression and diminished pulmonary and splenic ICAM-1 gene expression in HTS-treated as compared to RL-treated rats. Our experiments, however, did not demonstrate differences in ICAM-1 expression between the different resuscitation groups. This could indicate that this adhesion receptor may not be the principal target of HTS-mediated effects or that our model did not provide a sufficiently strong activating stimulus to produce such effects in the cremaster muscle. A

'two-hit' model of hemorrhage followed by an infectious insult (15, 23), for example, may have allowed for greater PMN activation and enabled us to demonstrate differential adhesion molecule expression. Animal ICAM-1 knockout models and adhesion molecule blockade may be more definite methods of establishing the true effects of HTS on ICAM-1 or other adhesion molecules. An additional evaluation of PMN activation and the upregulation of β_2 integrins would have better completed the adhesion molecule evaluation but was not conducted as all blood was used for hemoglobin and differential anlysis.

Resuscitation of hemorrhagic shock, particularly when using RL, has also been associated with reductions in circulating leukocytes (20). Certain authors suggest that this may be the result of increased interactions of PMNs with the vessel wall (21). Consequently, it could be argued that fewer EC-PMN interactions secondary to HTS-resuscitation explain higher circulating neutrophil levels. The present study confirmed a tendency to neutrophilia in HTS-resuscitated animals and a relative neutropenia in RL-resuscitated animals.

There are a few potential sources of error in our resuscitated hemorrhagic shock model. Since we did not label the circulating PMNs, and since other leukocytes (55) are known to interact with endothelium, we cannot be certain that all interacting leukocytes were neutrophils; nevertheless, studies of interacting leukocytes in similar models indicate that the vast majority of such visible leukocytes are indeed PMNs (29). We chose not to count transmigrated cells as the system we employed did not allow for long term air exposure of the delicate tissue. Thus this model cannot confirm that differences in adherent and rolling cells were necessarily translated into similar differences in tissue transmigration of PMNs in vivo. Animals in the RL group were resuscitated with volumes ten-fold greater than the HTS animals in addition to reinfused blood (mean 1.12 ± 0.12 vs 0.13 ± 0.003 ml, respectively, $p<0.001$). This large intravascular fluid challenge may

have effectively fluid overloaded the animals resuscitated with RL, at least initially, so that an additional detrimental mechanical effect on the vessel wall of these animals cannot be entirely discounted. Moreover, related to the different resuscitation volumes, RL animals took longer to resuscitate than HTS animals, effectively prolonging the duration of hypotension and possibly of ischemia. In contrast, HTS and RL but not SHAM animals displayed a gradually decreasing blood pressure 30 minutes following resuscitation (Figure 1). The differences in salt loads of the two resuscitation regimens were considerable but direct effects of these loads were not evaluated independent of hemodynamic parameters. We did not elect to investigate further the effects of salt load in this study. We also did not evaluate the adequacy of resuscitation in either group and the question of whether both groups were equally well resuscitated could be raised. Nonetheless, differences in PMN rolling and adhesion were already seen prior to this time point, beginning shortly after resuscitation and indicating an early differential effect. Furthermore, protocols using resuscitation with shed blood and once/twice that volume of RL or 4cc/kg 7% HTS, in addition to shed blood, have been used by several other groups on different rodent models (15, 21, 23).

In summary, compared to Ringers lactate, hypertonic saline resuscitation of a single-hit hemorrhagic shock murine model results in faster moving post-capillary venular neutrophils, with diminished rolling and adherence to endothelial cells. Furthermore, RL-resuscitated animals displayed an early and sustained elevation in vascular leakage *in vivo* that was not observed in HTS-resuscitated animals. These novel findings further contribute to the understanding of the natural history of hemorrhagic shock and the sequential steps that can lead its victims to organ failure and subsequent death. The future use of HTS-resuscitation and its immunomodulation following hemorrhagic shock may potentially reduce PMN-mediated tissue injury leading to organ failure in trauma victims and severely ill patients.

References to Manuscript #2

1. Velanovich V. Crystalloid versus colloid fluid resuscitation: a meta-analysis of mortality. Surgery 1989; 105(1):65-71.
2. Nakayama S, Sibley L, Gunther RA, *et al.* Small-volume resuscitation with hypertonic saline (2,400 mOsm/liter) during hemorrhagic shock. Circ Shock 1984; 13(2):149-159.
3. Shackford SR, Bourguignon PR, Wald SL, *et al.* Hypertonic saline resuscitation of patients with head injury: a prospective, randomized clinical trial. J Trauma 1998; 44(1):50-58.
4. Mattox KL, Maningas PA, Moore EE, *et al.* Prehospital hypertonic saline/dextran infusion for post-traumatic hypotension. The U.S.A. Multicenter Trial. Ann Surg 1991; 213(5):482-491.
5. Velasco IT, Pontieri V, Rocha e Silva M, Jr., Lopes OU. Hyperosmotic NaCl and severe hemorrhagic shock. Am J Physiol 1980; 239(5):H664-673.
6. Kreimeier U, Thiel M, Peter K, Messmer K. Small-volume hyperosmolar resuscitation. Acta Anaesthesiol Scand Suppl 1997; 111:302-306.
7. Wade CE, Kramer GC, Grady JJ, *et al.* Efficacy of hypertonic 7.5% saline and 6% dextran-70 in treating trauma: a meta-analysis of controlled clinical studies. Surgery 1997; 122(3):609-616.
8. Botha AJ, Moore FA, Moore EE, *et al.* Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. J Trauma 1995; 39(3):411-417.
9. Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989; 320(6):365-376.
10. Botha AJ, Moore FA, Moore EE, *et al.* Base deficit after major trauma directly relates to neutrophil CD11b expression: a proposed mechanism of shock-induced organ injury. Intensive Care Med 1997; 23(5):504-509.
11. Bevilacqua MP, Nelson RM. Selectins. J Clin Invest 1993; 91(2):379-387.
12. Etzioni A. Integrins--the glue of life. Lancet 1999; 353(9150):341-343.
13. Ahmed N, Christou N. Systemic inflammatory response syndrome: interactions between immune cells and the endothelium. Shock 1996; 6(Suppl 1):S39-42.
14. Cochrane CG, Aikin BS. Polymorphonuclear leukocytes in immunologic reactions. The destruction of vascular basement membrane in vivo and in vitro. J Exp Med 1966; 124(4):733-752.
15. Rizoli SB, Kapus A, Fan J, *et al.* Immunomodulatory effects of hypertonic resuscitation on the development of lung inflammation following hemorrhagic shock. J Immunol 1998; 161(11):6288-6296.
16. Hampton MB, Chambers ST, Vissers MC, Winterbourn CC. Bacterial killing by neutrophils in hypertonic environments. J Infect Dis 1994; 169(4):839-846.
17. Ciesla DJ ME, Zallen G, Biffl WL, Elzi DJ, Silliman CC. Hypertonic saline attenuation of neutrophil cytotoxic function is reversed upon return to normotonicity. Surgical Forum 1999; L:189-191.
18. Ciesla DJ, Moore EE, Zallen G, *et al.* Hypertonic saline attenuation of polymorphonuclear neutrophil cytotoxicity: timing is everything. J Trauma 2000; 48(3):388-395.
19. Rhee P, Wang D, Ruff P, *et al.* Human neutrophil activation and increased adhesion by various resuscitation fluids. Crit Care Med 2000; 28(1):74-78.
20. Angle N, Hoyt DB, Cabello-Passini R, *et al.* Hypertonic saline resuscitation reduces neutrophil margination by suppressing neutrophil L selectin expression. J Trauma 1998; 45(1):7-12.
21. Angle N, Hoyt DB, Coimbra R, *et al.* Hypertonic saline resuscitation diminishes lung injury by suppressing neutrophil activation after hemorrhagic shock. Shock 1998; 9(3):164-170.
22. Sun LL, Ruff P, Austin B, *et al.* Early up-regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in rats with hemorrhagic shock and resuscitation. Shock 1999; 11(6):416-422.
23. Coimbra R, Hoyt DB, Junger WG, *et al.* Hypertonic saline resuscitation decreases susceptibility to sepsis after hemorrhagic shock. J Trauma 1997; 42(4):602-606.
24. Bauer M, Marzi I, Ziegenfuss T, *et al.* Comparative effects of crystalloid and small volume hypertonic hyperoncotic fluid resuscitation on hepatic microcirculation after hemorrhagic shock. Circ Shock 1993; 40(3):187-193.
25. Vollmar B, Lang G, Menger MD, Messmer K. Hypertonic hydroxyethyl starch restores hepatic microvascular perfusion in hemorrhagic shock. Am J Physiol 1994; 266(5 Pt 2):H1927-1934.
26. Corso CO, Okamoto S, Ruttinger D, Messmer K. Hypertonic saline dextran attenuates leukocyte accumulation in the liver after hemorrhagic shock and resuscitation. J Trauma 1999; 46(3):417-423.
27. Baez S. An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. Microvasc Res 1973; 5(3):384-394.
28. Coimbra R, Junger WG, Hoyt DB, *et al.* Hypertonic saline resuscitation restores hemorrhage-induced immunosuppression by decreasing prostaglandin E2 and interleukin-4 production. J Surg Res 1996; 64(2):203-209.
29. Tangelder GJ, Janssens CJ, Slaaf DW, *et al.* In vivo differentiation of leukocytes rolling in mesenteric postcapillary venules. Am J Physiol 1995; 268(2 Pt 2):H909-915.

30. Giaid A, Michel RP, Stewart DJ, *et al.* Expression of endothelin-1 in lungs of patients with cryptogenic fibrosing alveolitis. Lancet 1993; 341(8860):1550-1554.
31. Vassar MJ, Fischer RP, O'Brien PE, *et al.* A multicenter trial for resuscitation of injured patients with 7.5% sodium chloride. The effect of added dextran 70. The Multicenter Group for the Study of Hypertonic Saline in Trauma Patients. Arch Surg 1993; 128(9):1003-1011; discussion 1011-1003.
32. Holcroft JW, Vassar MJ, Perry CA, *et al.* Perspectives on clinical trials for hypertonic saline/dextran solutions for the treatment of traumatic shock. Braz J Med Biol Res 1989; 22(2):291-293.
33. Vassar MJ, Perry CA, Gannaway WL, Holcroft JW. 7.5% sodium chloride/dextran for resuscitation of trauma patients undergoing helicopter transport. Arch Surg 1991; 126(9):1065-1072.
34. Moore FA, Moore EE. Evolving concepts in the pathogenesis of postinjury multiple organ failure. Surg Clin North Am 1995; 75(2):257-277.
35. Smith RM, Giannoudis PV. Trauma and the immune response. J R Soc Med 1998; 91(8):417-420.
36. Junger WG, Hoyt DB, Davis RE, *et al.* Hypertonicity regulates the function of human neutrophils by modulating chemoattractant receptor signaling and activating mitogen-activated protein kinase p38. J Clin Invest 1998; 101(12):2768-2779.
37. Angle N, Cabello-Passini R, Hoyt DB, *et al.* Hypertonic saline infusion: can it regulate human neutrophil function? Shock 2000; 14(5):503-508.
38. Nolte D, Bayer M, Lehr HA, *et al.* Attenuation of postischemic microvascular disturbances in striated muscle by hyperosmolar saline dextran. Am J Physiol 1992; 263(5 Pt 2):H1411-1416.
39. Hartl R, Medary MB, Ruge M, *et al.* Hypertonic/hyperoncotic saline attenuates microcirculatory disturbances after traumatic brain injury. J Trauma 1997; 42(5 Suppl):S41-47.
40. Barone M, Jimenez F, Huxley VH, Yang XF. Morphologic analysis of the cerebral microcirculation after thermal injury and the response to fluid resuscitation. Acta Neurochir Suppl 1997; 70:267-268.
41. Mazzoni MC, Borgstrom P, Arfors KE, Intaglietta M. Dynamic fluid redistribution in hyperosmotic resuscitation of hypovolemic hemorrhage. Am J Physiol 1988; 255(3 Pt 2):H629-637.
42. Mazzoni MC, Borgstrom P, Warnke KC, *et al.* Mechanisms and implications of capillary endothelial swelling and luminal narrowing in low-flow ischemias. Int J Microcirc Clin Exp 1995; 15(5):265-270.
43. Corso CO, Okamoto S, Leiderer R, Messmer K. Resuscitation with hypertonic saline dextran reduces endothelial cell swelling and improves hepatic microvascular perfusion and function after hemorrhagic shock. J Surg Res 1998; 80(2):210-220.
44. Messmer K. Capillary functions and white cell interaction. Basel ; New York: Karger, 1991.
45. Steinbauer M, Harris AG, Messmer K. Effects of dextran on microvascular ischemia-reperfusion injury in striated muscle. Am J Physiol 1997; 272(4 Pt 2):H1710-1716.
46. Matsumiya A, Yamaguchi M, Nakano H, *et al.* Dextran sulfate inhibits E-selectin-mediated neutrophil adhesion to endotoxin-activated vascular endothelial cells. Life Sci 1999; 64(2):L9-17.
47. Saetzler RK, Jallo J, Lehr HA, *et al.* Intravital fluorescence microscopy: impact of light-induced phototoxicity on adhesion of fluorescently labeled leukocytes. J Histochem Cytochem 1997; 45(4):505-513.
48. McCuskey RS, Reilly FD. Hepatic microvasculature: dynamic structure and its regulation. Semin Liver Dis 1993; 13(1):1-12.
49. Bauer M, Zhang JX, Bauer I, Clemens MG. ET-1 induced alterations of hepatic microcirculation: sinusoidal and extrasinusoidal sites of action. Am J Physiol 1994; 267(1 Pt 1):G143-149.
50. Nishida J, McCuskey RS, McDonnell D, Fox ES. Protective role of NO in hepatic microcirculatory dysfunction during endotoxemia. Am J Physiol 1994; 267(6 Pt 1):G1135-1141.
51. de Carvalho H, Matos JA, Bouskela E, Svensjo E. Vascular permeability increase and plasma volume loss induced by endotoxin was attenuated by hypertonic saline with or without dextran. Shock 1999; 12(1):75-80.
52. Moon PF, Hollyfield-Gilbert MA, Myers TL, *et al.* Fluid compartments in hemorrhaged rats after hyperosmotic crystalloid and hyperoncotic colloid resuscitation. Am J Physiol 1996; 270(1 Pt 2):F1-8.
53. Rizoli SB, Kapus A, Parodo J, *et al.* Hypertonic immunomodulation is reversible and accompanied by changes in CD11b expression. J Surg Res 1999; 83(2):130-135.
54. Oreopoulos GD, Hamilton J, Rizoli SB, *et al.* In vivo and in vitro modulation of intercellular adhesion molecule (ICAM)-1 expression by hypertonicity . Shock 2000; 14(3):409-414; discussion 414-405.
55. Broide DH, Stachnick G, Castaneda D, *et al.* Inhibition of eosinophilic inflammation in allergen-challenged TNF receptor p55/p75--and TNF receptor p55-deficient mice. Am J Respir Cell Mol Biol 2001; 24(3):304-311.

Preface to Manuscript #3

Thus, immediately after hypertonic saline resuscitation of hemorrhagic shock, neutrophil rolling and adhesion is attenuated and concurrently, microvascular integrity is better maintained than with RL resuscitation. The current model used may or may not have reflected more profound conditions of severe hemorrhagic shock (more blood loss for a longer hypotensive period). More importantly, it is unknown whether these in vivo changes occur in more clinically relevant 'two-hit' conditions. Resuscitated trauma patients who survive the initial injury will often be transferred to an intensive care unit where they will invariably suffer subsequent minor 'insults' such as an aspiration, a mild gastrointestinal bleed or minor surgery. These 'two-hit' conditions are particularly conducive for host progression to systemic inflammation and organ dysfunction.

Additionally, the above novel results require ex-vivo validation – Is hypertonic saline resuscitation truly reducing activated neutrophil sequestration and transmigration in tissue as our fewer demonstrated EC/PMN interactions would indicate? Furthermore, it is important to establish whether attenuated PMN sequestration in the lung by HTS resuscitation occurs concurrently with, or subsequently to, reduced in vivo EC/PMN interactions.

The 3rd manuscript describes a variation of the initial murine model, adapted to simulate two-hit conditions. Furthermore, it uses a more profound and longlasting hemorrhagic shock, making it more clinically relevant by reproducing classs III shock which is routinely treated with blood in addition to crystalloid in humans. Using this new model diminished neutrophil sequestration (by myeloperoxidase assay) and transmigration (by lung histologic PMN localization) is demonstrated in HTS resuscitated animals. Furthermore, these same animals show concurrent in vivo attenuations in neutrophil adherence to endothelium hours earlier as compared to RL-resuscitated counterparts.

Manuscript #3

Hypertonic Saline Resuscitation Attenuates Neutrophil Lung Sequestration and Transmigration by Diminishing Leukocyte-Endothelial Interactions in a Two-hit Model of Hemorrhagic Shock and Infection

José L. Pascual MD, Kosar A. Khwaja MD, Lorenzo E. Ferri MD, Betty Giannias BSc, David C. Evans MD, FRCS(C), Tarek Razek MD FRCS(C), René P. Michel MD, FRCP(C) and Nicolas V. Christou, MD, PhD, FRCS(C), FACS

Presented at the 62nd Annual Meeting of the American Association for the Surgery of Trauma
September 24, 2002, Orlando, Florida.
Published in the *Journal of Trauma*, Vol. 54, 121-132 (2003).

Keywords: Neutrophil, hypertonic saline, Ringer's lactate, adhesion, myeloperoxidase, intravital microscopy, multiple organ failure, hemorrhagic shock, resuscitation, Two-hit theory, PMN transmigration, lung histology,

Structured Abstract

Background: Hypertonic saline (HTS) attenuates polymorphonuclear neutrophil (PMN)-mediated tissue injury after hemorrhagic shock. We hypothesized that HTS resuscitation reduces early *in vivo* endothelial cell (EC)/PMN interactions and late lung PMN sequestration in a 2-hit model of hemorrhagic shock followed by mimicked infection.

Methods: Thirty-two mice were hemorrhaged (40mmHg) for 60 minutes and then given intra-tracheal lipopolysaccharide (LPS) (10μg) 1 hour after resuscitation with shed blood and either **HTS** (4cc/kg 7.5%NaCl) or Ringer's lactate (**RL**) (twice shed blood volume). Eleven **controls** were not manipulated. Cremaster intravital microscopy quantified 5-hour EC-PMN adherence, myeloperoxidase assay assessed lung PMN content (2½ and 24 hours) and lung histology determined 24-hour PMN transmigration.

Results: Compared with RL, HTS animals displayed 55% less 5-hour EC-PMN adherence (p=0.01), 61% lower 24-hour lung myeloperoxidase (p=0.007), and 57% lower mean 24-hour lung histologic score (p=0.027).

Conclusion: Compared with RL, HTS resuscitation attenuates early PMN-EC adhesion and late lung PMN accumulation in hemorrhagic shock followed by inflammation. HTS resuscitation may attenuate PMN-mediated organ damage.

The hemodynamic effects of hypertonic saline resuscitation in hemorrhagic shock are well documented. Resuscitation with hypertonic saline (HTS) restores hemodynamic parameters and effective circulating volume, in part through vasodilatation of precapillary resistance vessels and increases in cardiac preload[1]. Several large randomised human trials have shown that HTS compares favourably to Ringer's lactate (RL) in the safe and efficient resuscitation of hemorrhagic shock in trauma patients[2-4]. Results from a large multicenter study have suggested additional, non-hemodynamic advantages of HTS resuscitation, describing reductions in the posttraumatic incidence of acute respiratory distress syndrome (ARDS), coagulopathies and renal failure[4]. More recently, reports of hypertonicity-related alterations of the immune system, particularly with respect to polymorphonuclear neutrophil (PMN) structure and function, have renewed interest in hypertonic saline resuscitation of hemorrhagic shock.

The PMN is believed to be a key mediator of tissue damage leading to organ injury and multiple organ dysfunction after hemorrhagic shock and other systemic inflammatory conditions[5-7]. Under physiological conditions, passage of PMNs from the microcirculation into tissue involves an ordered sequence of steps characterized by PMN rolling, adhesion to endothelium and transmigration. This process may become altered and uncontrolled in certain conditions. Following resuscitated hemorrhagic shock, aberrant and unbridled neutrophil-endothelial interactions are believed to contribute to inappropriate neutrophil activation with the release of toxic oxygen free radicals and proteinases that promote organ injury[6, 8]. As reflected in the high proportion of trauma patients that subsequently develop ARDS, the lung is particularly susceptible to secondary injury by these systemically released products[9]. Severe hemorrhagic shock is believed to render the host vulnerable to a second minor inflammatory stimulus, the so-called 2nd-hit, contributing to the inappropriate activation of neutrophils and endothelium and leading to the development of systemic organ dysfunction[5, 10, 11].

Neutrophil exposure to hypertonic media *in vitro* reduces their bactericidal activity[12-14] and the expression of surface adhesion molecules[15, 16]. *In vivo* models of hemorrhagic shock have demonstrated diminished lung injury and lung PMN sequestration when animals were resuscitated with HTS instead of lactated Ringer's[17]. More clinically relevant 'two-hit' models where HTS resuscitation of hemorrhagic shock (1^{st} hit) is followed by a subsequent mild inflammatory insult (2^{nd} hit), have similarly demonstrated diminished PMN adhesion molecule expression and pulmonary sequestration. In addition, they have shown reductions in lung capillary leakage, lung and liver histologic injury, and incidence of positive bacterial cultures[18, 19]. Shrinkage of neutrophils and cytoskeletal alterations through differential phosphorylation of membrane kinases are believed to be some of the possible mechanisms by which HTS may promote these changes in neutrophil function and structure[16, 20, 21].

Although it has been assumed that reduced adhesion molecule expression leads to diminished leukocyte-endothelial interactions (rolling, adhesion), it was not until the advent of intravital microscopy that this phenomenon was observed with HTS infusions. Hemorrhage and resuscitation with hypertonic regimens by our group and others have used intravital microscopy to demonstrate diminished adhesion and rolling of neutrophils in cremaster or hepatic models[22-24].

To date, no study has correlated HTS-related reductions in leukocyte adhesion *in vivo* with subsequent diminished tissue sequestration and transmigration of neutrophils *ex vivo*. Furthermore, no hemorrhagic shock intravital microscopy study has evaluated microcirculatory effects of HTS after a second inflammatory insult.

We thus hypothesized that, compared with RL, HTS resuscitation of hemorrhagic shock would diminish leukocyte-endothelial interactions 4 hours after a subsequent inflammatory insult, and that this would result in diminished lung PMN sequestration and transmigration the next day. To evaluate this hypothesis, we used a two-hit murine

model where hemorrhagic shock (1st hit) was differentially resuscitated with either RL or HTS followed by lipopolysaccharide (LPS) (2nd hit) instilled intratracheally one hour after the completion of resuscitation. According to the two-hit theory, the second hit would trigger activation of systemic PMN primed by antecedent resuscitated shock. *In vivo* EC/PMN interactions would thus become more pronounced allowing for more subtle differences to emerge. Hence, intravital microscopy of the microcirculation 5 hours after resuscitation was used to evaluate leukocyte-endothelial interactions, pulmonary PMN sequestration was evaluated by lung myeloperoxidase analysis before microscopy (at 2½ hours) and at 24 hours, and PMN transmigration and tissue edema were determined by blinded histologic scoring of H&E stained lung sections also at 24 hours after resuscitation.

Materials and Methods

Reagents

Lactated Ringer's [RL] and normal saline [NS] solutions were purchased from Baxter Corporation (Toronto, Ontario), heparin (10 000 USP units/ml) from Organon Teknika (Toronto, ON), ketamine from Wyeth-Ayerst (Guelph, ON) and xylazine from Bayer (Etobicoke, ON). Potassium phosphate [KH_2PO_4], was from Merck & Co. (Montréal, QC), 3,3',5,5'-tetramethyl-benzidine [TMB] and lipopolysaccharide [LPS, Escherichia coli 0127:B8] from Sigma Chemical Company (St-Louis, MO) and hexadecyltrimethyl-ammonium bromide [HTAB], N,N,-dimethyl formamide [DMF] and 30% hydrogen peroxide [H_2O_2] were from Fisher Scientific (Fair Lawn, NJ). 70 % glutaraldehyde was purchased from Canemco Inc. (St-Laurent, QC). Phosphate Buffered solution [PBS] (Bicarbonate buffered solution [BBS] (NaCl 132 mM, KCl 4.7 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1.2 mM, NaOH 18mM, pH 7.4), 3% glutaraldehyde [GA] (70%

stock diluted in 0.1M KH_2PO_4 [pH7.4]) and hypertonic saline [HTS] (7.5% NaCl in dH_2O) were constituted in our laboratory.

Animal model

Approval was obtained from the McGill University Animal Care Committee prior to experiments. CD1 male mice (Charles River: St-Constant, QC), 25-30gm, were fed *ad libitum* and housed in standard care facilities for 3-5 days prior to study. Acclimated mice were anaesthetized by intra-peritoneal (i/p) injection (xylazine = 6.7mg/kg, ketamine = 13.4mg/kg) and the right carotid artery was cannulated with polyethylene (PE)-10 tubing. The carotid artery catheter was used to monitor blood pressure continuously through a pressure transducer (Living Systems Instrumentation: Burlington, VT), for blood withdrawal, for administration of blood, resuscitation fluids, and intermittent 50µl boluses of xylazine/ketamine solution to maintain anaesthesia.

Hemorrhagic shock was induced by withdrawing blood from the carotid artery over 5 to 7 minutes into a tuberculin syringe previously flushed with 25U heparin until mean arterial pressure (MAP) reached 40 mmHg. Hypotension was maintained for 60 minutes, by further blood withdrawals if MAP rose above 45 mmHg, or by re-infusions of withdrawn blood if MAP fell below 35 mmHg. Following the hypotension period, animals were resuscitated with a crystalloid regimen in addition to shed blood. The carotid artery was then ligated, the cervical incision closed and the animals returned to their cages.

One hour after resuscitation, intraperitoneal (i/p) anaesthesia was administered as above and the cervical incision was reopened. Pretracheal muscles were spread bluntly, exposing the tracheal rings and 20µl LPS (500µg/ml) was injected into the trachea under direct vision using a 0.5cc insulin syringe. Again, the cervical incision was closed and the animals were returned to their cages. Control animals did not undergo

any manipulation. Animals were subsequently re-anaesthetised at different times depending on the protocol arm to which they had been assigned.

Study groups and experimental protocol

Forty-three animals were randomized to one of three arms: **HTS** (shock and resuscitation with 4cc/kg of 7.5% hypertonic saline immediately followed by all shed blood [n=18]); **RL** (shock and resuscitation with lactated Ringer's, [twice the volume of withdrawn blood] immediately followed by all shed blood [n=14]); and **control** (no surgical manipulation, hemorrhage or resuscitation [n=11]). Both resuscitation regimens have been demonstrated to adequately resuscitate mice after such hemorrhagic shock conditions[14, 19, 25]. Once resuscitation was completed, animals were returned to their cages and received intratracheal LPS one hour later. Animals from each resuscitation arm were then evaluated at three time intervals after completion of resuscitation and LPS administration: 2½ hours after resuscitation, lung myeloperoxidase (MPO) analysis (n=18) was used to evaluate tissue PMN sequestration; at 5 hours, cremaster intravital microscopy (n=10) was used to evaluate *in vivo* PMN adhesion to endothelium; and at 24 hours, lung histology (n=8) was used to evaluate lung interstitial edema and PMN transmigration and lung MPO (n=8) again were used to determine PMN sequestration. Control animals were evaluated for lung MPO (n=9) and for histology (n=4) without prior manipulation.

Intravital microscopy

Four hours after receiving intratracheal LPS, resuscitated animals received intraperitoneal anaesthesia as above and underwent right jugular canulation. The jugular line was used to administer anaesthetic solution. The mouse cremaster was

prepared for intravital microscopy as described previously[26]. Briefly, after the animal was immobilized by attachment of all four limbs, the cremaster muscle was exteriorized through a small scrotal incision, opened and dissected free of the testicle and epididymis. Using five-point fixation with 4-0 silk, the cremaster was splayed open and fixed to a plexiglass stage. For the remainder of the experiment the exteriorized cremaster was continuously perfused with thermostat-controlled (37° C) BBS. The stage was placed on a Nikon TE 300 inverted microscope (Nikon Canada: Montréal, QC), and live tissue microcirculation was imaged at 2120X magnification.

An optical Doppler velocimeter (Microcirculation Research Institute: College Station, TX) was used to directly measure central red blood cell velocity (V_{RBC}) in post-capillary venules (PCVs). To minimize intersample shear stress variability, only non-branching PCVs with a diameter of 20-40μm and a V_{MEAN} of 1.25–2.50 mm/sec were chosen. Mean vessel flow velocity (V_{MEAN}) was calculated from V_{RBC} using the formula: $V_{MEAN} = V_{RBC}/1.6$. Shear rate (γ), in sec^{-1}, was calculated using the formula: $\gamma = 8 \times (V_{RBC}/D_V)$, where D_V is the venular diameter measured directly off line using calipers. Shear stress was calculated using the formula: shear stress = 0.25 X shear rate.

Live microscopic footage of chosen post capillary venules was captured with a high definition black and white video camera (CCD High Performance Camera, COHU, San Diego, CA), transferred to a monitor (Trinitron Color monitor – SSM-14NE, Sony: Toronto, ON), and recorded on videotape with a video recorder (SVHS HrS3910u, JVC Company of America: Wayne, NJ). A video Time-Date Generator (model WJ-810, Panasonic: Toronto, ON) projected the time, date, and stopwatch function onto the monitor at all times. Body temperature was maintained at 37° C with a radiant heat lamp throughout the study period. All animals were sacrificed by xylazine/ketamine overdose followed by cervical dislocation at the completion of experiments.

Quantification of EC-PMN interactions

In each animal, seven-minute video footage of each of three different post capillary venules per animal were captured after a stabilization period following the completion of cremaster surgery. These recordings were subsequently played back off line by another member of the laboratory who quantified leukocyte-endothelial cell interactions without prior knowledge of treatment.

To avoid alteration of PMN adhesive properties, neutrophils were not labeled with fluorescent markers. Nonetheless, available evidence suggests that the vast majority of leukocytes visible in such an IVM model were likely PMNs[27]. Leukocyte rolling was defined as the number of leukocytes crossing a line perpendicular to the long axis of the vessel, that were moving at a rate slower than erythrocytes over a period of two minutes. Leukocyte adherence was defined as the number of cells stationary for a minimum of 30 seconds in a 100 μm length of venule during a five-minute period and pre-adherence was defined as the number of immobile neutrophils in the same 100 μm vessel section at the initiation of counting. Total neutrophil adherence was the sum of neutrophil adherence and pre-adherence for a given vessel footage.

Myeloperoxidase Assay

One and ½ hours and 23 hours after LPS, animal lungs underwent MPO analysis. After induction into anaesthesia by intraperitoneal ketamine/xylazine, animals underwent sternotomy and the entire tracheobronchial tree was excised by sharp dissection. The right lung was dissected free of diaphragmatic and pleural tissue and subsequently weighed while the left lung was prepared for histology. The right lung vasculature and bronchial tree weer not flushed prior to excision. The excised right lung sample was then homogenized (PT10 00 Polytron Homogenizer, Kinematica GMBH:

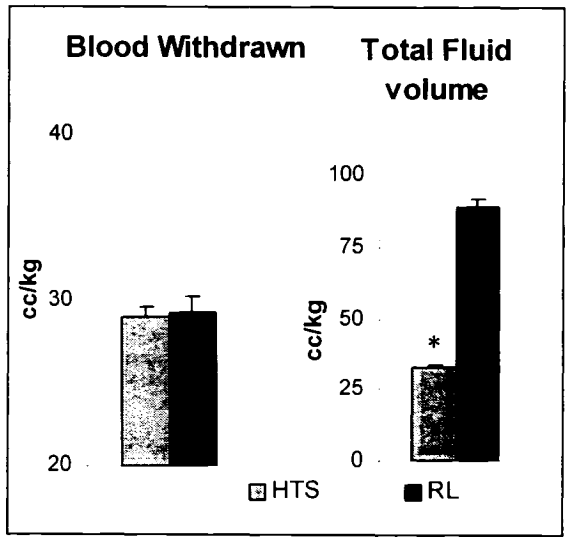

FIGURE 1: There were no differences in withdrawn blood volumes between resuscitated groups ($p>0.05$). RL animals received twice the shed blood volume in addition to shed blood whereas HTS animals received 4cc/kg 7.5% saline in addition to shed blood (* $p<0.001$ vs RL).

Luzern, Switzerland) for 1 minute in 3cc of 10mM KH_2PO_4 [pH7.4] buffer and centrifuged at 12 000g for 20 minutes at 4° C. The supernatant was discarded and the pellet resuspended and homogenized in 50mM KH_2PO_4 [pH6.0] buffer containing 0.5% HTAB. The homogenate was kept frozen at -80° C and at a later time, it was thawed, rehomogenized for 1 minute and sonicated (Sonic Dismembrator Model 300, Fisher: Farmingdale, New York) at 40 W for 1 minute. After centrifugation as described above the supernatant was diluted (X3) and 50µl was added to 350µl of 220mM KH_2PO_4 in 110mM NS and reacted 45 seconds later with 50µl 16mM TMB in DMF prior to blanking the spectrophotometer (DU 640, Beckman Coulter: Fullerton, California) at 37° C. The colorimetric reaction was then read at 655nm after the addition of 50µl of 3mM H_2O_2. The absorbance change over 180 seconds was used as a measure of MPO activity. Results were expressed as MPO activity per gram of lung tissue.

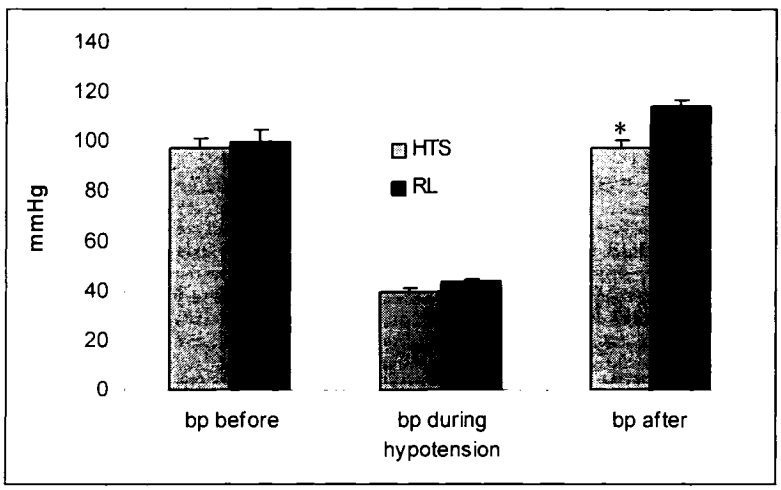

FIGURE 2: Blood pressure did not differ significantly between resuscitated groups before and during hypotension ($p>0.05$) but was significantly higher after resuscitation in RL compared with HTS animals (* $p<0.01$ vs. RL).

Histologic Lung Analysis

As described above, at 24 hours anaesthetized animals underwent sternotomy and the entire tracheobronchial tree was excised by sharp dissection. The

right lung was used for MPO evaluation. The left lung was fixed by instillation of 3% glutaraldehyde into the left mainstem bronchus using a 22-gauge angiocatheter and fixed overnight. The following day, the lung was sliced, sections were processed using routine histological techniques and embedded in paraffin. From these, 5 μm-thick sections were cut and stained with hematoxylin and eosin. The slides were subsequently graded by one of the authors (R.P.M.) using a modified histologic score[28] without prior knowledge of treatment groups. The following histologic parameters were graded: marginated intravascular neutrophils, interstitial neutrophils, alveolar neutrophils, and interstitial edema. For a given sample, each parameter received a score of zero (none), one (scant), two (moderate) or three (extensive) points. Total lung histology scores were calculated as the sum of individual parameter scores for a given sample.

Statistical Analysis:

All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Differences between groups were compared using analysis of varience (ANOVA) with Bonferroni correction using Systat 10 data analysis software (SPSS, Chicago, Illinois). When data were not normally distributed, the Kruskal-Wallis Test was applied to determine significance. Twenty four-hour survival was compared using confidence interval analysis for differences in binomial proportions. Values of $p < 0.05$ were deemed statistically significant.

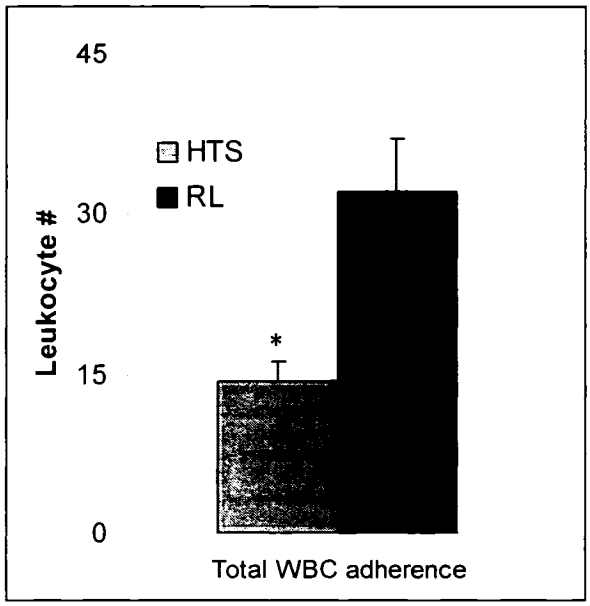

FIGURE 3: Total leukocyte adherence to endothelium in the cremaster circulation 4 hours after intratracheal LPS administration was greater in RL than HTS animals (*p=0.01 vs. RL).

Results

Vascular and hemodynamic mechanics

Blood volume withdrawn (mean 29.1 ± 0.56 cc/kg) did not differ between resuscitation groups but total fluid administered (blood + crystalloid) was significantly greater in RL-resuscitated animals compared to HTS animals (88.6 ± 2.7 vs 33.0 ± 0.7 cc/kg, respectively, $p<0.01$) (Figure 1). Systolic blood pressure before (mean 98.9 ± 3.15 mmHg) and during hypotension (mean 41.2 ± 1.43 mmHg) was not different between groups whereas RL-resuscitated animals had a significantly higher blood pressure than HTS counterparts at the completion of resuscitation (114.6 ± 2.4 vs 97.7 ± 3.1 mmHg, respectively; $p<0.01$) (Figure 2). Post-capillary venular diameter (mean 29.8 ± 1.0μm), V_{RBC} (mean 3.22 ± 0.19mm/sec), V_{MEAN} (mean 2.01 ± 0.12mm/sec), shear rates (mean 0.65 ± 0.075sec^{-1}) and shear stress (mean 0.16 ± 0.019sec^{-1}) did not differ statistically between both resuscitation groups ($p>0.05$).

Effect of hypertonic saline on leukocyte interactions with endothelium

Total leukocyte adhesion to ECs was significantly lower in HTS-resuscitated animals than in RL counterparts [14.4 ± 1.82 leukocytes/100μm vs 32.1± 4.96 leukocytes/100μm, respectively, $p=0.01$] (Figure 3). There were no statistically significant differences in leukocyte rolling between groups (data not shown). Figure 4 contrasts an image displaying several adherent leukocytes (A) taken from footage of a RL-resuscitated animal 4 hours after LPS administration with another showing few interacting leukocytes (B) taken from an HTS animal at the same time interval.

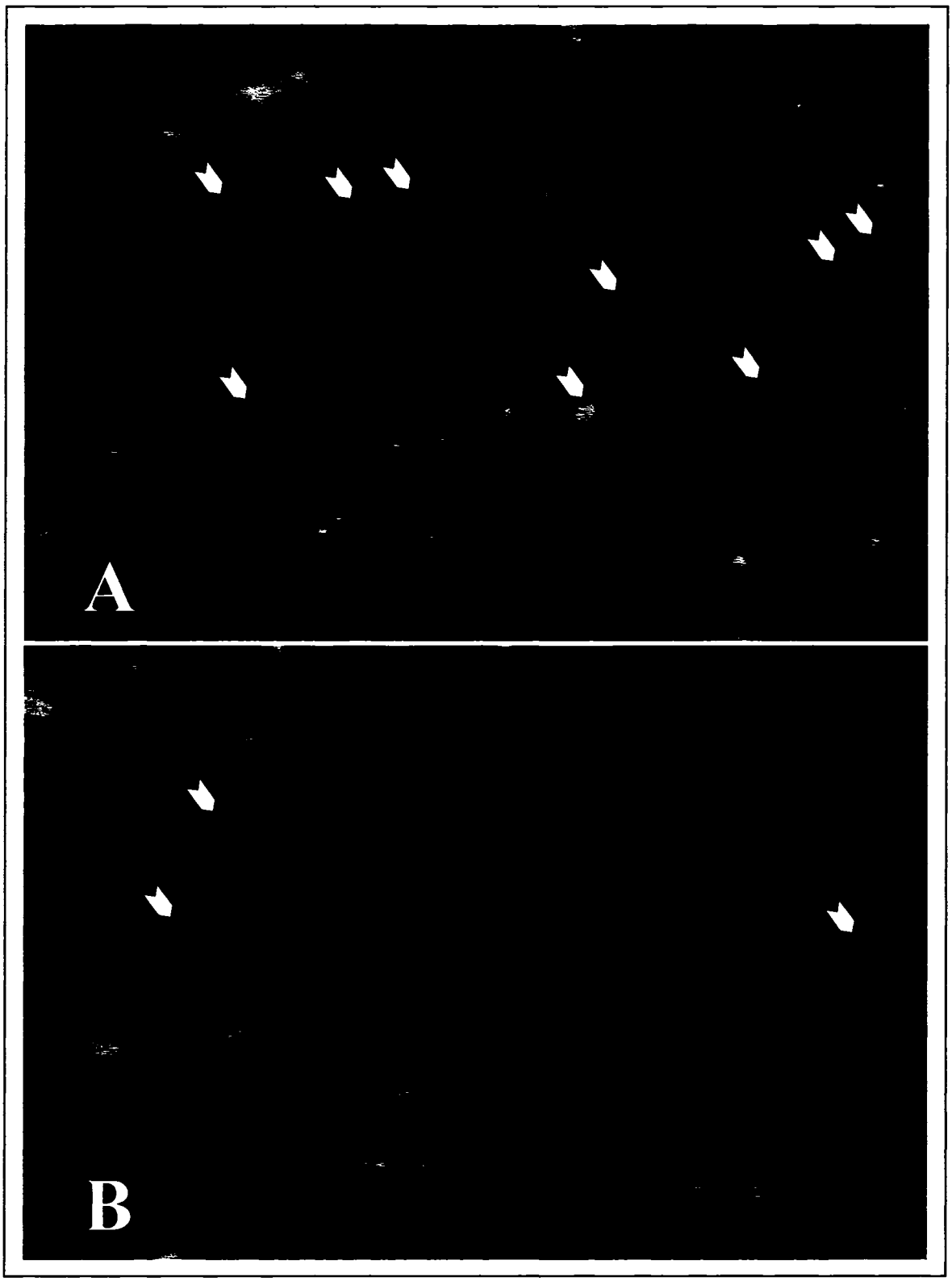

FIGURE 4: A representative image demonstrating a post capillary venule with several adherent leukocytes (some indicated with arrowheads) taken from a RL resuscitated animal 4 hours after LPS (A) is contrasted with one displaying few interacting leukocytes (B), taken from an animal resuscitated with HTS at the same time interval.

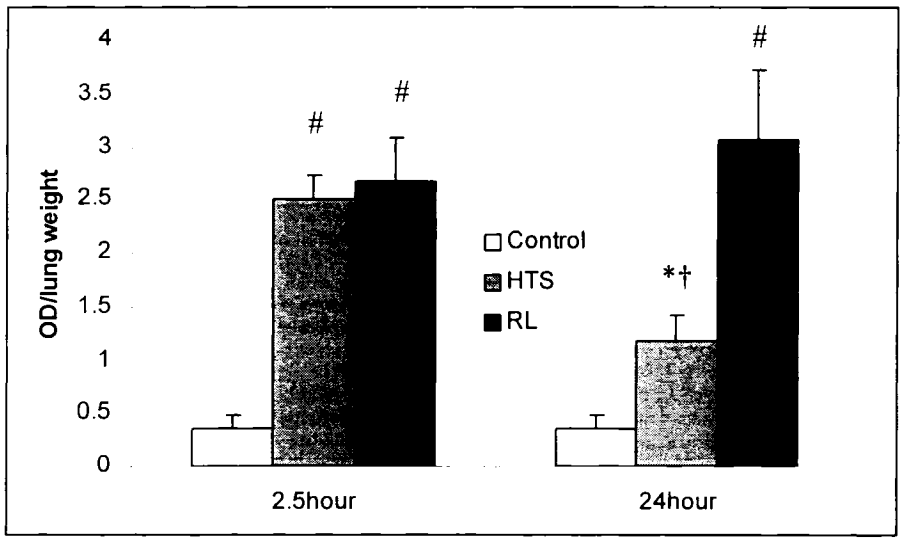

FIGURE 5: Pulmonary myeloperoxidase levels were similar in HTS and RL animals ($p>0.05$) but greater than control 2½ hours after resuscitation (#$p<0.001$vs. control in the same time interval). At 24 hours, lung MPO in HTS animals was similar to that of controls ($p>0.05$) but significantly lower than in RL animals (*$p=0.007$ vs. RL in the same time interval). Changes in lung MPO between 2½ and 24 hours following LPS was not significant in RL animals but diminished by half in HTS animals (†$p=0.005$ vs HTS at 2½ hours).

Neutrophil sequestration in lung is decreased in HTS-resuscitated animals 24 hours after resuscitation

One and a half hours following LPS administration, lung myeloperoxidase levels were similar in HTS and RL animals [2.51 ± 0.22 ΔOD/g vs 2.70 ± 0.43 ΔOD/g, respectively, $p>0.05$] (Figure 5). At this time, control animals displayed significantly lower lung MPO than either resuscitation group [0.35 ± 0.13 vs RL and HTS, $p<0.001$]. At 24 hours, lung MPO in HTS animals was similar to that of controls but 61% lower than in RL animals (**HTS**:1.19 ± 0.23 ΔOD/g; **RL**:3.06 ± 0.66 ΔOD/g, $p=0.007$). Lung MPO changes in RL animals were not significant between 2½ and 24 hours after resuscitation, whereas in HTS animals, lung MPO decreased by more than 50% during that time interval ($p=0.005$). Thus,

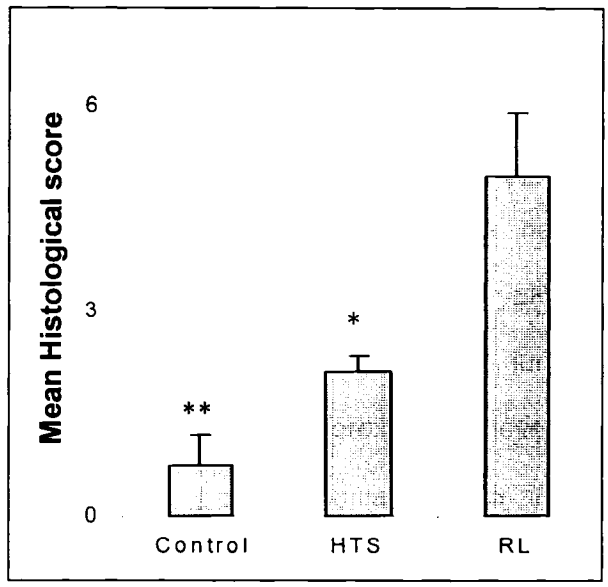

Figure 6: Histologic lung transmigration scores 24 hours after resuscitation were significantly higher in RL than in HTS or control animals (*$p=0.027$ vs RL, ** $p=0.002$ vs RL). Histologic score was the sum of all parameter scores (marginated PMN, interstitial PMN, alveolar PMN, and interstitial edema)

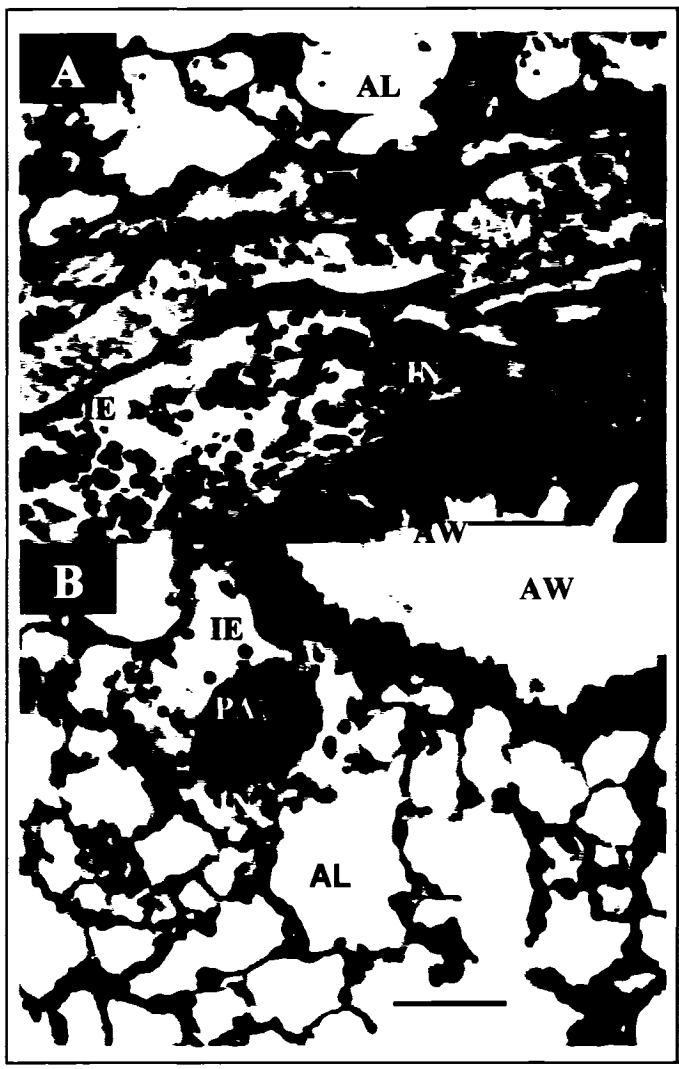

FIGURE 7: High power light photomicrographs of lung sections from a RL-resuscitated mouse 24 hours after resuscitation. (A) Detail showing a longitudinal section of small pulmonary artery (PA) flanked by numerous interstitial neutrophils (IN) and associated interstitial edema (IE) adjacent to the airway (AW) and alveoli (AL). (B) Small artery (PA) filled with numerous marginated neutrophils, also seen in the surrounding edematous periarterial interstitial space. Bars indicate 50 μm.

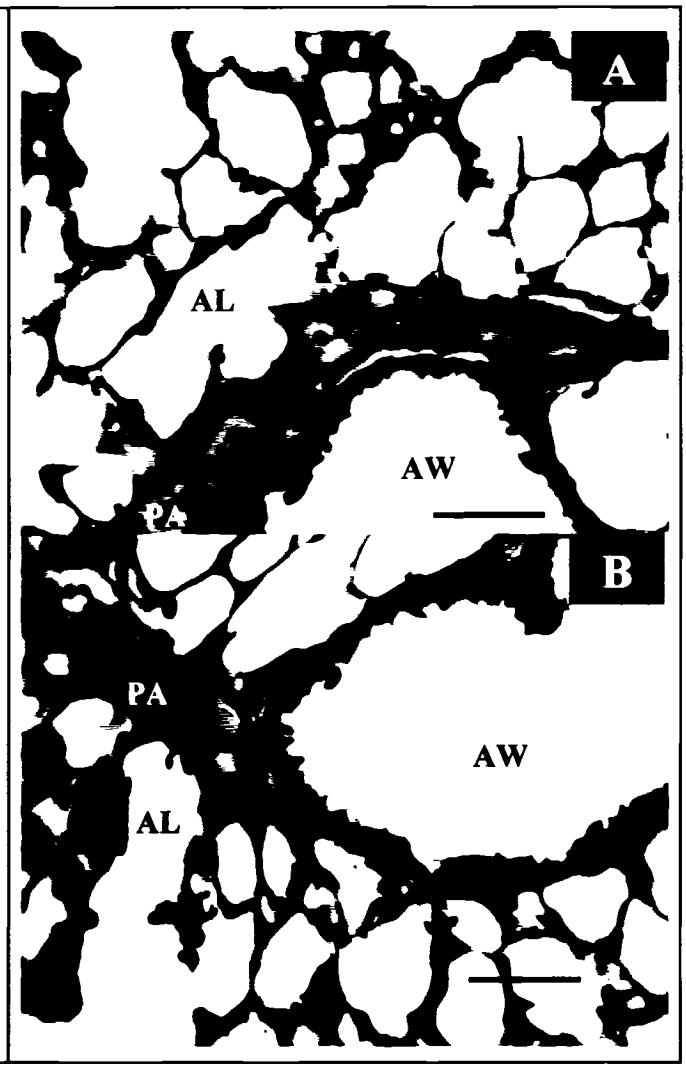

FIGURE 8: High power light photomicrographs of lung sections from an HTS-resuscitated mouse 24 hours after resuscitation. (A) Detail showing a longitudinal section of small artery between airway and alveoli (AL). No edema or neutrophils are present. (B) Normal airway (AW), small pulmonary artery (PA) devoid of periarterial interstitial edema, and with only rare marginated and interstitial neutrophils. Bars indicate 50 μm.

neutrophil accumulation in lung tissue as evaluated by MPO reflected PMN in lung parenchyma but also sequestered in vessels ('trapped' or otherwise) and the bronchial tree. Therefore, total PMN lung content of all resuscitated animals was greater than in controls shortly after LPS administration but 24 hours after resuscitation, pulmonary

PMN content of HTS animals returned to control levels but remained elevated in RL counterparts.

Lung histologic scores are decreased in HTS-resuscitated animals 24 hours after resuscitation

Twenty-four hours after resuscitation, lung histologic scores were significantly higher in RL animals [5.0 ± 0.9] compared with controls [0.7 ± 0.4, p=0.002 vs RL] and HTS animals [2.1 ± 0.2, p=0.027 vs RL] (Figure 6). Differences in mean score were mostly attributable to differences in the degree of marginated and interstitial neutrophils between resuscitation groups. Control and HTS animals did not differ significantly in total and individual parameter scores. Figure 7 shows representative photomicrographs of lung with high histology score taken from a RL-resuscitated animal displaying numerous marginated and interstitial neutrophils flanked by interstitial edema. In figure 8 lung photomicrographs taken from an HTS-resuscitated animal show normal airways, small pulmonary arteries devoid of periarterial interstitial edema, and with only rare marginated and interstitial neutrophils. Thus, 24 hours after resuscitation, lungs from HTS resuscitated animals displayed half the histologic transmigration score of lungs from RL animals.

Twenty four-hour survival

Four of 10 (40%) RL animals and 4 of 5 (80%) HTS animals survived 24 hours after resuscitation but this was not statistically significant (p>0.05).

Discussion

For over 30 years, trauma research has sought evidence for the best fluid for resuscitation of hemorrhagic shock. Although massive isotonic crystalloid resuscitation popularized in the Vietnam War was a major advance in the treatment of hemorrhagic shock, the incidence of subsequent pulmonary edema (Da Nang Lung[29], adult respiratory distress syndrome [ARDS]) was considerable. Concerns that isotonic resuscitation might be harmful in itself gave rise to interest in small volume hypertonic resuscitation[30]. The administration of much smaller volumes (4cc/kg) of 7.5% saline solution in hypovolemic conditions results in the rapid improvement of mean arterial pressure, cardiac output and peripheral perfusion[1, 31]. Large, randomized, controlled human trials have established the safety and efficiency of HTS resuscitation of hypovolemic trauma patients[4, 32]. Nonetheless, a significant proportion of trauma deaths are not directly attributable to inadequate volume restoration during the initial resuscitation phase; indeed, over 50% of deaths that occur more than one week after injury are related to complications of host immunity leading to multiple organ failure (MOF)[33, 34].

The dichotomous nature of host immunity in the context of trauma involves, on the one hand, host protection from pathogen invasion, and on the other hand, an ability to cause host tissue damage resulting in multiple organ injury and possibly death[35].

The polymorphonuclear neutrophil is a key effector of host immunity that perfectly illustrates this dichotomy. In physiologic conditions, the neutrophil is summoned to areas of pathogen invasion or tissue injury reaching its target through an orderly series of steps. First marginating out of central blood flow, the neutrophil begins to interact weakly with endothelium through fragile interactions between surface selectins (L, E and P)[36] and ligands of the Sialyl Lewis X (sLe^X) family. This process slows the neutrophil out of central vessel flow and imparts on it a rolling motion permitting a greater proximity to

endothelial cells. The resulting close association between the two cell types allows for firm coupling between PMN surface integrins (CD11a, CD11b)[37] and endothelial receptors of the immunoglobulin superfamily (ICAM-1, ICAM-2). These latter interactions lead to arrest of the neutrophil on the endothelium (adherence). The neutrophil is then able to transmigrate between endothelial cells to the interstitial environment and migrate down a chemotactic gradient to destroy pathogens and scavenge necrotic tissue debris[38].

The injurious side of neutrophil behavior can be seen in conditions of systemic inflammation such as hemorrhagic shock, where this orderly process of neutrophil trafficking is disrupted. When hemorrhagic shock is followed by a subsequent inflammatory insult (2nd hit), the aberrant upregulation of these neutrophil-endothelial interactions is believed to cause tissue sequestration of neutrophils and an indiscriminate release of toxic oxygen radicals and enzymes resulting in tissue damage and subsequent organ injury.

The importance of the neutrophil in hemorrhagic shock is underscored in two-hit models. The two-hit (two-insult) theory maintains that neutrophils are first primed by an insult such as hemorrhage and resuscitation and that with a second subsequent usually innocuous stimulus, (aspiration, urinary tract infection), neutrophils are activated, releasing cytotoxic substances leading to further activation, interactions with endothelium and tissue injury[35]. The distinctness of neutrophil priming and activation has been demonstrated *in vitro* in studies where activated but not primed human neutrophils were capable of enhanced superoxide release, tissue sequestration and injury to endothelial cells[39]. Although *in vivo* animal studies have also demonstrated how two spaced insults resulted in microcirculatory compromise[40], evaluations of leukocyte-endothelial interactions in such conditions are still lacking.

Multiple *in vitro* experiments have demonstrated that hypertonic saline alters neutrophil structure and function. Neutrophils exposed to hypertonicity prior to *in vitro* activation display diminished oxidative burst[14, 15, 41], phagocytosis[13] and cytotoxicity[13, 20]. In addition, primed and unprimed neutrophils incubated in hypertonic media have demonstrated diminished CD11b expression[15, 18, 41, 42] but varying alterations in the expression of L-selectin[18, 42]. Neutrophils from healthy humans infused with HTS *in vivo* displayed diminished CD11b but increased L-selectin after activation *in vitro*[43, 44]. These and other similar reports of adhesion molecule modulation by HTS have lead to the proposal that HTS attentuates leukocyte-endothelial interactions *in vivo*.

In vivo studies involving single-hit hemorrhagic shock in mice have demonstrated diminished bronchioalveolar PMN accumulation, lung neutrophil sequestration and lung histologic injury 24 hours after resuscitation when HTS was the initial or the only resuscitation fluid given[14, 17]. Yet, these and other studies from the same authors showed contradictory alterations of CD11b and L-selectin expression following HTS resuscitation[45]. Interestingly, 2-hit *in vivo* models of hemorrhagic shock followed by inflammation have demonstrated extensive *ex-vivo* benefits. In a rat model of resuscitated hemorrhagic shock followed by intratracheal LPS, Rizoli and colleagues[18] demonstrated reductions in lung capillary leakage, neutrophil sequestration (MPO), lung injury (histology), lung ICAM-1 and PMN CD11b/L-selectin levels with HTS compared to RL resuscitation. Similarly, but in mice, Coimbra *et al*[19] found that cecal ligation and puncture (CLP) 24 hours after resuscitation resulted in diminished pulmonary and hepatic histologic injury 2 and 24 hours later with hypertonic saline but not RL resuscitation. In the present study, we confirmed through *ex-vivo* pulmonary myeloperoxidase analysis that 24 hours after resuscitation, neutrophil lung sequestration in HTS animals was 39% that of RL counterparts. However, lung MPO levels at 2½ hours were similar in both resuscitated groups. These early MPO elevations likely

reflect edematous, non-deformable leukocytes becoming passively trapped in narrowed lung capillary passages following ischemia and reperfusion in all resuscitated animals[46]. The concept that poor neutrophil pliability early in the course of systemic inflammation promotes mechanical trapping in the microvasculature of organs with a high number of capillary segments is not novel[47, 48]. Histologic evaluations in sepsis have demonstrated that in lungs, early neutrophil sequestration may be less dependent on adhesion molecules and interactions with endothelium than on changes in physical parameters such as lack of deformability and stiffness of leukocytes[47, 49, 50]. Lung neutrophil sequestration persisting 24 hours later better reflected increased neutrophil-endothelial interactions causing receptor-mediated neutrophil adherence and transmigration into lung tissue[50].

While MPO levels illustrate total tissue content of PMNs, they do not determine the anatomic location where the majority of neutrophils are sequestered. Our evaluation of lung histologic sections not only confirmed lower total amounts of neutrophils in the lungs of HTS animals but also brought the missing information on their anatomic distribution within lung tissue. Marginated intravascular and transmigrated interstitial neutrophils were the principal contributors to differences in histologic scores demonstrating more than three times the marginated and interstitial PMNs in RL compared with HTS animals. These histologic findings agree with previously reported results from studies evaluating lung histopathology in 2-hit hemorrhagic shock models[18, 19]. However, none of these studies correlated these histologic changes with *in vivo* differences in leukocyte/endothelial interactions related to HTS resuscitation.

Single and double hit hemorrhagic shock studies have suggested that attenuation in leukocyte-endothelial interactions by HTS resuscitation is implied by reduced adhesion molecule expression. We believe that the current study provides novel evidence linking *in vivo* changes in leukocyte-endothelium interactions with diminished lung neutrophil

sequestration and transmigration after HTS-resuscitation of hemorrhagic shock. Using intravital microscopy, our group and others have previously reported diminished neutrophil adhesion and rolling following HTS-resuscitation of shock (one-hit)[22-24]. Although none of these studies evaluated *ex vivo* neutrophil sequestration or histologic transmigration, our evaluation concurrently demonstrated diminished *in vivo* macromolecular leakage 2 hours after resuscitation of hemorrhagic shock with HTS compared with RL[22]. The current study further demonstrates that HTS resuscitation also reduces neutrophil adhesion in a two-hit model of resuscitated hemorrhagic shock followed by a subsequent inflammatory lung stimulus concurrent with diminished tissue PMN sequestration and margination. Variability in leukocyte rolling within each resuscitation group was a possible reason that the trend of diminished rolling in HTS animals was not significant. Any mechanical capillary neutrophil trapping that may have been occurring during IVM analysis was not apparent as we evaluated much larger venules (20 to 40μm) unlikely able to 'trap' even swollen neutrophils, normally 5-12μm in diameter[51].

We have analysed our model for possible confounding variables. We chose not to label neutrophils with fluorescent markers as sometimes is done in intravital microscopy studies because fluorescent tags are known to affect their adhesive properties[52]. Thus, leukocytes that were counted as adherent to endothelial cells may not all have been neutrophils. Furthermore, evidence from other studies suggests that the majority of visible leukocytes in such microscopic analysis are, in fact, neutrophils[27]. Additional study groups such as SHAM animals undergoing cremaster surgery but not hemorrhage or LPS tracheal instillation or animals undergoing LPS administration without shock would have added relevandce to the study but were omitted for the interest of simplicity. Blood pressure was significantly higher at the completion of resuscitation with lactated Ringer's compared to that in animals resuscitated with HTS

(figure 2). While this higher blood pressure could have augmented microvascular shear stress and impacted on leukocyte-endothelial interactions, by controlling v_{RBC} and vessel diameter, shear stress in the chosen vessels was kept constant and the bias of this variable unlikely affected our results. The large volume of fluid administered to RL animals compared to HTS counterparts may have, in itself, contributed to microvascular injury. Yet, any injury from excessive volume administration would again have occurred through increased shear stress which was controlled for by selection of uniform diameter vessels for microscopic analysis. Furthermore, although higher shear stress would have implied diminished leukocyte adhesion to endothelium, we still found that RL animals demonstrated higher adhesion levels. Nonetheless, protocols with the fluid regimens administered in the current study have been shown to offer equally satisfactory resuscitation in several other hemorrhagic shock studies[14, 19, 45]. In addition, smaller volumes are inherent to clinical HTS resuscitation whereas much larger volumes are always required when using isotonic crystalloids. This study does not explore if the parallel decrease in cremaster EC/PMN interactions and lung PMN sequestration by HTS are necessarily related. While they follow the same pattern other non receptor-mediated mechanisms are known to occur in the lung capillary-tissue interphase.

In summary, the present study demonstrates that hypertonic resuscitation of hemorrhagic shock followed by an inflammatory lung stimulus attenuates leukocyte adhesion to endothelium at five hours, and is associated with reduced lung sequestration and transmigration of neutrophils at 24 hours. These findings help further elucidate the extent of immunomodulatory effects from hypertonic saline resuscitation in hemorrhagic shock. Further experiments will be needed to confirm HTS-related benefits in trauma patients suffering subsequent insults in the intensive care unit and at risk of developing neutrophil-mediated organ injury.

References to Manuscript #3

1. Velasco IT, Pontieri V, Rocha e Silva M, Jr., Lopes OU. Hyperosmotic NaCl and severe hemorrhagic shock. *Am J Physiol* 1980; 239:H664-673.
2. Shackford SR, Bourguignon PR, Wald SL, Rogers FB, Osler TM, Clark DE. Hypertonic saline resuscitation of patients with head injury: a prospective, randomized clinical trial. *J Trauma* 1998; 44:50-58.
3. Wade CE, Kramer GC, Grady JJ, Fabian TC, Younes RN. Efficacy of hypertonic 7.5% saline and 6% dextran-70 in treating trauma: a meta-analysis of controlled clinical studies. *Surgery* 1997; 122:609-616.
4. Mattox KL, Maningas PA, Moore EE, *et al.* Prehospital hypertonic saline/dextran infusion for post-traumatic hypotension. The U.S.A. Multicenter Trial. *Ann Surg* 1991; 213:482-491.
5. Moore FA, Moore EE, Read RA. Postinjury multiple organ failure: role of extrathoracic injury and sepsis in adult respiratory distress syndrome. *New Horiz* 1993; 1:538-549.
6. Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. *J Trauma* 1995; 39:411-417.
7. Weiss SJ. Tissue destruction by neutrophils [see comments]. *N Engl J Med* 1989; 320:365-376.
8. Botha AJ, Moore FA, Moore EE, Peterson VM, Goode AW. Base deficit after major trauma directly relates to neutrophil CD11b expression: a proposed mechanism of shock-induced organ injury [see comments]. *Intensive Care Med* 1997; 23:504-509.
9. Faist E, Baue AE, Dittmer H, Heberer G. Multiple organ failure in polytrauma patients. *J Trauma* 1983; 23:775-787.
10. Swank DW, Moore SB. Roles of the neutrophil and other mediators in adult respiratory distress syndrome. *Mayo Clin Proc* 1989; 64:1118-1132.
11. DeCamp MM, Demling RH. Posttraumatic multisystem organ failure. *Jama* 1988; 260:530-534.
12. Ciesla DJ, Moore EE, Biffl WL, Gonzalez RJ, Silliman CC. Hypertonic saline attenuation of the neutrophil cytotoxic response is reversed upon restoration of normotonicity and reestablished by repeated hypertonic challenge. *Surgery* 2001; 129:567-575.
13. Hampton MB, Chambers ST, Vissers MC, Winterbourn CC. Bacterial killing by neutrophils in hypertonic environments. *J Infect Dis* 1994; 169:839-846.
14. Murao Y, Hoyt DB, Loomis W, *et al.* Does the timing of hypertonic saline resuscitation affect its potential to prevent lung damage? *Shock* 2000; 14:18-23.
15. Rhee P, Wang D, Ruff P, *et al.* Human neutrophil activation and increased adhesion by various resuscitation fluids. *Crit Care Med* 2000; 28:74-78.
16. Rizoli SB, Kapus A, Parodo J, Rotstein OD. Hypertonicity prevents lipopolysaccharide-stimulated CD11b/CD18 expression in human neutrophils in vitro: role for p38 inhibition. *J Trauma* 1999; 46:794-798; discussion 798-799.
17. Angle N, Hoyt DB, Coimbra R, *et al.* Hypertonic saline resuscitation diminishes lung injury by suppressing neutrophil activation after hemorrhagic shock. *Shock* 1998; 9:164-170.
18. Rizoli SB, Kapus A, Fan J, Li YH, Marshall JC, Rotstein OD. Immunomodulatory effects of hypertonic resuscitation on the development of lung inflammation following hemorrhagic shock. *J Immunol* 1998; 161:6288-6296.
19. Coimbra R, Hoyt DB, Junger WG, *et al.* Hypertonic saline resuscitation decreases susceptibility to sepsis after hemorrhagic shock. *J Trauma* 1997; 42:602-606; discussion 606-607.
20. Junger WG, Hoyt DB, Davis RE, *et al.* Hypertonicity regulates the function of human neutrophils by modulating chemoattractant receptor signaling and activating mitogen-activated protein kinase p38. *J Clin Invest* 1998; 101:2768-2779.
21. Ciesla DJ, Moore EE, Musters RJ, Biffl WL, Silliman CA. Hypertonic saline alteration of the PMN cytoskeleton: implications for signal transduction and the cytotoxic response. *J Trauma* 2001; 50:206-212.
22. Pascual JL, Ferri, L.E., Seely, A.J.E., Campisi, Chaudhury, P., G.,Giannias, B., Evans, D.C., Razek, T., Michel, R.P., Christou, N.V. Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces *in vivo* vascular leakage. *Annals of Surgery* 2002; 236: 634-642.
23. Vollmar B, Lang G, Menger MD, Messmer K. Hypertonic hydroxyethyl starch restores hepatic microvascular perfusion in hemorrhagic shock. *Am J Physiol* 1994; 266:H1927-1934.
24. Corso CO, Okamoto S, Ruttinger D, Messmer K. Hypertonic saline dextran attenuates leukocyte accumulation in the liver after hemorrhagic shock and resuscitation. *J Trauma* 1999; 46:417-423.

25. Coimbra R, Junger WG, Hoyt DB, Liu FC, Loomis WH, Evers MF. Hypertonic saline resuscitation restores hemorrhage-induced immunosuppression by decreasing prostaglandin E2 and interleukin-4 production. *J Surg Res* 1996; 64:203-209.
26. Baez S. An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc Res* 1973; 5:384-394.
27. Tangelder GJ, Janssens CJ, Slaaf DW, oude Egbrink MG, Reneman RS. In vivo differentiation of leukocytes rolling in mesenteric postcapillary venules. *Am J Physiol* 1995; 268:H909-915.
28. Bachofen M, Weibel ER. Structural alterations of lung parenchyma in the adult respiratory distress syndrome. *Clin Chest Med* 1982; 3:35-56.
29. Pearce FJ, Lyons WS. Logistics of parenteral fluids in battlefield resuscitation. *Mil Med* 1999; 164:653-655.
30. Nakayama S, Sibley L, Gunther RA, Holcroft JW, Kramer GC. Small-volume resuscitation with hypertonic saline (2,400 mOsm/liter) during hemorrhagic shock. *Circ Shock* 1984; 13:149-159.
31. Schmall LM, Muir WW, Robertson JT. Haemodynamic effects of small volume hypertonic saline in experimentally induced haemorrhagic shock. *Equine Vet J* 1990; 22:273-277.
32. Younes RN, Aun F, Ching CT, *et al.* Prognostic factors to predict outcome following the administration of hypertonic/hyperoncotic solution in hypovolemic patients. *Shock* 1997; 7:79-83.
33. Rotstein OD. Novel strategies for immunomodulation after trauma: revisiting hypertonic saline as a resuscitation strategy for hemorrhagic shock [In Process Citation]. *J Trauma* 2000; 49:580-583.
34. Sauaia A, Moore FA, Moore EE, *et al.* Epidemiology of trauma deaths: a reassessment. *J Trauma* 1995; 38:185-193.
35. Moore FA, Moore EE. Evolving concepts in the pathogenesis of postinjury multiple organ failure. *Surg Clin North Am* 1995; 75:257-277.
36. Bevilacqua MP, Nelson RM. Selectins. *J Clin Invest* 1993; 91:379-387.
37. Etzioni A. Integrins--the glue of life. *Lancet* 1999; 353:341-343.
38. Ahmed N, Christou N. Systemic inflammatory response syndrome: interactions between immune cells and the endothelium. *Shock* 1996; 6:S39-42.
39. Partrick DA, Moore FA, Moore EE, Barnett CC, Jr., Silliman CC. Neutrophil priming and activation in the pathogenesis of postinjury multiple organ failure. *New Horiz* 1996; 4:194-210.
40. Spain DA, Kawabe T, Keelan PC, Wilson MA, Harris PD, Garrison RN. Decreased alpha-adrenergic response in the intestinal microcirculation after "two-hit" hemorrhage/resuscitation and bacteremia. *J Surg Res* 1999; 84:180-185.
41. Ciesla DJ, Moore EE, Gonzalez RJ, Biffl WL, Silliman CC. Hypertonic saline inhibits neutrophil (PMN) priming via attenuation of p38 MAPK signaling. *Shock* 2000; 14:265-269; discussion 269-270.
42. Thiel M, Buessecker F, Eberhardt K, *et al.* Effects of hypertonic saline on expression of human polymorphonuclear leukocyte adhesion molecules. *J Leukoc Biol* 2001; 70:261-273.
43. Angle N, Cabello-Passini R, Hoyt DB, *et al.* Hypertonic saline infusion: can it regulate human neutrophil function? *Shock* 2000; 14:503-508.
44. Khwaja KA, Gholipuor C, Pascual JL, *et al.* Hypertonic Saline in Humans Reduces L-Selectin Shedding, Trauma Association of Canada, Whistler, British Columbia, Canada, 2002.
45. Angle N, Hoyt DB, Cabello-Passini R, Herdon-Remelius C, Loomis W, Junger WG. Hypertonic saline resuscitation reduces neutrophil margination by suppressing neutrophil L selectin expression. *J Trauma* 1998; 45:7-12; discussion 12-13.
46. Hanger CC, Wagner WW, Jr., Janke SJ, Lloyd TC, Jr., Capen RL. Computer simulation of neutrophil transit through the pulmonary capillary bed. *J Appl Physiol* 1993; 74:1647-1652.
47. Yodice PC, Astiz ME, Kurian BM, Lin RY, Rackow EC. Neutrophil rheologic changes in septic shock. *Am J Respir Crit Care Med* 1997; 155:38-42.
48. Hogg JC. Neutrophil kinetics and lung injury. *Physiol Rev* 1987; 67:1249-1295.
49. Doyle NA, Bhagwan SD, Meek BB, *et al.* Neutrophil margination, sequestration, and emigration in the lungs of L- selectin-deficient mice. *J Clin Invest* 1997; 99:526-533.
50. Kubo H, Doyle NA, Graham L, Bhagwan SD, Quinlan WM, Doerschuk CM. L- and P-selectin and CD11/CD18 in intracapillary neutrophil sequestration in rabbit lungs. *Am J Respir Crit Care Med* 1999; 159:267-274.
51. Carlson RH, Gabel CH, Chan SS, Austin RH, Brody JP, Winkleman JW. Self-Sorting of White Blood Cells in a Lattice. *Physical Review Letters* 1997; 79:2149-2152.
52. Saetzler RK, Jallo J, Lehr HA, *et al.* Intravital fluorescence microscopy: impact of light-induced phototoxicity on adhesion of fluorescently labeled leukocytes. *J Histochem Cytochem* 1997; 45:505-513.

Preface to manuscript #4

Thus, in two hit conditions hypertonic saline resuscitation of hemorrhagic shock attenuates early in vivo PMN adhesion to endothelium and reduces its late transmigration and sequestration in lung tissue. Clearly, persistent attenuation of histologic PMN margination and transmigration up to a day after HTS resuscitation would suggest that EC/PMN interactions remain altered despite return to normotonicity. Yet, some in vitro reports suggest that HTS effects on neutrophil adhesion molecules may be transient, lasting less than a day. Could this prolonged HTS effect relate more to endothelial expression of adhesion receptors? By the same token, the above post mortem findings and those of others demonstrate less lung edema a day after HTS resuscitation. Is this secondary to less interstitial edema incurred during the resuscitation phase or rather an enduring reduction in microvascular leakiness with greater vessel wall integrity by HTS? No in vivo evaluation of microvascular permeability is available to determine the duration of reduced vessel permeability with HTS resuscitation. Most importantly, do these reductions in EC/PMN interactions and in tissue edema translate into attenuated tissue injury after HTS resuscitation of hemorrhagic shock?

The fourth manuscript uses the previously established two-hit model to demonstrate persistent HTS attenuation of in vivo neutrophil adhesion and venular permeability a day after resuscitation. Simultaneously, MPO analysis demonstrates a concurrent reduction in lung neutrophil sequestration while blinded histologic analysis of lung and cremaster sections confirms a tendency to HTS mediated attenuations in tissue injury. Furthermore, this study shows improved survival with HTS resuscitation.

Manuscript #4

Hypertonic saline resuscitation of hemorrhagic shock: persistent in vivo attenuation of neutrophil-endothelial interactions and microvascular permeability one day after shock

José Pascual, MD, Lorenzo Ferri, MD, FRCP(C), Kosar Khwaja, MD, Betty Giannias, BSc, Shea T. Chia, MD, Renée Bernatchez, MSc, David Evans, MD, FRCS(C), René Michel, MD, FRCP(C) and Nicolas Christou, MD, FRCS(C), FACS

Presented in part at the 88th Annual Clinical Congress Surgical Forum of the American College of Surgeons, October 9, 2002, San Francisco, California.
Submitted for publication to the *American Journal of Physiology-Heart*, March 2004

Keywords: Two-hit theory, hemorrhagic shock, resuscitation, fluids, hypertonic saline, lung injury, histology, neutrophil, intravital microscopy, adhesion, permeability, myeloperoxidase, sICAM-1

Abstract

Introduction: Compared to Ringer's lactate (RL), hypertonic saline (HTS) resuscitation of hemorrhagic shock is known to diminish early endothelial/neutrophil (EC/PMN) interactions, reducing late PMN tissue accumulation. We hypothesized that in vivo attenuations of EC/PMN interactions by HTS persist a day after resuscitation and are accompanied by reduced vessel permeability, circulating sICAM-1 levels, lung injury and PMN accumulation.

Methods: Blinded intravital microscopy quantification of *in vivo* PMN/EC interactions and venular leakage (epifluorescence after 50mg/kg FITC-albumin) 22 hours post-resuscitation were evaluated on cremaster tissue in a two-hit murine hemorrhagic shock model (blood loss with 1 hour hypotension [40mmHg] followed 4 hours later by 1.5μg intra-tracheal LPS). At 23 hours, lung myeloperoxidase (MPO) assessed PMN accumulation, ELISA determined circulating sICAM-1 levels and blinded lung and cremaster histologic analysis ascertained tissue injury. Resuscitation groups were: **HTS** (4cc/kg 7.5%HTS + shed blood), **RL** (RL [2X shed blood volume] + shed blood), **sham** (surgery but no hemorrhage/resuscitation) and **controls** (no manipulation). Mortality was evaluated at 22 hours. Bonferroni corrected ANOVA, Chi square or the Kruskal-Wallis Test were used to determined significance.

Results: Survival in HTS animals was 81.8% vs 40.0% for RL counterparts ($p=0.025$). Compared to RL, HTS animals displayed 45% fewer rolling PMN, 70% fewer adherent PMN, 28% less venular leakage and 55% less MPO ($p<0.05$, all comparisons). sICAM-1 levels and histologic injury were also reduced in HTS animals.

Conclusions: Compared to RL, HTS resuscitation in hemorrhagic shock followed by an inflammatory insult attenuates PMN-EC interactions, vascular permeability and PMN accumulation up to 22 hours later imparting a survival advantage. HTS resuscitation has immunomodulatory benefits that persist beyond the hours following resuscitation and may be useful for reducing persistent PMN-mediated tissue injury in hemorrhagic shock patients.

The hemodynamic effects of hypertonic saline (HTS) in hemorrhagic shock have been extensively investigated. Several large, randomized, controlled, studies and subsequent meta-analyses have demonstrated that HTS is at least as safe and efficacious as standard isotonic resuscitation(1, 2). These and other studies have demonstrated improved hemodynamic parameters necessitating less fluid and blood product requirements with a tendency to improved survival, particularly in subgroups of surgical and head injured patients(3-6).

The polymorphonuclear neutrophil (PMN) appears to be a key element in the tissue destruction that occurs following standard large volume isotonic resuscitation of hemorrhagic shock(7, 8). Experimental 'two-hit' models, where hemorrhagic shock is subsequently followed by a second insult such as an infection illustrate well the key role neutrophils play in tissue injury leading to systemic inflammation and multiple organ dysfunction(9).

In physiologic conditions, the neutrophil undergoes an ordered and controlled sequence of steps to proceed from the microcirculation to the interstitial milieu (neutrophil rolling, adhesion to endothelial cells [ECs] and transmigration). Once it has reached the area of parenchymal invasion or injury the PMN destroys pathogens and tissue debris through degranulation and phagocytosis. In conditions of systemic inflammation, this orderly sequence may become inappropriately amplified and unbridled resulting in the intravascular release of proteinases and reactive oxygen species. This inadvertent release of toxic neutrophil byproducts may result in microvascular endothelial cell injury and tissue edema leading to organ malfunction(9).

Hypertonic saline has been shown to exert a profound influence on microcirculatory elements, particularly on neutrophils and endothelium. Both in vitro and in vivo studies have shown hypertonicity–mediated changes in the structure and function of neutrophils and endothelial cells(10-14). More importantly, the reduced expression of

adhesion molecules by hypertonic saline in these experiments has been substantiated by intravital microscopy (IVM) animal models demonstrating live reductions in venular interactions between neutrophils and endothelium shortly after HTS resuscitation(15-17).

Ex vivo evaluations of tissue injury, microvascular permeability, tissue bacterial contamination and tissue neutrophil sequestration have confirmed that HTS resuscitation of hemorrhagic shock confers end-organ benefits, well beyond the first few hours following resuscitation(18-20). Yet, to date no study has evaluated whether HTS-mediated alterations of in vivo EC/PMN interactions endure extended periods of time. Only a handful of studies report HTS mediated alterations in neutrophil or endothelial adhesion molecule expression beyond the immediate hours following resuscitation. Furthermore, no investigations have ever validated histologic reductions in neutrophil mediated tissue injury by demonstrating concurrent in vivo effects of hypertonic saline resuscitation at the same time interval.

We propose that, compared to hemorrhagic shock resuscitated with lactated Ringer's, resuscitation with hypertonic saline causes persistent blunting of neutrophil and endothelial activation and function beyond the first hours after resuscitation. We thus hypothesized that 22 hours after hemorrhagic shock resuscitation, HTS would not only diminish lung neutrophil sequestration and histologic injury but would also, noticeably reduce in vivo neutrophil interactions with endothelium and better maintain vascular integrity. To evaluate this hypothesis, we used our established two-hit murine model of resuscitated severe hemorrhagic shock followed by intratracheal administration of lipopolysaccharide (LPS). A day later, animals were evaluated for neutrophil-endothelial (PMN-EC) interactions and microvascular permeability using cremaster intravital microscopy. Animal serum was removed for soluble endothelial adhesion receptor assessment while lungs and cremaster were harvested for ex vivo evaluation of tissue

injury and neutrophil sequestration. Though not an end-point originally intended for study, mortality analysis was also determined at the time of animal sacrifice.

Materials and Methods

Reagents

Lactated Ringer's [RL] and normal saline [NS] solutions were purchased from Baxter Corporation (Toronto, Ontario), heparin (10 000 USP units/ml) from Organon Teknika (Toronto, ON), ketamine from Wyeth-Ayerst (Guelph, ON) and xylazine from Bayer (Etobicoke, ON). Potassium phosphate [KH_2PO_4], was from Merck & Co. (Montréal, QC), 3,3',5,5'-tetramethyl-benzidine [TMB], bovine fluorescein isothiocyanate (FITC)-labeled albumin and lipopolysaccharide [LPS, Escherichia coli 0127:B8] from Sigma Chemical Company (St-Louis, MO) while hexadecyltrimethyl-ammonium bromide [HTAB], N,N,-dimethyl formamide [DMF] and 30% hydrogen peroxide [H_2O_2] were from Fisher Scientific (Fair Lawn, NJ). Seventy percent glutaraldehyde was purchased from Canemco Inc. (St-Laurent, QC). Phosphate buffered solution [PBS], bicarbonate buffered solution [BBS] (NaCl 132 mM, KCl 4.7 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1.2 mM, NaOH 18mM, pH 7.4), 3% glutaraldehyde [GA] (70% stock diluted in 0.1M KH_2PO_4 [pH7.4]) and hypertonic saline [HTS] (7.5% NaCl in dH_2O) were constituted in our laboratory.

Animal model

Approval was obtained from the McGill University Animal Care Committee prior to experiments. CD1 male mice (Charles River: St-Constant, QC), 25-30gm, were fed *ad libitum* and housed in standard care facilities for 3-5 days prior to study. Acclimated mice were anaesthetized by intra-peritoneal injection (xylazine = 6.7mg/kg, ketamine =

13.4mg/kg) and the right carotid artery was cannulated with polyethylene (PE)-10 tubing. This catheter was used to monitor blood pressure continuously through a pressure transducer (Living Systems Instrumentation: Burlington, VT), for blood withdrawal and for administration of blood, resuscitation fluids, and intermittent 50µl boluses of xylazine/ketamine solution to maintain anaesthesia.

After a brief stabilization period following surgery, hemorrhagic shock was induced by withdrawing blood from the carotid artery over 8 to 12 minutes into a tuberculin syringe previously flushed with 25U heparin until mean arterial pressure (MAP) reached 40 mmHg. Hypotension was maintained for 60 minutes, by further blood withdrawals if MAP rose above 45 mmHg, or by re-infusions of withdrawn blood if MAP fell below 35 mmHg. After the hypotension period, animals were resuscitated with a given crystalloid regimen in addition to shed blood. The carotid artery was then ligated, the cervical incision closed and the animals returned to their cages.

Four hours after resuscitation, intraperitoneal anaesthesia was administered as above and the cervical incision was reopened. Pretracheal muscles were spread bluntly exposing the tracheal rings and 30µl LPS (50µg/ml) were injected into the trachea under direct vision using a 0.5cc insulin syringe. Again the cervical incision was closed, 2cc of normal saline were injected subcutaneously in the animal's flank for long-term hydration and the animal was again returned to its cage for the night. Animals were subsequently re-anaesthetized the next day, 22 hours after resuscitation to undergo intravital microscopy, and subsequent sacrifice by cervical dislocation for immediate organ harvesting. Sham animals underwent carotid and jugular cannulation as well as cremaster dissection but were neither hemorrhaged nor resuscitated. Control animals did not undergo any manipulation.

Study groups and experimental protocol

Thirty-four animals were randomized to one of three arms: **HTS:** Shock and resuscitation with 4cc/kg of 7.5% hypertonic saline immediately followed by all shed blood (n=8); **RL**: Shock and resuscitation with lactated Ringer's, (twice the volume of withdrawn blood) immediately followed by all shed blood (n=8) and **sham**: Carotid and jugular cannulation, cremaster dissection but neither hemorrhage nor resuscitation (n=9). Both resuscitation regimens have been demonstrated to adequately resuscitate mice after such hemorrhagic shock conditions(19, 21, 22). **Control** animals underwent no surgical manipulation (n=9). Sham animals were only evaluated for IVM while control animals were only evaluated for lung histology and myeloperoxidase (MPO) assay. Once resuscitation was completed, animals were returned to their cages and received intratracheal LPS 4 hours later as above. Twenty-two animals underwent cremaster intravital microscopy for determination of EC-PMN interactions (HTS:n=6, RL:n=7, sham:n=9) out of which 13 also underwent determination of in vivo venular permeability following administration of fluorescent albumin (HTS:n=5, RL:n=4, sham:n=4). Immediately after IVM, anaesthetized mice underwent cardiac puncture prior to sacrifice and blood was withdrawn for sICAM-1 determination (HTS:n=6, RL:n=5) as well as for chemistry and hematology analysis (HTS:n=3, RL:n=4, sham:n=5). The cremaster and both lungs were then harvested for cremaster histology (HTS:n=6, RL:n=4), lung histology (control:n=9, HTS:n=7, RL:n=6) and lung myeloperoxidase analysis (control:n=6, HTS:n=8, RL:n=8) to determine tissue PMN sequestration.

Intravital microscopy

Twenty-two hours after resuscitation study animals and sham counterparts were re-anesthetized as above and underwent right jugular vein cannulation with PE-10 tubing for anaesthesia and FITC-albumin administration. Cremaster preparation was then

conducted for intravital microscopy as described previously(23). Briefly, after the animal was immobilized by attachment of all four limbs, the cremaster muscle was exteriorized through a small scrotal incision, opened and dissected free of the testicle and epididymis. Using five-point fixation with 4-0 silk, the cremaster was splayed open and fixed to a Plexiglas stage. For the remainder of the experiment the animal was warmed under a radiant heat lamp and its exteriorized cremaster was continuously perfused with thermostat-controlled (37° C) BBS. The transparent stage was placed on a Nikon TE 300 inverted microscope (Nikon Canada: Montréal, QC), and live tissue microcirculation was imaged at 2120X magnification.

An optical Doppler velocimeter (Microcirculation Research Institute: College Station, TX) was used to directly measure central red blood cell velocity (V_{RBC}) in post-capillary venules (PCVs). Based on Newtonian definitions(24), mean vessel flow velocity (V_{MEAN}) was calculated from V_{RBC} using the formula: $V_{MEAN} = V_{RBC}/1.6$. To minimize intersample wall shear stress variability, only non-branching PCVs with a diameter of 20-40μm and a V_{MEAN} of 1.25–2.50 mm/sec were chosen. Wall shear rate (γ), in sec^{-1}, was calculated using the formula: $\gamma = 8 \times (V_{MEAN}/D_V)$, where D_V is the venular diameter measured directly off line using calipers. Shear stress was calculated using the formula: shear stress = 0.25 X shear rate.

Live microscopic footage of chosen post capillary venules was captured with a high definition black and white video camera (CCD High Performance Camera, COHU, San Diego, CA), transferred to a monitor (Trinitron Color monitor – SSM-14NE, Sony: Toronto, ON), and recorded on videotape with a video recorder (SVHS HrS3910u, JVC Company of America: Wayne, NJ). A video Time-Date Generator (model WJ-810, Panasonic: Toronto, ON) projected the time, date, and stopwatch function onto the monitor at all times.

Quantification of EC-PMN interactions

In each animal, seven-minute video footage of each of three different selected post capillary venules per animal were captured after a stabilization period following the completion of cremaster surgery. Another member of the laboratory without prior knowledge of treatment subsequently played back these recordings off line and quantified neutrophil-endothelial cell interactions. To avoid alteration of PMN adhesive properties, neutrophils were not labeled with fluorescent markers. Available evidence suggests that the vast majority of leukocytes visible in such IVM models are PMNs(25). Neutrophil rolling was defined as the number of neutrophils crossing a line perpendicular to the long axis of the vessel, that were moving at a rate slower than erythrocytes over a period of two minutes. Neutrophil adherence was defined as the number of cells stationary for a minimum of 30 seconds in a 100 μm length of venule during a five-minute period and pre-adherence was defined as the number of immobile neutrophils in the same 100 μm vessel section at the initiation of counting. Total neutrophil adherence was the sum of neutrophil adherence and pre-adherence for a given vessel footage.

Fluorescent quantification of vascular permeability

Following the completion of video recordings, animals underwent additional fluorescent IVM. Fifty mg/kg of fluorescein isothiocyanate (FITC)–labeled bovine serum albumin was injected intravenously through the jugular catheter. Five minutes later, epiluminescence microscopy was performed with the same inverted microscope using a high-pressure mercury fluorescence lamp (HB 10103AF, Nikon Canada: Toronto, Canada). Moving images captured by the COHU video camera, were transferred to a computer (Dimension XPS B866r, Dell Canada: Toronto, Canada) and recorded by a frame grabber from Scion Image processing software (Scion Image for Windows, Scion Corporation: Frederick, MD). Frozen digital images of fluorescent post-capillary venules

were analysed by software capable of determining gray levels [0 (black) to 256 (white)] in six equal areas within the venule (venular light intensity, I_v), as well as six separate equal areas in the perivenular space (perivenular light intensity, I_p) (Figure 1). Using the means of I_p and I_v measurements, vascular leakage (permeability index, PI) was calculated using the formula I_p / I_v =PI for each PCV. PI was determined in this way for three different venules in each animal which were then averaged to estimate the given animal's microvascular permeability index.

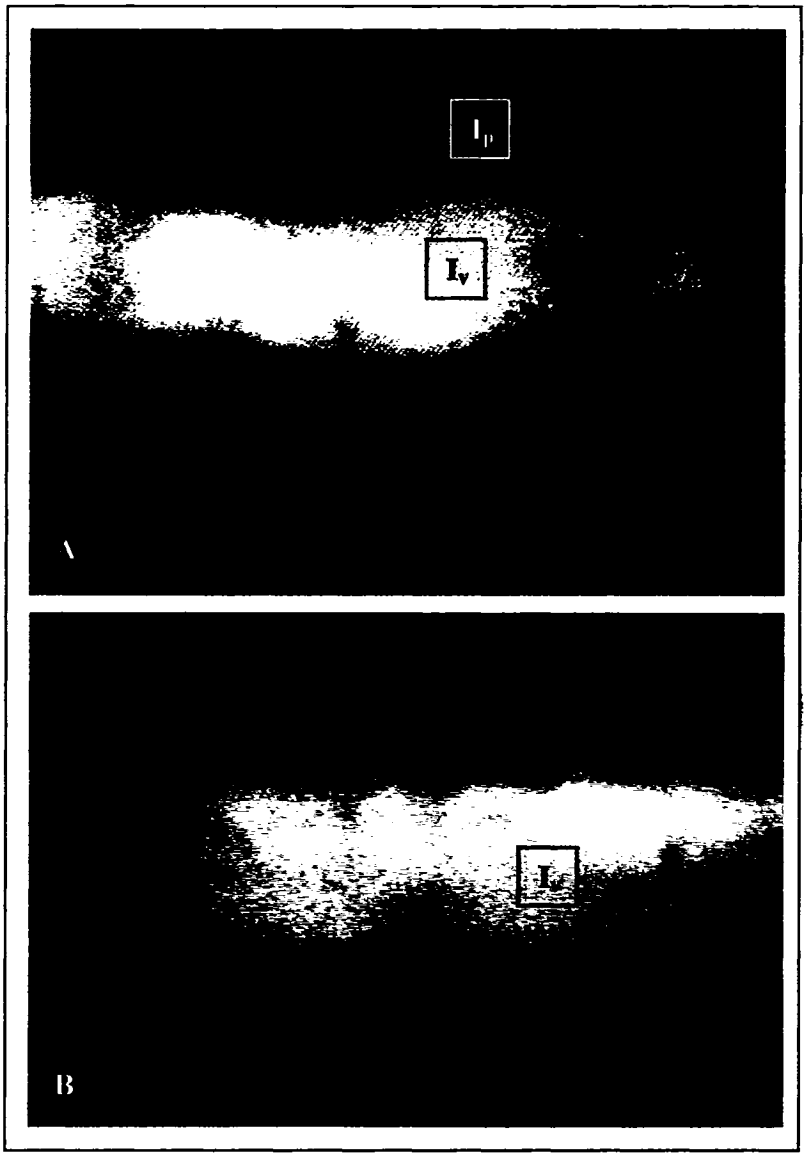

FIGURE 1: Representative frozen images of post capillary venules from fluorescent intravital microscopy footage contrasting a lower amount of FITC labeled albumin leakage (22 hours following HTS resuscitation) [A] and a greater amount of albumin leakage (same time interval following RL resuscitation) [B]. Ip (perivenular intensity) and Iv (venular intensity) represent typical areas evaluated for fluorescent light intensity.

Circulating soluble-ICAM-1 levels

Immediately following IVM, anesthetized animals underwent cardiac puncture for withdrawal of 1.0ml of venous blood. Five hundred microliters were immediately centrifuged at 500g for 10 minutes. Serum was extracted, placed in a labeled polypropylene vial and frozen at -80°C for

analysis at a later time. When sufficient samples had been collected, serum samples were thawed and analysed for soluble intercellular adhesion molecule-1 (sICAM-1) using an enzyme-linked immunosorbent assay (ELISA) microplate reader (ELx808-I, Bio-Tek instruments, Inc.: Winooski, VT) with a specific ELISA test kit for sICAM-1 (Endogen Inc.: Woburn, MA). Sandwich-type technique and analysis (KC4v3.1 software Bio-Tek instruments, Inc.: Winooski, VT) was conducted according to the manufacturer's instructions. Normal range for murine sICAM-1 has been reported at 10300 to 15000 ng/ml and the kit detection limit was 5ng/mL(26).

Systemic complete blood count and serum chemistry:

The remaining 500 µl of withdrawn blood was analysed for complete blood count (CBC) and differential, serum sodium and osmolality. The McGill University Health Center hematology and chemistry laboratory used an Advia Hematology System (model 120, Bayer Systems: Hialeah, FL) for CBC and differential, a Synchron Clinical System (model LX20PRO, Beckman Coulter: Fullerton, CA) for serum sodium and an Advanced Micro Osmometer (model 330, Advanced Instruments Inc.: Norwood, MA) for serum osmolality determinations.

Lung myeloperoxidase assay

Immediately prior to sacrifice, animal lungs were harvested for histology and myeloperoxidase (MPO) analysis. Anesthetized animals underwent sternotomy and the entire tracheobronchial tree was excised by sharp dissection. The right lung was dissected free of diaphragmatic and pleural tissue and subsequently weighed, while the left lung was prepared for histologic evaluation. The excised right lung sample was then homogenized (PT10 00 Polytron Homogenizer, Kinematica GMBH: Luzern, Switzerland) for 1 minute in 3cc of 10mM KH_2PO_4 [pH7.4] buffer and centrifuged at 12 000g for 20

minutes at 4° C. The supernatant was discarded and the pellet resuspended and homogenized in 50mM KH_2PO_4 [pH6.0] buffer containing 0.5% HTAB. The homogenate was kept frozen at -80° C and at a different time, it was thawed, rehomogenized for 1 minute and sonicated (Sonic Dismembrator Model 300, Fisher: Farmingdale, New York) at 40 W for 1 minute. After centrifugation as described above, the supernatant was diluted (X3) and 50µl were added to 350µl of 220mM KH_2PO_4 in 110mM NS and reacted 45 seconds later with 50µl 16mM TMB in DMF prior to blanking the spectrophotometer (DU 640, Beckman Coulter: Fullerton, California) at 37° C. The colorimetric reaction was then read at 655nm after the addition of 50µl of 3mM H_2O_2. The absorbance change over 180 seconds was used as a measure of MPO activity. Results were expressed as change in optical density (ΔOD) per gram of lung tissue.

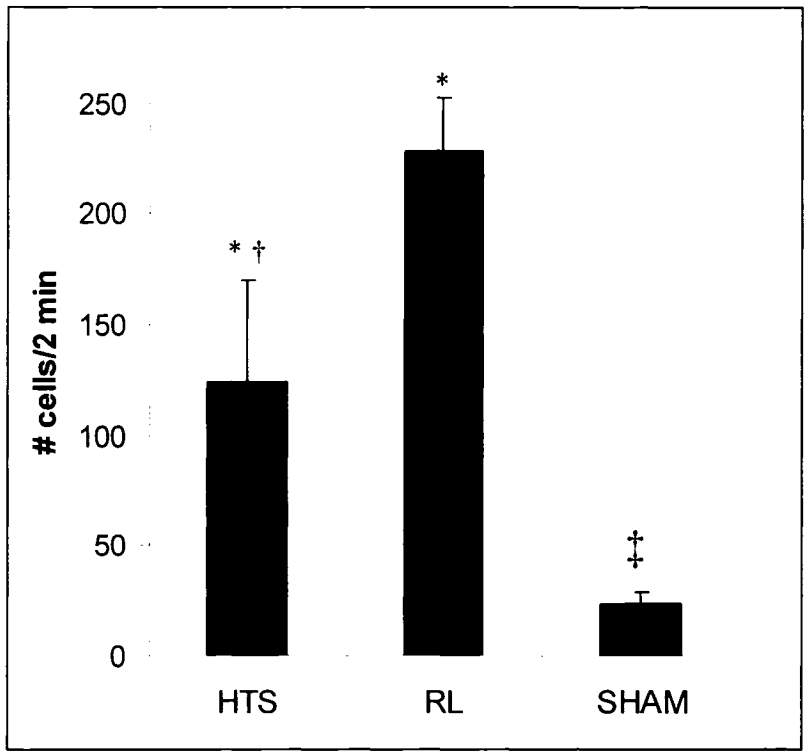

FIGURE 2: Neutrophil rolling (number of rolling neutrophils crossing a line perpendicular to the vessel axis which were traveling slower than red cells for a period of 2 minutes) was greater in RL animals than in HTS or sham animals 22 hours following resuscitation. (*p<0.05 vs. SHAM, †p<0.05 vs. RL, ‡p<0.001 vs.RL)

Histologic lung injury analysis

As described above, anesthetized animals underwent sternotomy and excision of their tracheobronchial tree. The left lung was flushed by instillation of 3% glutaraldehyde into the left mainstem bronchus using a 22-gauge angiocatheter and was subsequently fixed overnight. The following day, the lung was sliced; sections were processed using routine histologic techniques and embedded in paraffin. From these, 5 μm-thick sections were cut and stained with hematoxylin and eosin (H & E). The slides were subsequently graded by one of the authors (R.P.M.) without prior knowledge of treatment groups using a modified histologic injury score(27). The following histologic injury parameters were graded: marginated intravascular neutrophils, interstitial neutrophils, alveolar neutrophils, alveolar hemorrhage, interstitial edema and alveolar debris. For a given sample, each parameter received a score of zero (none), one (scant), two (moderate) or three (extensive). Total lung histologic injury scores were calculated as the sum of individual parameter scores for a given sample.

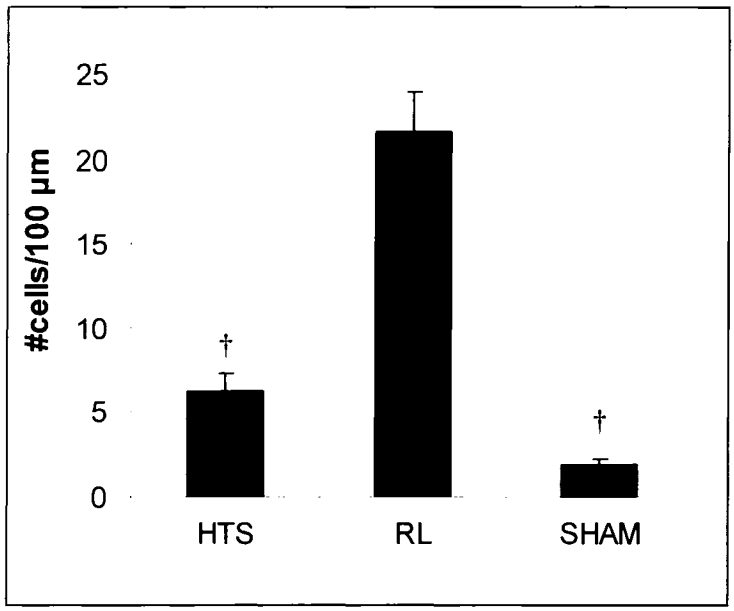

FIGURE 3:Total neutrophil adhesion (number of neutrophils stationary for a minimum of 30 seconds in a 100 μm length of venule during a five-minute period added to the number of immobile neutrophils at the initiation of counting) at 22 hours was not significantly different in HTS and sham animals but greater in RL counterparts. (†$p<0.001$ vs. RL).

Histologic cremaster injury analysis

Prior to sternotomy, anaesthetized animals had their exposed cremaster transected and placed in 3% glutaraldehyde for overnight fixation. As with lung tissue, cremaster samples were embedded, sliced and stained with H & E. The sections were also graded for histologic injury by one of the authors (R.P.M.), without prior knowledge of treatment groups. The following histologic injury parameters were graded in cremaster sections: marginated intravascular neutrophils, interstitial neutrophils, interstitial edema, and interstitial hemorrhage. For a given sample, each parameter received a score of zero (none), one (scant), two (moderate) or three (extensive). Total cremaster histologic injury scores were calculated as the sum of individual parameter scores for a given sample.

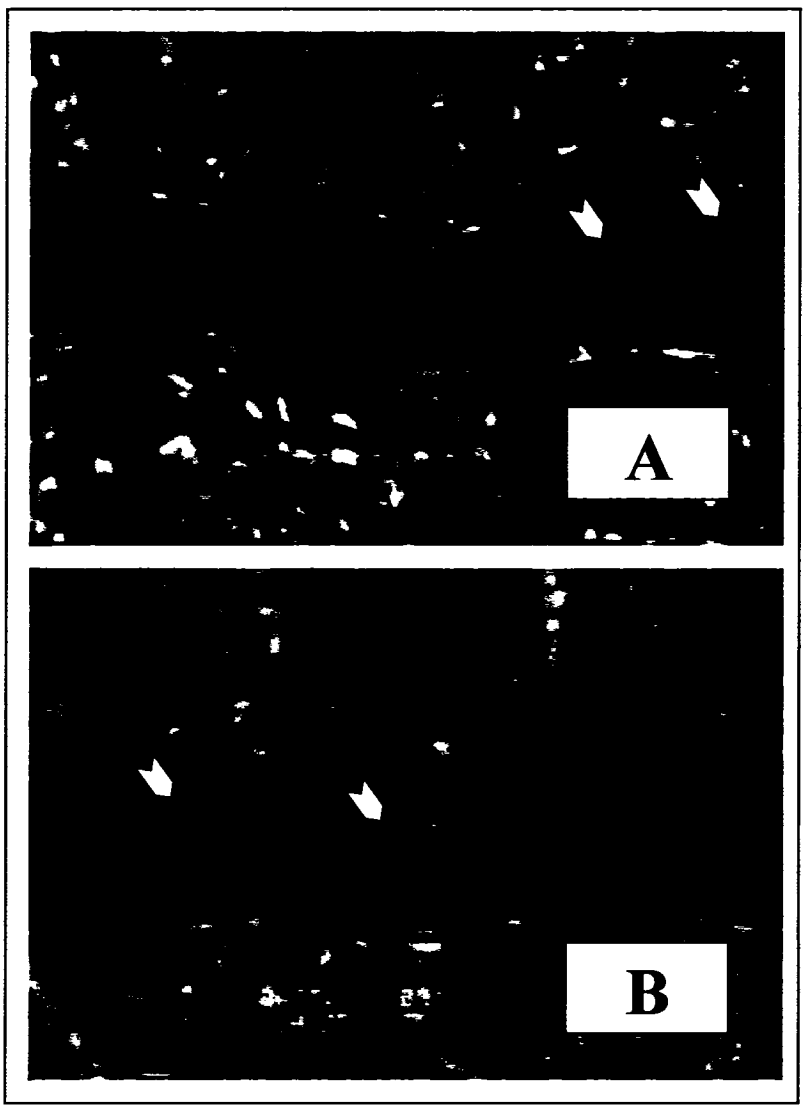

FIGURE 4: Representative images of post-capillary venules from cremaster intravital microscopy displaying few rolling and adherent neutrophils (arrow) from an animal resuscitated with HTS, 22 hours earlier (A) and another displaying greater numbers of adherent neutrophils (arrows) from an RL resuscitated animal (B) at the same interval.

Twenty two-hour survival analysis:

While survival was not an end point originally intended on, we evaluated whether animals were dead or alive at two different time points. Time intervals were as follows: immediately prior to LPS administration (4 hours after resuscitation) and at the time of IVM preparation (22 hours).

Statistical analysis:

All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Differences between groups were compared using analysis of variance (ANOVA) with Bonferroni correction using Systat 10 data analysis software (SPSS, Chicago, Illinois). When data was not normally distributed, the Kruskal-Wallis Test was applied to determine significance. Twenty four-hour survival was compared using Chi square and confidence interval analysis for differences in binomial proportions. Probabilities < 0.05 were deemed statistically significant.

Results

Vascular and hemodynamic mechanics

Withdrawn blood volume (mean 28.13 ± 2.81 cc/kg) did not differ between resuscitation groups but total fluid administered (blood +

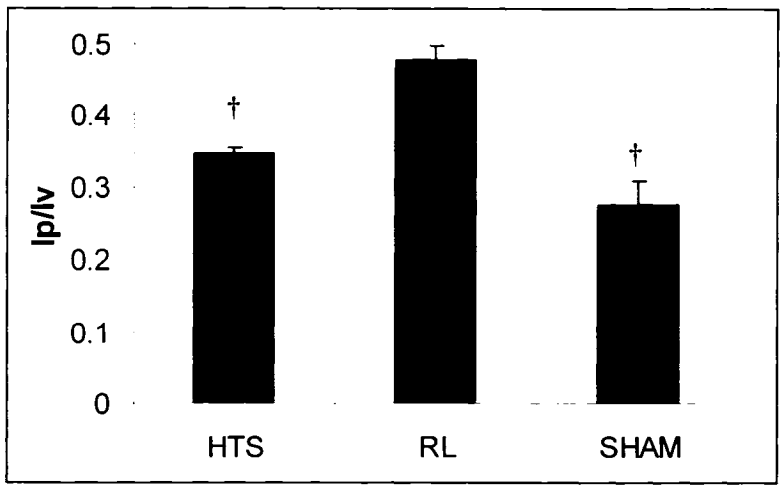

FIGURE 5: Venular permeability (Ip/Iv, where Ip is perivenular light intensity and Iv is venular light intensity) was greater in RL animals than either sham or HTS (†vs RL, $p<0.01$), 22 hours following resuscitation.

crystalloid) was significantly greater in RL-resuscitated animals compared to HTS animals (85.99 ± 3.18 vs. 31.64 ± 1.54 cc/kg, respectively, $p<0.001$). Systolic blood pressure before (mean 109.24 ± 2.48 mmHg), and after resuscitation (mean 109.38 ± 2.64 mmHg) was not different between the three groups but at the hypotension time interval both hemorrhaged groups had significantly lower blood pressure (mean 39.50 ± 1.33 mmHg) than sham animals (mean 113.44 ± 4.66 mmHg, $p<0.001$ vs either RL or HTS groups). Red blood cell velocity (V_{RBC}) (mean 2.84 ± 0.12 mm/sec), V_{MEAN} (mean 1.78 ± 0.07 mm/sec), shear rates (mean 0.53 ± 0.028 sec^{-1}) and shear stress (mean 0.13 ± 0.007 sec^{-1}) during intravital microscopy capture did not statistically differ between any of the study groups ($p>0.05$).

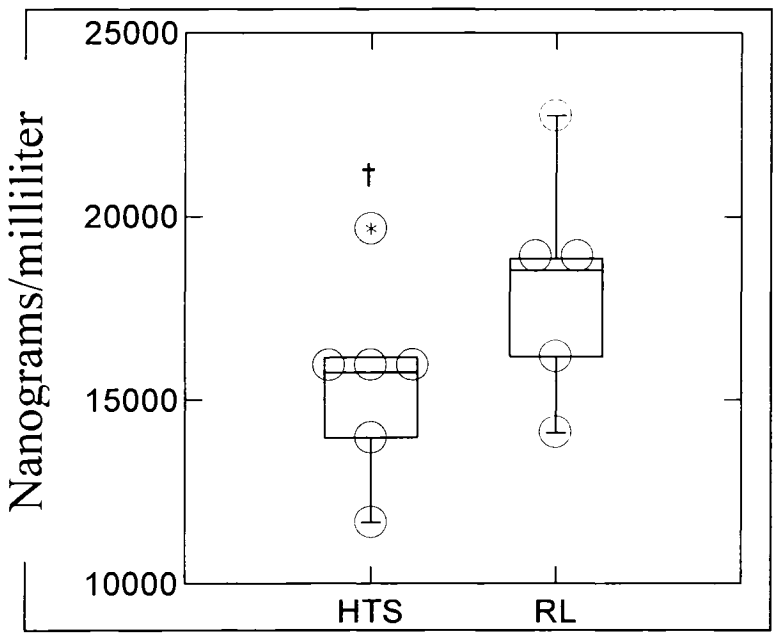

FIGURE 6: Plasma soluble ICAM-1 levels 23 hours after resuscitation were lower in HTS animals compared to RL counterparts. The statistical outlier (marked) was hypotensive prior to hemorrhage. If excluded from calculations † $p=0.047$ vs RL.

Hypertonic saline blunts neutrophil interactions with endothelium

Twenty-two hours after resuscitation neutrophil rolling was significantly lower in HTS animals compared to RL counterparts [123.7 ± 46.4 vs. 228.7 ± 23.7 neutrophils/100μm/2 minutes, respectively, $p=0.039$] (Figure 2). Both resuscitated groups displayed greater neutrophil rolling than sham animals [23.5 ± 5.6 leukocytyes/100μm/2 minutes, $p<0.05$ vs either RL or HTS groups]. Total neutrophil adhesion to EC was

similar in sham and HTS animals [1.9 ± 0.3 vs 6.3 ± 1.0 neutrophil/100μm, respectively, $p>0.05$] but significantly lower than in RL-resuscitated animals [21.6 ± 2.4 neutrophil/100μm, $p<0.001$ vs either sham or HTS groups] (Figure 3). There were no statistically significant differences in neutrophil rolling velocity between resuscitation groups (data not shown). Figure 4 contrasts an image displaying few adherent neutrophils (A) taken from footage of a HTS-resuscitated animal 18 hours after LPS administration with another showing several interacting neutrophils (B) taken from an RL animal at the same time interval. Thus, HTS animals displayed less in vivo neutrophil rolling and adhesion to endothelium twenty-two hours following resuscitation, to levels slightly higher than sham animals.

Macromolecular leakage is reduced in HTS-resuscitated animals

Vascular permeability (PI) was slightly higher in HTS than in sham mice (34.5 % ± 1.0 and 27.4 % ± 3.4, respectively, $p>0.05$) but both groups displayed significantly less leakage than RL mice (47.8 % ± 1.9, $p<0.01$ vs either HTS or sham) (Figures 1 and 5). Therefore, concurrent to diminished neutrophil-endothelial interactions, post-capillary venular macromolecular leakage was lower with HTS than RL 22 hours after resuscitation.

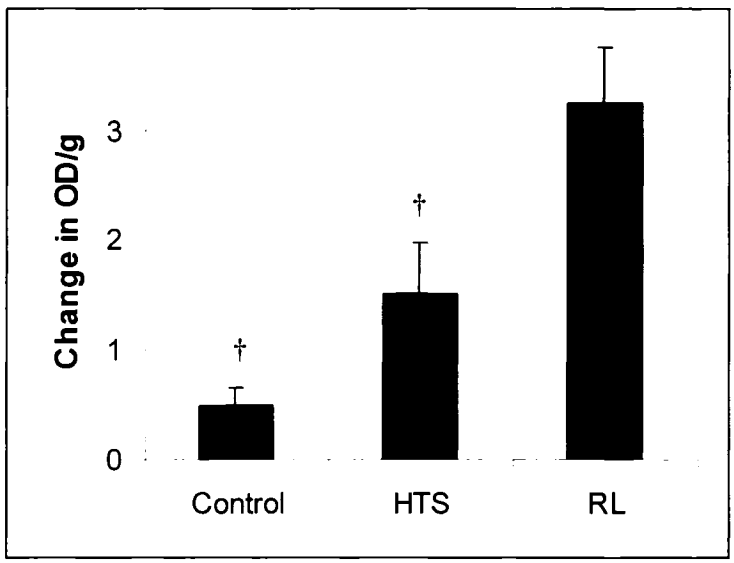

FIGURE 7: The right lung was harvested for myeloperoxidase assay immediately following IVM, 23 hours after resuscitation. HTS animals displayed similar neutrophil sequestration as controls but lower levels than RL counterparts (†vs RL, $p<0.05$).

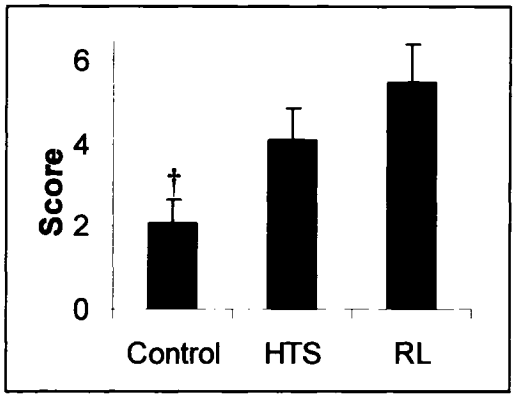

FIGURE 8: Lung injury scores 23 hours after resuscitation were higher in RL than in control animals (†vs RL, $p<0.01$). There was no significant difference between the scores of HTS and controls. Lung injury scores were calculated as the sum of individual parameter scores for: marginated intravascular neutrophils, interstitial neutrophils, alveolar neutrophils, alveolar hemorrhage, interstitial edema and alveolar debris. Each parameter was graded as follows: zero (none), one (scant), two (moderate) or three (extensive).

Serum sICAM-1 levels are reduced in HTS-resuscitated animals

Twenty-three hour sICAM-1 circulating levels in HTS resuscitated animals were lower than those in RL counterparts (15504 ± 1083 ng/ml and 18094 ± 1448 ng/ml, respectively, $p=0.14$) (Figure 6). Statistical analysis software found an HTS animal to be an outlier (marked) and reviewing the records, this animal was noted to be hypotensive prior to hemorrhage. (If excluded from calculations HTS: 14669 ± 845 ng/ml, $p<0.047$ vs. RL)

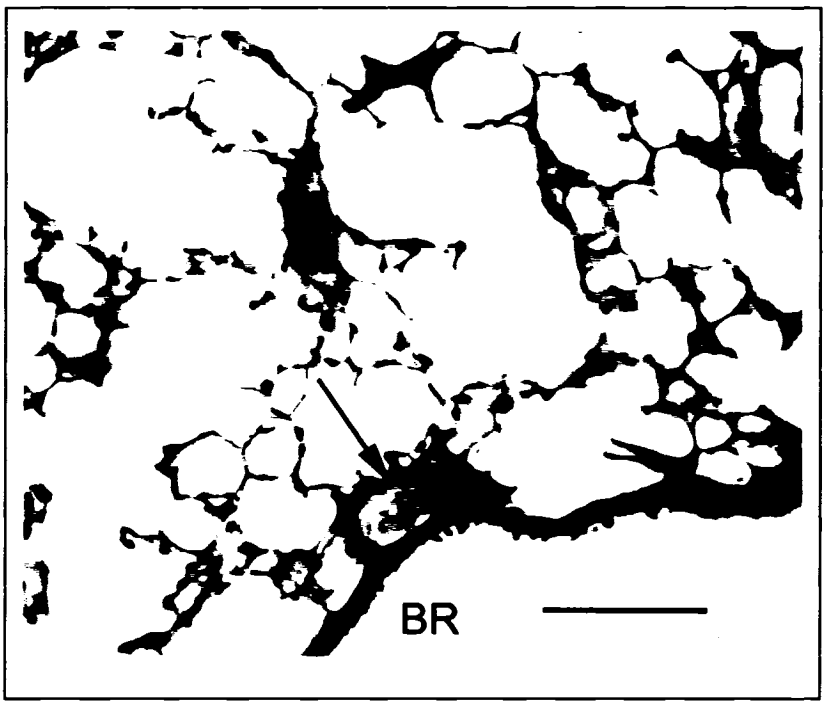

FIGURE 9: Medium power light micrograph of lung from control mouse showing normal alveoli, bronchiole (BR), and small pulmonary artery (arrow). Neutrophils are rare, interstitial and alveolar edema is absent. Hematoxylin & eosin stain. Bar indicates 100 µm.

Circulating Neutrophils are diminished with RL but not HTS; serum chemistry is unchanged.

The proportion of circulating leukocytes that were neutrophils was similar in HTS, sham and control animals, yet more than 45% lower in RL animals (4.03 %, 3.80 %, 3.90 % and 1.87 %, respectively) but this was not significant ($p>0.05$) (data not shown). Serum hemoglobin

(mean 106.8 ± 8.6 g/L), platelets (mean 630.4 ± 33.6 X 10^9/L), sodium (mean 148.0 ± 0.5 mmol/L) and osmolality (mean 315.5 ± 4.5 mmol/kg) levels did not differ between groups at 23 hours. Thus, while blood sodium levels were unchanged, RL animals tended to have fewer circulating neutrophils compared to HTS, sham and control animals.

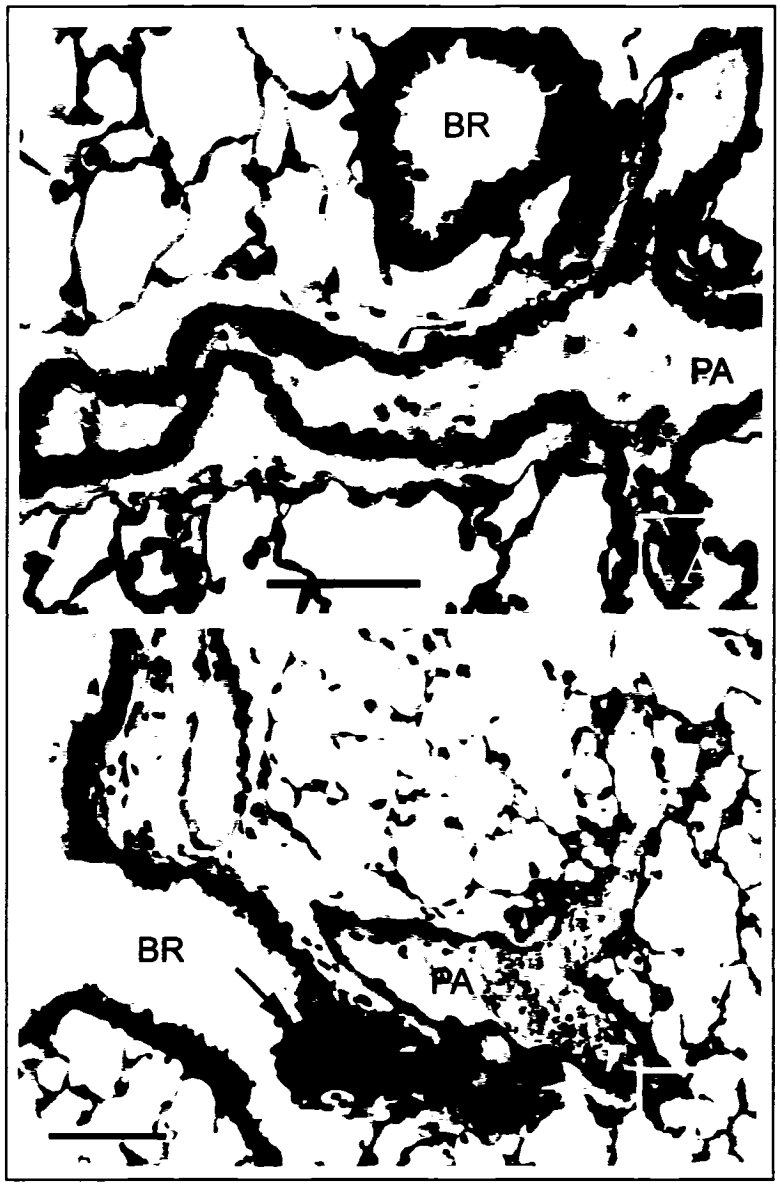

FIGURE 10: High power light micrographs showing details of lungs from mice resuscitated with RL, showing (A) a pulmonary artery (PA) with several marginated neutrophils in single file and a moderate amount of interstitial edema around the artery. Detail (B) showing numerous transmigrated neutrophils (arrow) clustered in the interstitial space between the bronchiole (BR) and the pulmonary arterial branch (PA). There is interstitial edema predominantly around the artery. Hematoxylin & eosin stain. Bars indicate 50 μm.

Neutrophil sequestration in lung is decreased in HTS-resuscitated animals 23 hours after resuscitation

Twenty-three hours after resuscitation, lung myeloperoxidase levels in HTS animals were similar to that of controls [1.51 ± 0.46 vs. 0.49 ± 0.17 ΔOD/g, p>0.05] but 55% lower than in RL animals [3.26 ± 0.50 ΔOD/g, p=0.02 vs. HTS, p=0.001 vs. control] (Figure 7). Thus, total neutrophil accumulation in lung tissue was reduced to near control levels at 23 hours following resuscitation with HTS, less

than half found in RL counterparts.

Decreased lung tissue injury following HTS resuscitation

Twenty-three hours after resuscitation, histologic lung injury scores were higher in RL animals [5.5 ± 0.9] compared with controls [2.0 ± 0.6, p=0.01 vs RL]. HTS animals had scores that did not differ significantly from control animals [4.1 ± 0.8, p>0.05 vs control]. HTS animals tended to have lower lung scores than RL animals but this was not significant [p>0.05] (Figure 7). This trend predominantly reflected the greater degree of histologic neutrophil margination in RL as compared to HTS animals [1.2 ± 0.20 vs. 0.5 ± 0.15, respectively, p=0.018]. Figure 8 shows typical lung tissue obtaining a low histologic injury score which was taken from a control animal 23 hours after resuscitation. Of note are the normal alveoli, bronchiole (BR), and pulmonary artery (arrow) with an absence of neutrophils, interstitial and alveolar edema. Figure 9 shows typical lung sections scored high for histologic injury which were taken from RL-resuscitated animals at the same time interval. Note the pulmonary artery (PA) with marginated neutrophils in single file and the moderate amount of interstitial edema around the artery (Figure 9A). Figure 9B shows numerous

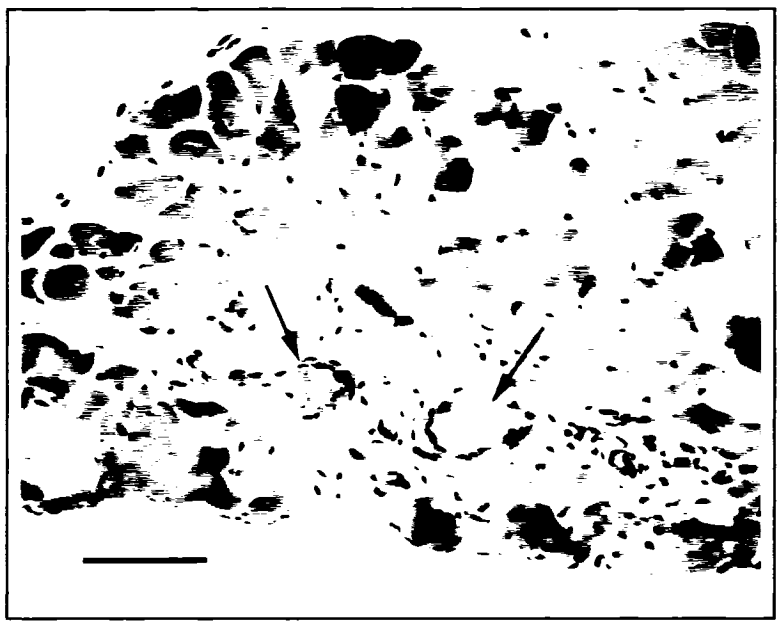

FIGURE 11: Medium power photomicrograph of cremaster muscle from mouse resuscitated with HTS showing normal skeletal muscle fibers supplied by small arteries (arrows). There are neither interstitial neutrophils nor any edema. Hematoxylin & eosin stain. Bar indicates 100 μm.

interstitial neutrophils surrounded by interstitial edema. Thus, 23 hours after resuscitation, lungs from HTS resuscitated animals displayed a reduced tissue injury compared to RL counterparts, which showed significantly worse scores than control animals.

HTS-resuscitated animals display less cremaster injury

Twenty-three hours after resuscitation, cremaster injury scores tended to be higher in RL [3.8 ± 1.1] compared to HTS animals [2.2 ± 0.6, $p>0.05$]. Figure 11 shows normal cremaster tissue taken from an animal 23 hours after HTS resuscitation, revealing normal skeletal muscle with few marginated neutrophils

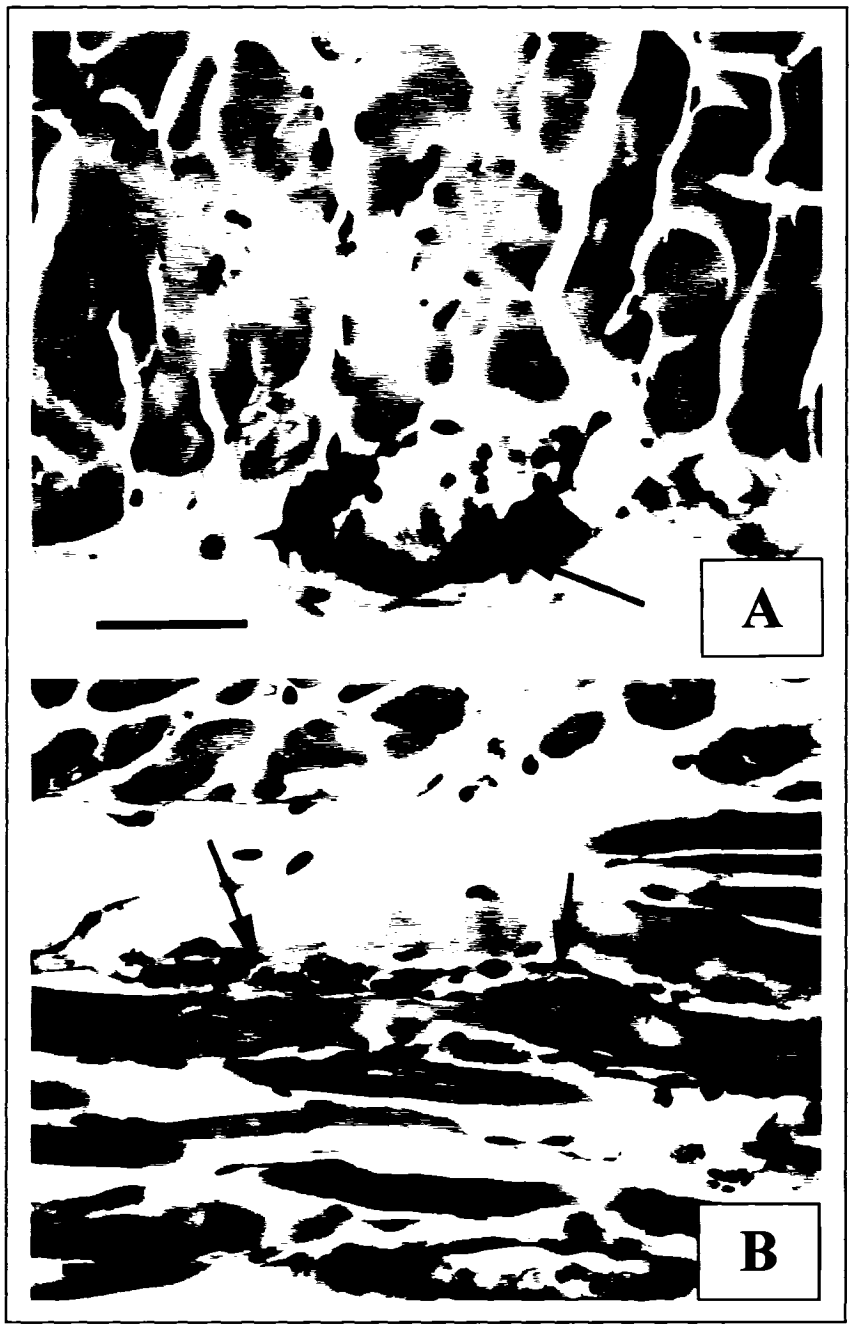

FIGURE 12: High power details of cremaster muscle from mouse resuscitated with RL showing a small vessel with prominent margination of neutrophils (arrow) (A). A second detail showing skeletal muscle fibers infiltrated by rows of neutrophils in the edematous interstitial space (arrows) (B). Hematoxylin & eosin stain. Bars indicate 50 μm.

and absent interstitial edema. Figure 12 shows details of cremaster sections from RL resuscitated animals. Prominent margination of neutrophils is seen in a small cremaster vessel (A) and skeletal muscle fibers are infiltrated by rows of neutrophils in edematous interstitium (B). Hence, as in the lungs, cremaster tissue tended to display less tissue injury in HTS animals compared to RL counterparts.

Twenty two-hour survival

Immediately prior to LPS administration survival of animals was recorded. At this time, 10 of 11 (90.0%) HTS-resuscitated animals were alive while 15 of 20 (75.0%) RL animals had survived (P>0.05). Twenty-two hours after resuscitation, prior to IVM analysis and sacrifice, 9 of 11 (81.8%) HTS and 8 of 20 (40.0 %) RL animals were still alive (p=0.025) (Figure 13).

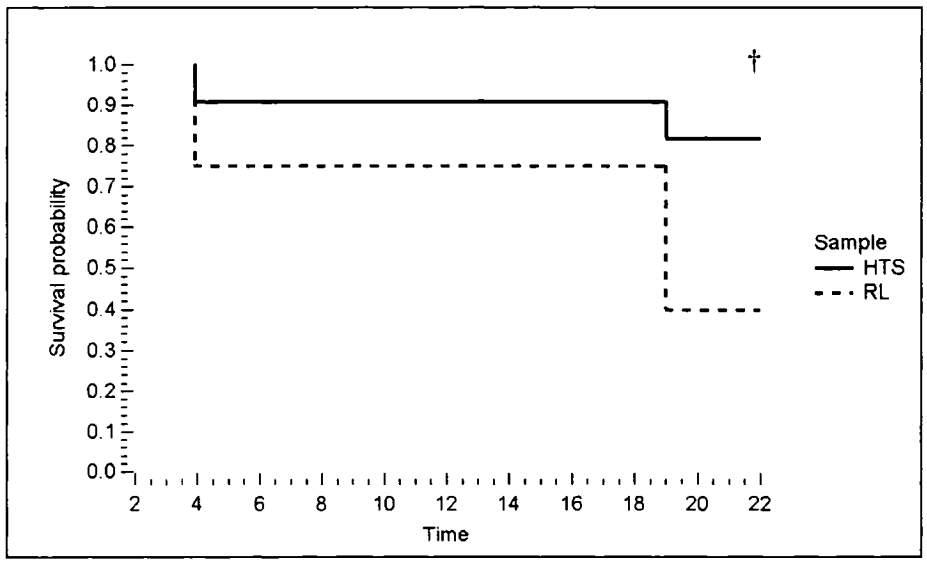

FIGURE 13: Animal mortality was recorded prior to LPS administration (T=4 hours) and the following day, immediately prior to IVM preparation (T=22 hours). At the second time point the HTS group demonstrated a higher survival than the RL group. (†p=0.025 vs RL)

Discussion:

For more than 30 years, administration of isotonic crystalloids has been the main treatment to restore effective circulating volume and re-establish cardiac and vascular hemodynamics in hypotensive conditions. Nonetheless, isotonic resuscitation often

requires the administration of large fluid volumes as the greater part of infused fluid readily leaks out of capillaries into the interstitial space. Such large fluid administration may lead to tissue edema, most evident in the lungs of resuscitated trauma victims who develop the acute respiratory distress syndrome (ARDS)(28).

Hypertonic crystalloid resuscitation, on the other hand, has the potential logistic benefits of greater portability and more rapid administration since much smaller quantities are required for rapid restoration of effective circulating volume. Contrarily to isotonic fluids, the fluid remains intravascular, drawing water from interstitial and intracellular spaces, making it an ideal alternative to avoid the systemic tissue edema of massive isotonic resuscitation. Several large, multicentric, randomized, controlled trials were launched in the early 1990s to evaluate whether this perceived advantage would improve the mortality and morbidity of hypotensive trauma patients(6, 29, 30). These and other trials confirmed the efficacy and safety of hypertonic saline resuscitation, demonstrating advantages over standard isotonic crystalloid in certain subgroups of surgical and head injured patients(1, 4-6). Additionally, hypertonic resuscitation appeared to reduce complications of resuscitated hemorrhagic shock such as coagulopathies, renal failure and ARDS(6). These and other studies have lead certain institutions to recommend HTS as the first line resuscitative fluid for hemorrhagic shock casualties on the battlefield(31).

The detrimental effects of hemorrhagic shock followed by standard resuscitation are underscored in 'two-hit' conditions. The two-hit theory maintains that a major systemic insult to the host (such as severe hemorrhagic shock) renders the host vulnerable to a subsequent apparently innocuous second insult (such as a gastrointestinal bleed, an aspiration or a urinary tract infection), which precipitates the host into the systemic inflammatory response syndrome (SIRS), frequently resulting in organ dysfunction and death(32). Experimental models emulating two-hit conditions

have implicated different immune cells in the over-exuberant host response that leads to systemic inflammation and the multiple organ dysfunction syndrome (MODS). The polymorphonuclear neutrophil (PMN), an essential member of the innate, non-specific immune system, appears to play a key role in the host tissue injury that occurs with systemic inflammation(7, 33).

In physiologic conditions, neutrophil phagocytosis and degranulation (the release of proteases and reactive oxygen species [ROS]) are part of the host's first line of defence against injury or pathogen invasion. To access tissue injury debris and invading pathogens, circulating neutrophils must undergo a sequence of controlled and organized steps to depart the intravascular space. First marginating out of central vessel flow, neutrophils interact weakly with endothelial cells through surface selectins (L, E and P) undergoing a torsional deceleration, which results in their rolling along the surface of the vessel wall(34). This close cellular proximity allows for stronger bonds to develop between neutrophil β-integrins (CD11a/CD11b) and endothelial surface adhesion molecules of the immunoglobulin superfamily (ICAM-1, ICAM-2)(35). These latter interactions result in the complete arrest of the neutrophil on the vessel wall (neutrophil adhesion), a critical step for neutrophil transmigration. Subsequent transit of the neutrophil between endothelial cells to the interstitial milieu involves other, less well characterised adhesion molecules (platelet endothelial cell adhesion molecule [PECAM] and others). Once in the interstitium, the PMN migrates down a chemotactic gradient to reach and destroy pathogens or engulf tissue debris(36).

This well-organized sequence of steps is thought to become generalized and aberrant in two hit conditions creating global inflammation. No longer under adequate control, neutrophil/endothelial interactions become over-exuberant and unbridled, causing the indiscriminate release of ROS and proteinases which damage the vessel wall, increasing microvascular permeability, tissue edema and organ injury(37). The

susceptibility of trauma patients to develop ARDS in the days that follow the initial injury attests to the particular vulnerability of the pulmonary system to systemic inflammation occurring in two-hit conditions(38).

Recent evaluations of hypertonic saline have demonstrated significant immune modulating effects on neutrophils and endothelial cells. In vitro hypertonicity blunts neutrophil cytotoxicity(39), phagocytosis(40, 41) and degranulation(42, 43). In vivo animal models of burns, pancreatitis and ischemia/reperfusion have further demonstrated HTS-related attenuation of tissue bacterial invasion as well as lung injury and sequestration of neutrophils(44-47). In vivo hemorrhagic shock models have also demonstrated diminished neutrophil oxidative burst as well as neutrophil sequestration and transmigration in lung(18, 22). Moreover, two-hit hemorrhagic shock models have also shown HTS-related reductions in tissue bacterial contamination, pulmonary neutrophil sequestration and transmigration as well as pulmonary capillary leakage several hours following resuscitation(11, 19, 20). Furthermore, these and other two-hit animal models correlate diminished neutrophil sequestration with attenuated tissue injury in lung and liver following HTS resuscitation.

The current study concurs with these results, demonstrating a trend to improved histologic injury in lung and cremaster 23 hours following resuscitation with HTS. More importantly, it provides novel in vivo confirmation of these long-term ex vivo results by revealing half the rolling and a third the adherent neutrophils in HTS animals compared to RL counterparts, at the same time interval. These findings were also corroborated by myeloperoxidase tissue levels, which concurrently demonstrated 54% less lung neutrophil sequestration in HTS than RL resuscitated animals. Furthermore, histologic score breakdown into component parameters substantiated in vivo attenuations in EC/PMN interactions by identifying 60% fewer marginating neutrophils (histologic equivalent of EC/PMN interactions) in the pulmonary and cremaster vessels of HTS

animals at the same time interval. In conjuction with our previous studies demonstrating in vivo attenuations of EC/PMN interactions by HTS occuring immediately following resuscitation and shortly after we can conclude that HTS attenuates early PMN interactions with endothelium, an effect which persists well beyong the initial post-resuscitative phase and which is associated with diminished lung injury and PMN sequestration at 24 hours.

Several hemorrhagic shock models (one and two-hit) have demonstrated diminished endothelial and neutrophil adhesion molecule expression with HTS resuscitation(10, 11, 13, 48, 49). In particular, ICAM-1 expression appears to be significantly influenced by hypertonicity. Both, ICAM-1 protein and mRNA expression was reduced in endothelial monolayers following in vitro exposure to hypertonic media(14). In the same study, hepatic ICAM-1 mRNA was attenuated in an in vivo hepatic ischemia/reperfusion model if pre-treatment with HTS instead of NS was employed. In another study, Sun and colleagues found elevated lung and spleen ICAM-1 protein and mRNA elevations with RL resuscitation of hemorrhagic shock which were not seen when hypertonic saline was used(13). In another two-hit model of hemorrhagic shock followed by intratracheal LPS, lung ICAM-1 expression was reduced by 50% in HTS animals compared to RL counterparts 7 hours after hemorrhage(11). Hypertonic effects on soluble ICAM-1, which is shed following severe endothelial activation has never been evaluated previously. Soluble ICAM-1, a circulating marker of endothelial activation has beeen emerging as an indicator of systemic inflammation. Several human studies have demonstrated sICAM-1 elevations with systemic inflammation, linking higher levels to worse outcomes. In patients with pancreatitis(50), aortic aneurysm repair(51), sepsis(52, 53) and trauma(54, 55), elevated levels of sICAM-1 were correlated with severity of disease, the development of multiple organ failure and even death. In the current study, 23-hour sICAM-1 levels were not evaluated in animals that

died prior to completing the study protocol. Due to a 60% mortality rate with RL resuscitation, levels were measured in only 8 animals. Despite this bias, sICAM-1 levels were 15% higher in RL than HTS animals. If dead animals had also been evaluated, perhaps the difference may have been much greater.

The advent of intravital microscopy (IVM) has permitted live observation of the microcirculation, enabling investigators to confirm that HTS not only attenuates PMN and EC adhesion molecules, but their in vivo interactions as well. Different burn, trauma and ischemia/reperfusion animal models employing IVM, have shown fewer PMN-EC interactions with HTS infusions(56-59). Similarly, single hit hemorrhagic shock IVM models have shown decreases in neutrophil rolling and adhesion to endothelium early on after hypertonic saline resuscitation(15-17). Our group previously reported that, in a similar two-hit IVM model of hemorrhagic shock followed by mimicked infection, HTS attenuated 5-hour neutrophil adhesion to endothelium and subsequently decreased 24-hour ex vivo lung neutrophil sequestration, margination and transmigration(20). The present study restates late ex vivo reductions in neutrophil sequestration, transmigration and tissue injury, but additionally, reveals continued in vivo attenuation of EC/PMN interactions one day after resuscitation with HTS.

The duration of HTS immune modulation remains ambiguous. Some investigators have suggested that HTS effects last only as long as the PMN is in a hyperosmolar milieu. In one study, in vitro experiments demonstrated that activated PMNs previously exposed to hypertonic media for 5 minutes displayed reduced CD11b expression and cytotoxicity (elastase and superoxide release) which returned to normal once normotonicity was restored(12). On the other hand, another study also evaluating in vitro CD11b expression by neutrophils exposed to hypertonicity found that PMN incubation in hypertonic media had to last much longer than 5 minutes, requiring a minimum of 4 hours before eliciting a blunted upregulation of CD11b(60). Unfortunately,

the in vitro nature of these studies excludes critical contributions from multiple other elements of the microcirculatory milieu, known to profoundly influence physiologic and pathologic neutrophil function and structure (endothelial cells, lymphocytes, macrophages, complement, cytokines, hormones etc.). Furthermore, in clinical conditions, while the initial sharp rise in serum sodium and osmolality after HTS infusion is short lived, a lesser degree of hyperosmolality is maintained for several hours later(11, 61). It is unclear how long this mild elevation in plasma tonicity lasts and when it returns to pre-infusion levels. An in vivo study using a two-hit rodent hemorrhagic shock model also investigated duration of HTS immune effects. The authors report that HTS resuscitation reduced broncoalveolar neutrophil sequestration and PMN CD11b expression shortly after resuscitation but that 18 hours later, both these parameters were indistinguishable to those found in RL resuscitated counterparts(60). Unfortunately, this evaluation only examined HTS effects on PMN CD11b and did not assess the endothelial counter ligand (ICAM-1) nor other adhesion molecules involved in neutrophil interactions with endothelium (selectins, CD11a, PECAM etc). Furthermore, the reported inability of HTS to maintain attenuated PMN tissue sequestration at this time interval is in contradistinction to several other studies demonstrating HTS-mediated such attenuations persisting for up to 72 hours after infusion(18-20).

Several authors have reported how the ability of HTS to attenuate neutrophil sequestration and transmigration into tissue persists well beyond the hours that follow resuscitation. In one hemorrhagic shock study, HTS resuscitation resulted in reductions of marginated (histology) and transmigrated (histology and bronchoalveolar lavage PMN) neutrophils in lung 24 and 72 hours following resuscitation(18). Another study using a two-hit model of hemorrhagic shock followed by cecal ligation and puncture resulted in fewer marginated and interstitial PMN (included as part of lung injury scores) at 24 hours if HTS had been used instead of RL(19). In the current study, this persistence of

attenuated histologic neutrophil-endothelial interactions one day after resuscitation has been demonstrated histologically after observing in vivo HTS-resuscitated animals with 55% the rolling and more strikingly, 29% the adherent neutrophils found in RL counterparts.

End organ benefits including less tissue leukosequestration, edema and injury are also known to last beyond the acute hyperosmolar phase that follows HTS resuscitation. In pancreatitis and ischemia/reperfusion models HTS resuscitated animals exhibit persistent reductions in lung leukosequestration, edema and macromolecular vascular leakage at 24 and 72 hours(45, 46). Coimbra and colleagues have demonstrated that mice subjected to hemorrhage and subsequent cecal ligation and puncture (CLP) continued to show improved lung and liver injury one day after HTS resuscitation(19). One hit hemorrhagic shock models also report persistent histopathologic improvements with hypertonic saline at 24 hours and beyond(18, 22). We additionally demonstrated in vivo vascular leakage to be 28% less with HTS resuscitation than with RL at 22 hours. Several ex vivo studies have previously demonstrated diminished tissue edema with HTS resuscitation in sepsis(62), burns(63), pancreatitis(45) and in ischemia/reperfusion(46). Intravital microscopic evaluation of macromolecular leakage also reveals HTS-related reductions in sepsis(64), burns(65) and ischemia reperfusion(59). Likewise, in hemorrhagic shock models, resuscitation with hypertonic regimens has shown diminished tissue edema(13, 18, 19, 22, 48) as well as decreased in vivo short term microvascular leakage of macromolecules(11, 15). The present study introduces in vivo substantiation that HTS decreases microvascular permeability up to a day following HTS resuscitation, confirming existing ex vivo evidence of reduced long term tissue edema. It further demonstrates evidence that these in vivo findings can be correlated with evidence of reduced EC/PMN interactions and PMN tissue sequestration by HTS using more traditional ex-vivo methods. The

mechanism by which this reduced accumulation of PMN in tissue occurs remains to be determined.

Taken together, these other studies coupled with findings of the present study suggest that HTS resuscitation continues to affect neutrophil-endothelial interactions and tissue neutrophil-mediated injury and edema beyond the hours that serum osmolality and tonicity are acutely elevated.

Our study also found no alterations in ratio of circulating neutrophils to leukocytes in HTS resuscitated animals 23 hours following resuscitation. As in control and sham counterparts, HTS animals had twice the ratio of circulating neutrophils as was found in RL counterparts. This could be explained in part by the greater PMN tissue sequestration in RL animals than in HTS mice as demonstrated by lung myeloperoxidase levels. Indeed, one could speculate that the greater proportion of intravascular neutrophils circulating freely in HTS animals corresponds to fewer interacting with endothelium at the vessel periphery at any given time interval.

Finally, HTS resuscitation resulted in twice the 22-hour survival as that found in RL animals. Unfortunately no autopsies were performed in dead animals which may have better revealed the cause of death. Coimbra and colleagues who also reported a fivefold survival advantage in hypertonic saline resuscitated animals using a two hit hemorrhage and abdominal infection model, did perform autopsies which demonstrated better containment of infection (abscess formation) and diminished hepatic and pulmonary histologic injury. We propose that the survival advantage associated with HTS resuscitation in our model originated from persistently attenuated neutrophil activation and interactions with endothelium resulting in less neutrophil mediated organ damage.

In summary the current study has brought novel in vivo evidence that hypertonic saline attenuates EC-PMN interactions and microvascular permeability which was confirmed ex vivo by reduced lung myeloperoxidase levels and less histologic injury well beyond the initial hours following resuscitation. Furthermore, these benefits were associated with improved survival with HTS resuscitation in this murine model. Additional clinical studies will be needed to establish these immune benefits in humans and to determine the immune advantages, if any, to administering hypertonic saline to trauma victims.

References to Manuscript #4

1. Wade CE, Kramer GC, Grady JJ, Fabian TC, Younes RN. Efficacy of hypertonic 7.5% saline and 6% dextran-70 in treating trauma: a meta-analysis of controlled clinical studies. Surgery 1997;122(3):609-16.
2. Bunn F, Roberts I, Tasker R, Akpa E. Hypertonic versus isotonic crystalloid for fluid resuscitation in critically ill patients. Cochrane Database Syst Rev 2000(4):CD002045.
3.Wade CE, Grady JJ, Kramer GC, Younes RN, Gehlsen K, Holcroft JW. Individual patient cohort analysis of the efficacy of hypertonic saline/dextran in patients with traumatic brain injury and hypotension. J Trauma 1997;42(5 Suppl):S61-5.
4. Holcroft JW, Vassar MJ, Perry CA, Gannaway WL, Kramer GC. Perspectives on clinical trials for hypertonic saline/dextran solutions for the treatment of traumatic shock. Braz J Med Biol Res 1989;22(2):291-3.
5. Vassar MJ, Perry CA, Gannaway WL, Holcroft JW. 7.5% sodium chloride/dextran for resuscitation of trauma patients undergoing helicopter transport. Arch Surg 1991;126(9):1065-72.
6.Mattox KL, Maningas PA, Moore EE, et al. Prehospital hypertonic saline/dextran infusion for post-traumatic hypotension. The U.S.A. Multicenter Trial. Ann Surg 1991;213(5):482-91.
7.Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. J Trauma 1995;39(3):411-7.
8. Patrick DA, Moore FA, Moore EE, Barnett CB, Silliman CC. Neutrophil priming and activation in the pathogenesis of postinjury multiple organ failure. New Horiz 1996;4(2):194-210.
9.Moore FA, Moore EE. Evolving concepts in the pathogenesis of postinjury multiple organ failure. Surg Clin North Am 1995;75(2):257-77.
10. Angle N, Hoyt DB, Cabello-Passini R, Herdon-Remelius C, Loomis W, Junger WG. Hypertonic saline resuscitation reduces neutrophil margination by suppressing neutrophil L selectin expression. J Trauma 1998;45(1):7-12; discussion -3.
11. Rizoli SB, Kapus A, Fan J, Li YH, Marshall JC, Rotstein OD. Immunomodulatory effects of hypertonic resuscitation on the development of lung inflammation following hemorrhagic shock. J Immunol 1998;161(11):6288-96.
12.Ciesla DJ, Moore EE, Biffl WL, Gonzalez RJ, Silliman CC. Hypertonic saline attenuation of the neutrophil cytotoxic response is reversed upon restoration of normotonicity and reestablished by repeated hypertonic challenge. Surgery 2001;129(5):567-75.
13.Sun LL, Ruff P, Austin B, et al. Early up-regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in rats with hemorrhagic shock and resuscitation. Shock 1999;11(6):416-22.
14. Oreopoulos GD, Hamilton J, Rizoli SB, et al. In vivo and in vitro modulation of intercellular adhesion molecule (ICAM)-1 expression by hypertonicity. Shock 2000;14(3):409-14; discussion 14-5.
15. Pascual JL, Ferri LE, Seely AJ, et al. Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces in vivo vascular leakage. Ann Surg 2002;236(5):634-42.

16. Bauer M, Marzi I, Ziegenfuss T, Seeck G, Buhren V, Larsen R. Comparative effects of crystalloid and small volume hypertonic hyperoncotic fluid resuscitation on hepatic microcirculation after hemorrhagic shock. Circ Shock 1993;40(3):187-93.
17. Corso CO, Okamoto S, Ruttinger D, Messmer K. Hypertonic saline dextran attenuates leukocyte accumulation in the liver after hemorrhagic shock and resuscitation. J Trauma 1999;46(3):417-23.
18.Angle N, Hoyt DB, Coimbra R, et al. Hypertonic saline resuscitation diminishes lung injury by suppressing neutrophil activation after hemorrhagic shock. Shock 1998;9(3):164-70.
19.Coimbra R, Hoyt DB, Junger WG, et al. Hypertonic saline resuscitation decreases susceptibility to sepsis after hemorrhagic shock. J Trauma 1997;42(4):602-6; discussion 6-7.
20. Pascual JL, Khwaja KA, Ferri LE, et al. Hypertonic Saline Resuscitation Attenuates Neutrophil Lung Sequestration and Transmigration by Diminishing Leukocyte-Endothelial Interactions in a Two-Hit Model of Hemorrhagic Shock and Infection. J Trauma 2003;54:In press.
21.Coimbra R, Junger WG, Hoyt DB, Liu FC, Loomis WH, Evers MF. Hypertonic saline resuscitation restores hemorrhage-induced immunosuppression by decreasing prostaglandin E2 and interleukin-4 production. J Surg Res 1996;64(2):203-9.
22.Murao Y, Hoyt DB, Loomis W, et al. Does the timing of hypertonic saline resuscitation affect its potential to prevent lung damage? Shock 2000;14(1):18-23.
23. Baez S. An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. Microvasc Res 1973;5(3):384-94.
24.House SD, Lipowsky HH. Leukocyte-endothelium adhesion: microhemodynamics in mesentery of the cat. Microvasc Res 1987;34(3):363-79.
25. Tangelder GJ, Janssens CJ, Slaaf DW, oude Egbrink MG, Reneman RS. In vivo differentiation of leukocytes rolling in mesenteric postcapillary venules. Am J Physiol 1995;268(2 Pt 2):H909-15.
26. Endogen I. Endogen Mouse soluble ICAM-1 (CD54) ELISA monograph. In. Woburn, Ma; 2000.
27. Bachofen M, Weibel ER. Structural alterations of lung parenchyma in the adult respiratory distress syndrome. Clin Chest Med 1982;3(1):35-56.
28.Pearce FJ, Lyons WS. Logistics of parenteral fluids in battlefield resuscitation. Mil Med 1999;164(9):653-5.
29.Younes RN, Aun F, Accioly CQ, Casale LP, Szajnbok I, Birolini D. Hypertonic solutions in the treatment of hypovolemic shock: a prospective, randomized study in patients admitted to the emergency room. Surgery 1992;111(4):380-5.
30.Vassar MJ, Perry CA, Holcroft JW. Prehospital resuscitation of hypotensive trauma patients with 7.5% NaCl versus 7.5% NaCl with added dextran: a controlled trial. J Trauma 1993;34(5):622-32; discussion 32-3.
31. Pope AFG, Longnecker. D. E. Editors. Fluid Resuscitation: State of the Science for Treating Combat Casualties and Civilian Injuries: National Academy Press; 1999.
32.Moore FA, Moore EE, Read RA. Postinjury multiple organ failure: role of extrathoracic injury and sepsis in adult respiratory distress syndrome. New Horiz 1993;1(4):538-49.
33.Partrick DA, Moore FA, Moore EE, Barnett CC, Jr., Silliman CC. Neutrophil priming and activation in the pathogenesis of postinjury multiple organ failure. New Horiz 1996;4(2):194-210.
34.Bevilacqua MP, Nelson RM. Selectins. J Clin Invest 1993;91(2):379-87.
35.Etzioni A. Integrins--the glue of life. Lancet 1999;353(9150):341-3.
36.Ahmed N, Christou N. Systemic inflammatory response syndrome: interactions between immune cells and the endothelium. Shock 1996;6(Suppl 1):S39-42.
37. Peitzman AB, Billiar TR, Harbrecht BG, Kelly E, Udekwu AO, Simmons RL. Hemorrhagic shock. Curr Probl Surg 1995;32(11):925-1002.
38. Sauaia A, Moore FA, Moore EE, et al. Epidemiology of trauma deaths: a reassessment. J Trauma 1995;38(2):185-93.
39.Gonzalez RJ, Moore EE, Ciesla DJ, Neto JR, Biffl WL, Silliman CC. Hyperosmolarity abrogates neutrophil cytotoxicity provoked by post- shock mesenteric lymph. Shock 2002;18(1):29-32.
40.Hampton MB, Chambers ST, Vissers MC, Winterbourn CC. Bacterial killing by neutrophils in hypertonic environments. J Infect Dis 1994;169(4):839-46.
41. Matsumoto T, van der Auwera P, Watanabe Y, et al. Neutrophil function in hyperosmotic NaCl is preserved by phosphoenol pyruvate. Urol Res 1991;19(4):223-7.
42.Ciesla DJ, Moore EE, Zallen G, Biffl WL, Silliman CC. Hypertonic saline attenuation of polymorphonuclear neutrophil cytotoxicity: timing is everything. J Trauma 2000;48(3):388-95.
43. Junger WG, Hoyt DB, Davis RE, et al. Hypertonicity regulates the function of human neutrophils by modulating chemoattractant receptor signaling and activating mitogen-activated protein kinase p38. J Clin Invest 1998;101(12):2768-79.
44.Assalia A, Bitterman H, Hirsh TM, Krausz MM. Influence of hypertonic saline on bacterial translocation in controlled hemorrhagic shock. Shock 2001;15(4):307-11.
45.Shields CJ, Winter DC, Sookhai S, Ryan L, Kirwan WO, Redmond HP. Hypertonic saline attenuates end-organ damage in an experimental model of acute pancreatitis. Br J Surg 2000;87(10):1336-40.

46. Shields CJ, Manning B, Winter DC, Wang JH, Kirwan WO, Redmond HP. Pulmonary injury due to ischemia - reperfusion is ameliorated by hypertonic saline infusion. In: First joint meeting Surgical Infection Society Surgical Infection Society - Europe; 2002 May 2-4, 2002; Westin Palace Hotel, Madrid, Spain; 2002. p. 151.
47.Shields CJ, Wang JH, Winter DC, Kirwan WO, Redmond HP. Hypertonic saline enhances host response to bacterial challenge by augmenting neutrophil intracellular superoxide formation. In: First joint meeting Surgical Infection Society Surgical Infection Society - Europe; 2002 May 2-4, 2002; Westin Palace Hotel, Madrid, Spain; 2002. p. 30.
48. Alam HB, Sun L, Ruff P, Austin B, Burris D, Rhee P. E- and P-selectin expression depends on the resuscitation fluid used in hemorrhaged rats. J Surg Res 2000;94(2):145-52.
49. Rizoli SB, Kapus A, Parodo J, Rotstein OD. Hypertonicity prevents lipopolysaccharide-stimulated CD11b/CD18 expression in human neutrophils in vitro: role for p38 inhibition. J Trauma 1999;46(5):794-8; discussion 8-9.
50.Kaufmann P, Demel U, Tilz GP, Krejs GJ. Time course of plasma soluble intercellular adhesion molecule-1 (sICAM-1) is related to severity of acute pancreatitis. Hepatogastroenterology 1999;46(28):2565-71.
51. Froon AH, Greve JW, Van der Linden CJ, Buurman WA. Increased concentrations of cytokines and adhesion molecules in patients after repair of abdominal aortic aneurysm. Eur J Surg 1996;162(4):287-96.
52.Jilma B, Blann A, Pernerstorfer T, et al. Regulation of adhesion molecules during human endotoxemia. No acute effects of aspirin. Am J Respir Crit Care Med 1999;159(3):857-63.
53.Sessler CN, Windsor AC, Schwartz M, et al. Circulating ICAM-1 is increased in septic shock. Am J Respir Crit Care Med 1995;151(5):1420-7.
54. Seekamp A, Jochum M, Ziegler M, van Griensven M, Martin M, Regel G. Cytokines and adhesion molecules in elective and accidental trauma-related ischemia/reperfusion. J Trauma 1998;44(5):874-82.
55. Gando S, Kameue T, Matsuda N, et al. Combined activation of coagulation and inflammation has an important role in multiple organ dysfunction and poor outcome after severe trauma. Thromb Haemost 2002;88(6):943-9.
56.Barone M, Jimenez F, Huxley VH, Yang XF. Morphologic analysis of the cerebral microcirculation after thermal injury and the response to fluid resuscitation. Acta Neurochir Suppl 1997;70:267-8.
57. Hartl R, Medary MB, Ruge M, Arfors KE, Ghahremani F, Ghajar J. Hypertonic/hyperoncotic saline attenuates microcirculatory disturbances after traumatic brain injury. J Trauma 1997;42(5 Suppl):S41-7.
58.Spera PA, Arfors KE, Vasthare US, Tuma RF, Young WF. Effect of hypertonic saline on leukocyte activity after spinal cord injury. Spine 1998;23(22):2444-8; discussion 8-9.
59. Nolte D, Bayer M, Lehr HA, et al. Attenuation of postischemic microvascular disturbances in striated muscle by hyperosmolar saline dextran. Am J Physiol 1992;263(5 Pt 2):H1411-6.
60. Rizoli SB, Kapus A, Parodo J, Fan J, Rotstein OD. Hypertonic immunomodulation is reversible and accompanied by changes in CD11b expression. J Surg Res 1999;83(2):130-5.
61.Angle N, Cabello-Passini R, Hoyt DB, et al. Hypertonic saline infusion: can it regulate human neutrophil function? Shock 2000;14(5):503-8.
62. Rabinovici R, Vernick J, Hillegas L, Neville LF. Hypertonic saline treatment of acid aspiration-induced lung injury. J Surg Res 1996;60(1):176-80.
63. Kinsky MP, Milner SM, Button B, Dubick MA, Kramer GC. Resuscitation of severe thermal injury with hypertonic saline dextran: effects on peripheral and visceral edema in sheep. J Trauma 2000;49(5):844-53.
64. de Carvalho H, Matos JA, Bouskela E, Svensjo E. Vascular permeability increase and plasma volume loss induced by endotoxin was attenuated by hypertonic saline with or without dextran. Shock 1999;12(1):75-80.
65.Barone M, Jimenez DF, Huxley VH, Yang XF. Cerebral vascular response to hypertonic fluid resuscitation in thermal injury. Acta Neurochir Suppl 1997;70:265-6.

Preface to manuscript #5

Thus, HTS resuscitation reduces tissue neutrophil accumulation and injury concomitantly attenuating in vivo endothelial neutrophil interactions and vascular leakage beyond the initial resuscitation phase.

Yet, it is unclear which of the multiple effects of HTS on the function and structure of neutrophils, endothelium and other cells is essential or most critical in reducing organ injury. Clearly, most late deaths in hemorrhagic shock victims occur secondary to systemic inflammation and organ injury. While several reports have demonstrated many immune alterations after HTS resuscitation, no study has conclusively shown which of these is directly responsible for HTS benefits in reducing lung injury. Is reduced EC or PMN adhesion molecule expression by HTS truly causative of attenuated lung injury or simply a concomitant but unrelated finding? Are fewer EC/PMN interactions simply associated with or are they essential for better histopathologic morphology in HTS resuscitated animals?

The fifth manuscript elucidates the importance of reduced adhesion molecule expression and fewer EC/PMN interactions found with HTS resuscitation. Through the artificial blockade of neutrophil adhesion in RL resuscitated animals we evaluated whether adhesion blockade is indeed an important mechanism by which HTS attenuates pulmonary injury. Live observation of the microcirculation in these animals confirms that the model is working and that the comparison is adequate. Surprisingly, RL supplementation with anti adhesion blockade does not reduce lung injury, on the contrary, tending to worsen histopathology compared to resuscitation with isotoic crystalloid alone. These findings indicate a less than essential role of the much discussed blunting of adhesion molecule expression and EC/PMN interactions by HTS resuscitation suggesting other functional effects of hypertoniciy as the basis of reductions in organ injury.

5.0 **Manuscript #5**

Immune Modulation of Hypertonic Saline in Hemorrhagic Shock: Blunting of Neutrophil Adhesion is not the Mechanism for Attenuated Lung Injury

José Pascual, MD, Lorenzo Ferri, MD, FRCP(C), Kosar Khwaja, MD, Betty Giannias, BSc, David Evans, MD, FRCS(C), René Michel, MD, FRCP(C) and Nicolas Christou, MD, FRCS(C), FACS

Submitted for publication to the *Journal of Immunology*, March 2004

Keywords: Two-hit theory, hemorrhagic shock, resuscitation, fluids, hypertonic saline, lung injury, histology, neutrophil, intravital microscopy, adhesion, permeability, myeloperoxidase, CD11b, L-selectin, monoclonal blockade,

Abstract

Compared to Ringer's lactate (RL), hypertonic saline (HTS) resuscitation of hemorrhagic shock diminishes endothelial cell (EC) and neutrophil (PMN) adhesion molecule expression and attenuates PMN mediated tissue injury. We hypothesized that hemorrhagic shock resuscitated with either RL supplemented with anti-adhesion monoclonal antibodies or HTS alone would be equally effective in reducing PMN activation, tissue sequestration and lung injury.

Methods: Murine PMNs activated in vivo by hemorrhagic shock resuscitated with shed blood and either 7.5% NaCl (4cc/kg) or RL (twice shed blood volume) were activated by LPS in vitro. Post-activation PMN L-selectin and CD11b were determined by flowcytometry. In separate experiments, blinded intravital microscopy quantification of *in vivo* PMN/EC interactions and venular leakage (epifluorescence after 50mg/kg FITC-albumin) 3 hours after resuscitation were evaluated on cremaster in a two-hit murine hemorrhagic shock model (blood loss with 1hour hypotension [40mmHg] followed by resuscitation and 1 hour later by 10 μg intra-tracheal LPS). Resuscitation was with HTS or RL (as above) supplemented with either monoclonal antibodies against CD11b & ICAM-1 (**HTSb,** n=9; **RLb**, n=9) or their isotype controls (**HTSn**, n=8; **RLn**, n=8). At 24 hours, animal activity was graded subjectively, lung myeloperoxidase (MPO) assessed PMN sequestration and lung and cremaster histologic analysis ascertained tissue injury.

Results: In vitro activation of primed HTS neutrophils demonstrated blunted upregulation of CD11b. Compared to RLn, HTSn and RLb equally reduced neutrophil adhesion (-74%*, -58%*, respectively) and lung neutrophil sequestration (-56%*, -47%*). HTSn was superior to RLb at reducing venular permeability (-42%*, -30%), and at improving subjective murine activity (+188%*, +97%). However, lung histological injury was only reduced by HTSn (-72%*) as RLb resulted in increased injury (+28%). HTSb did not differ to HTSn in any measured parameter. (*p≤0.05)

Conclusions: Compared to HTS, hemorrhagic shock resuscitation using RL supplemented with anti-adhesion blockade, results in similar reductions in PMN recruitment in the microcirculation and lung parenchyma. Yet, HTS additionally provides definitive improvements in microvascular integrity and murine activity. More importantltly, only HTS is able to reduce lung injury 24 hours after resuscitation. HTS resuscitation in hemorrhagic shock blunts tissue injury independent of its ability to functionally block EC and PMN adhesion molecules.

For several decades, it has been customary to resuscitate hemorrhagic shock with large volumes of isotonic solutions. The multiple drawbacks of requiring such large volume infusions have lead investigators to study alternatives such as hypertonic fluids which require a fraction of such volumes to obtain the same resuscitative effect. Four milliliters per kilogram of 7.5% sodium chloride (hypertonic saline, HTS) for the initial resuscitation of traumatic shock can be administered easily and rapidly, providing a significant logistic advantage, both in battlefield and urban trauma settings. Furthermore, the small volume required offers particular advantages in lung and head-injured patients as it may diminish third space fluid sequestration. Large, randomized clinical trials comparing hypertonic with standard isotonic resuscitation using Ringer's lactate (RL) were able to demonstrate equally safe and efficient restoration of systemic hemodynamics by both solutions[1-5]. Additionally, these and other studies conducted in the 1990s have demonstrated HTS-mediated reductions in post-resuscitation morbidity and survival advantages in subgroups of head-injured and surgical patients.

The recent resurgence of interest in hypertonic saline follows reports of its immune modulatory effects in the setting of systemic inflammation. Major host insults such as multiple trauma, sepsis, pancreatitis and severe hemorrhagic shock can result in uncontrolled systemic inflammation leading to multiple organ dysfunction syndrome (MODS) and death[6]. The so-called 'two-insult' or 'two-hit' model underscores the means by which this destructive sequence of events develops. This theory maintains that when the host survives a major systemic insult such as trauma, it may become particularly vulnerable to subsequent apparently innocuous insults (blood transfusion, pulmonary aspiration, minor gastro-intestinal bleed). The advent of this second stimulus or 'hit' triggers the host immune response which becomes self sustained and unbridled, causing globalized systemic inflammation and collateral injury to remote organs[7]. In trauma victims, the lung is particularly vulnerable to second 'insults' as is evidenced by the high

incidence of posttraumatic development of the acute respiratory distress syndrome (ARDS)[8]. Histopathologically, ARDS and other conditions of systemic inflammation display microvascular damage, tissue neutrophil infiltration, loss of capillary integrity, tissue edema, and parenchymal organ injury. The polymorphonuclear neutrophil (PMN), an essential constituent of the innate non-specific host immune response, and particularly its interactions with endothelial cells (ECs) appear to play a pivotal role in the development of organ injury in the setting of systemic inflammation.

In physiological conditions, the neutrophil must pass from the circulation to the interstitial space to reach invading pathogens or debris of tissue injury. This process begins with margination of the neutrophil out of central vessel flow, and its weak interaction with ECs through surface selectins (L, E, P-selectin) and their countereceptors (SLe^X)[9]. The resulting torsional force applied to its surface compels the neutrophil to roll on the vessel wall. PMN rolling permits stronger interactions to develop between neutrophil surface β-integrins (CD18/CD11a/CD11b) and endothelial adhesion molecules of the immunoglobulin superfamily (intercellular adhesion molecule 1 [ICAM-1], ICAM-2)[10]. These latter interactions result in firm adhesion of the neutrophil to the endothelial cell, an essential step for its subsequent extravascular transmigration and for its migration to the area of injury or pathogen invasion[11]. Once there, through phagocytosis and degranulation (the release of reactive oxygen species [ROS] and proteases), neutrophils engulf and destroy tissue debris and pathogens. While this process is orderly and controlled in physiologic conditions, in the setting of systemic inflammation it may become self-perpetuating, unbridled and destructive to the host itself. There, neutrophil-endothelial (PMN/EC) interactions become systemic and unrelenting, resulting in the sequestration of PMNs in capillary beds and their indiscriminate release of proteases and ROS, cytotoxic to endothelium. Endothelial injury compromises microvascular integrity, causing interstitial plasma leakage, tissue

edema, and eventual organ dysfunction. Two hit models have highlighted the tissue injury and organ dysfunction mediated by aberrant PMN/EC interactions[12-17].

The participation of uninhibited EC/PMN interactions in systemic inflammation can be further illustrated in studies where monoclonal antibodies against neutrophil or endothelial adhesion molecules blunt the degree of tissue damage incurred in different host injury models. For example, blockade of adhesion molecules responsible for neutrophil rolling (selectins, SLe^X) has been shown to attenuate EC/PMN interactions, improve liver, lung, adrenal and cardiac function while increasing survival in hemorrhagic shock models[18-24]. Likewise, monoclonal blockade against receptors responsible for the adhesion phase (CD11/18, ICAM) also blunts indiscriminate PMN/EC interactions and neutrophil accumulation in tissue[25-28]. Various host injury models have reproduced these beneficial effects of adhesion receptor blockade in sepsis[29-32], trauma[33], ischemia/reperfusion[34,35] and burns[36].

Hypertonic fluids have multiple effects on EC and PMN adhesion molecule expression. Selectins, which are responsible for neutrophil rolling are variably affected by HTS administration. In vitro experiments exposing neutrophils to hypertonic media have reported contradictory effects on L-selectin shedding[17,37]. In vivo models evaluating animals resuscitated from shock with hypertonic saline also report inconsistent results[17,38,39]. Yet, HTS administration to healthy human volunteers consistently demonstrates reductions in activated PMN L-selectin shedding[40,41]. Effects of hypertonicity on the expression of endothelial E and P-selectin also remain ambiguous[38,42-44].

Adhesion molecules involved in the adhesion phase of PMNs are more consistently altered by hypertonic saline. In most studies, CD11b expression is reduced by hypertonicity in vitro[17,45,46] and in vivo[17,40,41,47,48]. Endothelial expression of adhesion molecules responsible for firm PMN adhesion is likewise blunted by HTS[17,49,50]. Hence, it

appears that HTS primarily blocks the expression of adhesion molecules responsible for firm neutrophil adhesion to endothelial cells.

The hypertonic effect of functional blockade of receptors involved in EC-PMN interactions has been verified in live microcirculatory beds. Intravital microscopy (IVM) evaluating the microcirculation in ischemia/reperfusion, trauma and, burn models have confirmed diminished leukocyte interactions with endothelium following HTS infusions[51-54]. Similarly, IVM studies of hemorrhagic shock models by our group and others have demonstrated diminished leukocyte adhesion and interactions with endothelial cells following HTS resuscitation[13,55-58].

More importantly, hypertonic saline resuscitation of multiple conditions has consistently resulted in attenuated tissue-level injury. HTS administration in local or systemic host insults reduces pathogen translocation into tissues[59-65], and PMN mediated tissue injury[64,66-68]. In particular, HTS resuscitation results in attenuated tissue neutrophil sequestration and lung/liver injury in one and two-hit hemorrhagic shock models[13,17,39,42,47,65,69]. Furthermore, these and other injury models have shown HTS-mediated reductions in vascular leakage and tissue edema[55,70-72]. Taken together, this data suggests that attenuated tissue injury following HTS resuscitation, may be principally due to the functional blockade of PMN and EC adhesion molecules exerted by HTS.

We thus hypothesized that standard RL resuscitation supplemented with adhesion molecule blockade would result in reductions of EC/PMN interactions and tissue injury indistinguishable from that observed with HTS resuscitation alone. To study this hypothesis we used three series of experiments. First, to determine which PMN adhesion molecule was most important, activated L-selectin and CD11b expression was evaluated in PMN previously primed in vivo by hemorrhagic shock resuscitated with either HTS or RL. Demonstrating HTS effects primarily on CD11b and using the results

of a previous study on ICAM-1 lead us to postulate adhesion itself as the single EC/PMN interaction step most influenced by HTS. We thus chose MAbs which completely blocked the *step* of adhesion rather than either PMN or EC adhesion molecules responsible for adhesion. Subsequent experiments compared resuscitation with HTS alone to RL supplemented with monoclonal antibodies against EC and neutrophil receptors responsible for adhesion. In phase II, early in vivo leukocyte endothelial interactions and microcirculatory permeability in live cremaster tissue was compared in these study groups. Phase III completed the study by comparing long term (24-hour) animal activity, histologic tissue injury, and neutrophil sequestration with the different resuscitation regimens. Both latter phases employed a previously validated two hit murine model of resuscitated hemorrhagic shock followed by mimicked infection using intratracheal LPS.

Materials and Methods

Reagents

Lactated Ringer's [RL] and normal saline [NS] solutions were purchased from Baxter Corporation (Toronto, Ontario), heparin (10 000 USP units/ml) from Organon Teknika (Toronto, ON), ketamine from Wyeth-Ayerst (Guelph, ON) and xylazine from Bayer (Etobicoke, ON). Potassium phosphate [KH_2PO_4], was purchased from Merck & Co. (Montréal, QC), 3,3',5,5'-tetramethyl-benzidine [TMB], bovine fluorescein isothiocyanate (FITC)-labeled albumin, 0.5% bovine serum albumin [BSA] and lipopolysaccharide [LPS, Escherichia coli 0127:B8] from Sigma Chemical Company (St-Louis, MO) while hexadecyltrimethyl-ammonium bromide [HTAB], N,N,-dimethyl formamide [DMF], sodium azide 0.1% and 30% hydrogen peroxide [H_2O_2] were from

Fisher Scientific (Fair Lawn, NJ). Seventy percent glutaraldehyde was purchased from Canemco Inc. (St-Laurent, QC). Phosphate buffered solution [PBS] (pH 7.2) and fetal bovine serum [FBS] were bought from Gibco BRL (Burlington, ON). Flow cytometry monoclonal lysing solution waspurchased from BD PharMingen (Oakville, ON). Media B (PBS, 0.5% BSA, and sodium azide 0.1%), bicarbonate buffered solution [BBS] (NaCl 132 mM, KCl 4.7 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1.2 mM, NaOH 18mM, pH 7.4), 3% glutaraldehyde [GA] (70% stock diluted in 0.1M KH_2PO_4 [pH7.4]) and hypertonic saline [HTS] (7.5% NaCl in dH_2O) were constituted in our laboratory.

Monoclonal Antibodies

Monoclonal antibodies (MAbs) used in flow cytometry analysis were phycoerythrin (PE)-labeled anti-L-selectin purchased from Cedarlane, (Hornby, ON) and FITC-labeled anti-CD11b from Beckman-Coulter (Burlington, ON). Blocking monoclonal antibodies used in vivo were purified rat anti-mouse CD11b monoclonal antibody (clone M1/70, 1mg/ml) and purified rat IgG2bκ negative isotype standard antibody from BD Biosciences (Mississauga, ON). Functional grade purified rat anti-mouse ICAM-1 monoclonal antibody (clone YN1/1.7.4, 1mg/ml) and functional grade rat IgG2bκ isotype control antibody were from eBioscience (San Diego, CA). All antibody preparations were sodium azide-free and antibody lots were tested to ensure endotoxin levels were below 0.01ng/μg of protein.

Study groups and experimental protocols

Experiments were divided into three phases: To determine the effect of HTS on adhesion molecules, phase I involved in vitro LPS activation of murine PMNs previously primed in vivo by subjecting the animal to hemorrhagic shock and resuscitation with either HTS or RL. Using phase I results, phase II and III experiments attempted to shed

light on the mechanism by which HTS diminishes tissue injury. This was done by blocking a precise adhesion molecule (identified in phase I) and its ligand in a two-hit in vivo murine model of hemorrhagic shock/resuscitation followed by intratracheal LPS administration one hour later.

Phase I: Fourteen CD1 mice underwent hemorrhagic shock and were randomized to resuscitation by one of two resuscitation regimens: **RL**: resuscitation with lactated Ringer's, (twice the volume of withdrawn blood) immediately followed by all shed blood (n=7) or **HTS**: resuscitation with 4cc/kg of 7.5% hypertonic saline immediately followed by all shed blood (n=7). Animals were sacrificed 15 minutes after the end of resuscitation and their blood collected by cardiac puncture. Primed PMNs were then activated in vitro with subsequent adhesion molecule determination by flowcytometry analysis as delineated below.

Phase II and III: Fifty-two CD1 mice underwent hemorrhagic shock and were randomized to one of four resuscitation arms: **HTSb:** administration of 85µl of anti-CD11b MAb and 65µl of anti-ICAM-1 MAb prior to resuscitation with 4cc/kg of 7.5% hypertonic saline immediately followed by all shed blood (n=12), **HTSn:** administration of 150µl of isotype control MAb prior to resuscitation with 4cc/kg of 7.5% hypertonic saline immediately followed by all shed blood (n=10); **RLb**: administration of 85µl of anti-CD11b MAb and 65µl of anti-ICAM-1 MAb prior to resuscitation with lactated Ringer's, (twice the volume of withdrawn blood) immediately followed by all shed blood (n=12), **RLn**: hemorrhagic shock, administration of 150µl of isotype control MAb prior to resuscitation with lactated Ringer's, (twice the volume of withdrawn blood) immediately followed by all shed blood (n=10). Once resuscitation was completed, animals were returned to their cages and all received intratracheal LPS one hour later. Animals from each resuscitation arm were then evaluated at two time intervals depending on the phase they were assigned to. Phase II animals from all four resuscitation groups were evaluated 3 hours

after resuscitation with intravital microscopy analysis (n=23). Phase III animals were evaluated 24 hours after resuscitation for subjective functional animal activity, their lungs and cremaster were harvested and their serum analyzed for hematology and chemistry (n=24). **Control** animals underwent no surgical manipulation, hemorrhage nor resuscitation but were sacrificed for lung harvesting (n=8).

Hemorrhagic shock model (Phase I, II, and III)

Approval was obtained from the McGill University Health Center Animal Care Committee prior to experiments. CD1 male mice (Charles River: St-Constant, QC), 25-30gm, were fed *ad libitum* and housed in standard care facilities for 3-5 days prior to study. Acclimated mice were anaesthetized by intra-peritoneal (i/p) injection (xylazine: 6.7mg/kg, ketamine: 13.4mg/kg) and the right carotid artery was cannulated with polyethylene (PE)-10 tubing. This catheter was used for continuous blood pressure monitoring through a pressure transducer (Living Systems Instrumentation: Burlington, VT), as well as for blood withdrawal and for administration of blood, resuscitation fluids, and intermittent 50µl boluses of xylazine/ketamine solution to maintain anaesthesia.

Hemorrhagic shock was induced by withdrawing blood from the carotid artery over 5 to 7 minutes into a tuberculin syringe previously flushed with 25U heparin until mean arterial pressure (MAP) reached 40 mmHg. Hypotension was maintained for 60 minutes, by further blood withdrawals if MAP rose above 45 mmHg, or by re-infusions of withdrawn blood if MAP fell below 35 mmHg. Following the hypotension period all animals were resuscitated with a crystalloid regimen in addition to shed blood. Additionally, Phase II and III animals received assigned monoclonal antibodies immediately prior to resuscitation (see above). These fluid resuscitation regimens have been used extensively in the literature and have been shown to adequately resuscitate animals[13,39,55,69,73]. Animals that were still hypotensive after resuscitation for any reason

were discarded from data analysis. The carotid artery was then ligated, the cervical incision closed and the animals returned to their cages. Phase I animals were sacrificed 15 minutes later for blood collection while Phase II and III animals remained in their cages until one hour later when they received intratracheal LPS and were then evaluated and sacrificed at different times.

Intratracheal LPS instillation (Phase II and III)

Phase II and III animals underwent intraperitoneal (i/p) anaesthesia one hour after resuscitation from hemorrhagic shock, and their cervical incision was reopened. Pretracheal muscles were spread bluntly exposing the tracheal rings and 20μl LPS (500μg/ml) were injected into the trachea under direct vision using a 0.5cc insulin syringe. Animals where appropriate anatomic instillation into the trachea was in doubt were not used in data analysis. The cervical incision was then closed, 2cc of NS were injected subcutaneously in both flanks for long-term hydration and the animal was returned to its cage. Animals were subsequently re-anaesthetised at different times depending on the phase to which they had been assigned.

Phase I

In vitro PMN activation and determination of adhesion molecule expression

Phase I animals were sacrificed 15 minutes after the end of resuscitation following blood collection via cardiac puncture. For each blood sample, neutrophil surface adhesion molecule expression (CD11b and L-selectin) was determined before (pre-activation) and after (post-activation) LPS stimulation in vitro. One hundred microliters of whole blood was incubated in the dark with 10μl of FITC-labeled anti-CD11b MAbs for fifteen minutes at room temperature. Another 100 μl were added to 10 μl of PE-labeled anti-CD62L (L-selectin) and incubated in the dark at 4°C for thirty

minutes. Red cells from both samples were then lysed with 2 ml of monoclonal lysing solution and the samples were incubated in the dark at room temperature for ten minutes. After sedimenting the neutrophils (400g at 4°C for 5 minutes), the CD11b samples were washed with phosphate buffered solution containing 0.1% sodium azide and 1% FBS, while L-selectin samples were washed with ice-cold PBS solution. Cells from the CD11b samples were then suspended in 0.5ml 0.5% paraformaldehyde while the L-selectin sample cells were resuspended in 0.5 ml of ice-cold Media B and both were promptly analysed with an argon ion laser flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). Data was collected and analyzed with Cell Quest computer software (Becton Dickinson). Cells were plotted on forward vs. side scatter and were gated to isolate neutrophil populations. Using histogram plots from FL1 (CD11b) and FL2 (L-selectin) detectors (488 and 550 nm excitation wavelengths respectively), the mean intensity of fluorescence (mean channel fluorescence, MCF) for each receptor (which is directly proportional to the density of surface receptors per cell) was recorded for each PMN population. A minimum of 5 000 events were analyzed per sample. Results were expressed as mean channel fluorescence. In vitro PMN activation was conducted by immediately incubating 300 µl of whole blood with 18 µl (500µg/ml) of LPS for 15 min in a 37°C shaking water bath. The above protocol was then applied to determine surface CD11b and L-selectin levels in post LPS activation blood. Adhesion receptor change was calculated as the ratio of post to pre-activation adhesion molecule expression levels for either adhesion molecule.

Phase II

Intravital microscopy

Two hours after receiving intratracheal LPS, resuscitated phase II animals received intraperitoneal anaesthesia as above and underwent right jugular vein

cannulation. The jugular line was used to administer anaesthetic solution and FITC-albumin. The mouse cremaster was prepared for intravital microscopy as described previously[74]. Briefly, after the animal was immobilized by attachment of all four limbs, the cremaster muscle was exteriorized through a small scrotal incision, opened and dissected free of the testicle and epididymis. Using five-point fixation with 4-0 silk, the cremaster was splayed open and fixed to a plexiglass stage. For the remainder of the experiment the exteriorized cremaster was continuously perfused with thermostat-controlled (37° C) BBS. The stage was placed on a Nikon TE 300 inverted microscope (Nikon Canada: Montréal, QC), and live tissue microcirculation was imaged at 2120X magnification.

An optical Doppler velocimeter (Microcirculation Research Institute: College Station, TX) was used to directly measure central red blood cell velocity (V_{RBC}) in post-capillary venules (PCVs). Based on Newtonian definitions[75], mean vessel flow velocity (V_{MEAN}) was calculated from V_{RBC} using the formula: $V_{MEAN} = V_{RBC}/1.6$. Shear rate (γ), in sec^{-1}, was calculated using the formula: $\gamma = 8 \times (V_{RBC}/D_V)$, where D_V is the venular diameter measured directly off line using calipers. Shear stress (Φ) in sec^{-1}, was calculated using the formula: $\Phi = \gamma/4$. To minimize intersample shear stress variability, non-branching PCVs with a diameter of 25-40μm and a V_{MEAN} of 1.25–3.75 mm/sec were chosen. Animals that were unable to fulfill these predetermined conditions were not used for analysis of leukocyte-endothelial interactions.

Live microscopic footage of chosen post capillary venules was captured with a high definition black and white video camera (CCD High Performance Camera, COHU, San Diego, CA), transferred to a monitor (Trinitron Color monitor – SSM-14NE, Sony: Toronto, ON), and recorded on videotape with a video recorder (SVHS HrS3910u, JVC Company of America: Wayne, NJ). A video Time-Date Generator (model WJ-810,

Panasonic: Toronto, ON) projected the time, date, and stopwatch function onto the monitor. Body temperature was maintained at 37° C with a radiant heat lamp throughout the study period. In each animal, seven-minute video footage of each of three different post capillary venules per animal were captured after a stabilization period following the completion of cremaster surgery. These recordings were subsequently played back off line by another member of the laboratory without prior knowledge of treatment who quantified leukocyte-endothelial cell interactions. All animals were sacrificed by xylazine/ketamine overdose followed by cervical dislocation at the completion of experiments.

Quantification of EC-LEU interactions

To avoid alteration of PMN adhesive properties, neutrophils were not labeled with fluorescent markers. Nonetheless, available evidence suggests that the vast majority of leukocytes visible in such IVM models are likely PMNs[76]. Leukocyte rolling was defined as the number of leukocytes crossing a line perpendicular to the long axis of the vessel, that were moving at a rate slower than erythrocytes over a period of two minutes. Leukocyte rolling velocity was calculated as the mean transit time of ten neutrophils over a given 100 μm length of post-capillary venule. Leukocyte adherence was defined as the number of cells stationary for a minimum of 30 seconds in a 100 μm length of venule during a five-minute period and pre-adherence was defined as the number of immobile neutrophils in the same 100 μm vessel section at the initiation of counting. Total neutrophil adherence was the sum of neutrophil adherence and pre-adherence for a given vessel footage.

Fluorescent quantification of vascular permeability

Following the completion of all video recordings, phase II animals underwent fluorescent videomicroscopy. Fifty mg/kg of fluorescein isothiocyanate (FITC)–labeled bovine serum albumin were injected IV through the jugular catheter. Five minutes later, epiluminescence microscopy was performed on the same splayed cremaster using the Nikon inverted microscope illuminated with a high-pressure mercury fluorescence source lamp (HB 10103AF, Nikon Canada: Toronto, Canada). Moving images were captured by a frame grabber and transferred to Scion Image processing software (Scion Image for Windows, Scion Corporation: Frederick, MD) on a computer (Dimension XPS B866r, Dell Canada: Toronto, Canada). The captured frozen digital image of fluorescent post-capillary venules, were evaluated to determine light intensity in levels of gray [0 (black) to 256 (white)] in six equal areas within the venule (venular intensity, I_v), as well as six separate equal areas in the perivenular space (perivenular intensity, I_p). Using the means of I_p and I_v measurements, vascular leakage (permeability index, PI) was calculated with the formula I_p / I_v =PI for each venule. PI was determined in this way for three different venules in each animal which were then averaged to yield the given animal's mean permeability index.

Phase III

Subjective animal activity

At 24 hours, when phase III animals were to be anesthetized for organ harvest, their performance of normal murine activities was graded while still in their cages. The following parameters were evaluated and assigned one point if present: active feeding, active drinking, spontaneous movement, stimulated movement, active grooming, active playing, standing, urinating and the presence of shiny/smooth coats. Activity was assigned a score of 0 (none), 1 (minimal), 2 (moderate) and 3 (normal) while

stroke symptoms were assigned a score 0 (severe), 1 (moderate), 2 (minimal) and 3 (none). The points obtained for each parameter were subsequently summed to yield the total subjective activity score for each animal. The greater the score the closer the animal was to behaving normally

Lung sequestration of PMN

Twenty-four hours after resuscitation, phase III animals were sacrificed for lung and cremaster harvest. After induction into anaesthesia by intraperitoneal ketamine/xylazine, animals underwent sternotomy and their entire tracheobronchial tree was excised by sharp dissection. The right lung was dissected free of diaphragmatic and pleural tissue and subsequently weighed while the left lung was prepared for histology. The excised right lung sample was then homogenized (PT10 00 Polytron Homogenizer, Kinematica GMBH: Luzern, Switzerland) for 1 minute in 3cc of 10mM KH_2PO_4 [pH7.4] buffer and centrifuged at 12 000g for 20 minutes at 4° C. The supernatant was discarded and the pellet resuspended and homogenized in 50mM KH_2PO_4 [pH6.0] buffer containing 0.5% HTAB. The homogenate was kept frozen at -80° C and, at a different time, it was thawed, rehomogenized for 1 minute and sonicated (Sonic Dismembrator Model 300, Fisher: Farmingdale, New York) at 40 W for 1 minute. After centrifugation as described above the supernatant was diluted (X3) and 50µl were added to 350µl of 220mM KH_2PO_4 in 110mM NS and reacted 45 seconds later with 50µl 16mM TMB in DMF prior to blanking the spectrophotometer (DU 640, Beckman Coulter: Fullerton, California) at 37° C. The colorimetric reaction was then read at a wavelength of 655nm after the addition of 50µl of 3mM H_2O_2. The absorbance change over 180 seconds was used as a measure of MPO activity. Results were expressed as MPO activity per gram of lung tissue.

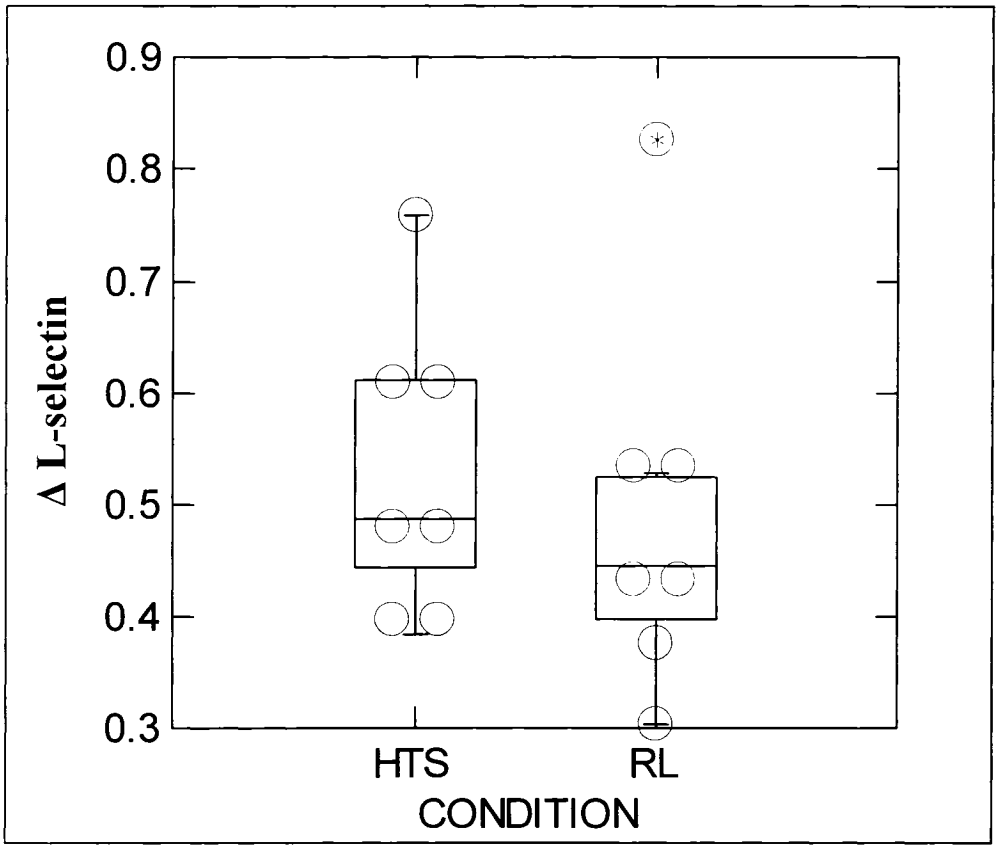

FIGURE 1: Following LPS activation in vitro, neutrophil surface L-selectin was shed more readily in PMNs from animals resuscitated from hemorrhagic shock with RL than with HTS. ($p>0.05$)

Histologic Lung Injury

As described above, at 24 hours anaesthetized animals underwent sternotomy and the entire tracheobronchial tree was excised by sharp dissection. The left lung was fixed by instillation of 3% glutaraldehyde into the left mainstem bronchus using a 22-gauge angiocatheter and was fixed overnight. The following day, the lung was sliced, sections were processed using routine histological techniques and embedded in paraffin. From these, 5 μm-thick sections were cut and stained with hematoxylin and eosin. The slides were subsequently graded by one of the authors (R.P.M.) without prior knowledge of treatment groups using a modified histological injury score[77]. The following histological parameters were graded: marginated intravascular neutrophils, interstitial transmigrated neutrophils, alveolar transmigrated neutrophils, and interstitial edema, septal hyperplasia, alveolar edema/hemorrhage. For a given sample, each parameter received a score of zero (none), one (scant), two (moderate) or three (extensive) points.

Total histologic lung injury scores were calculated as the sum of individual parameter scores for each animal.

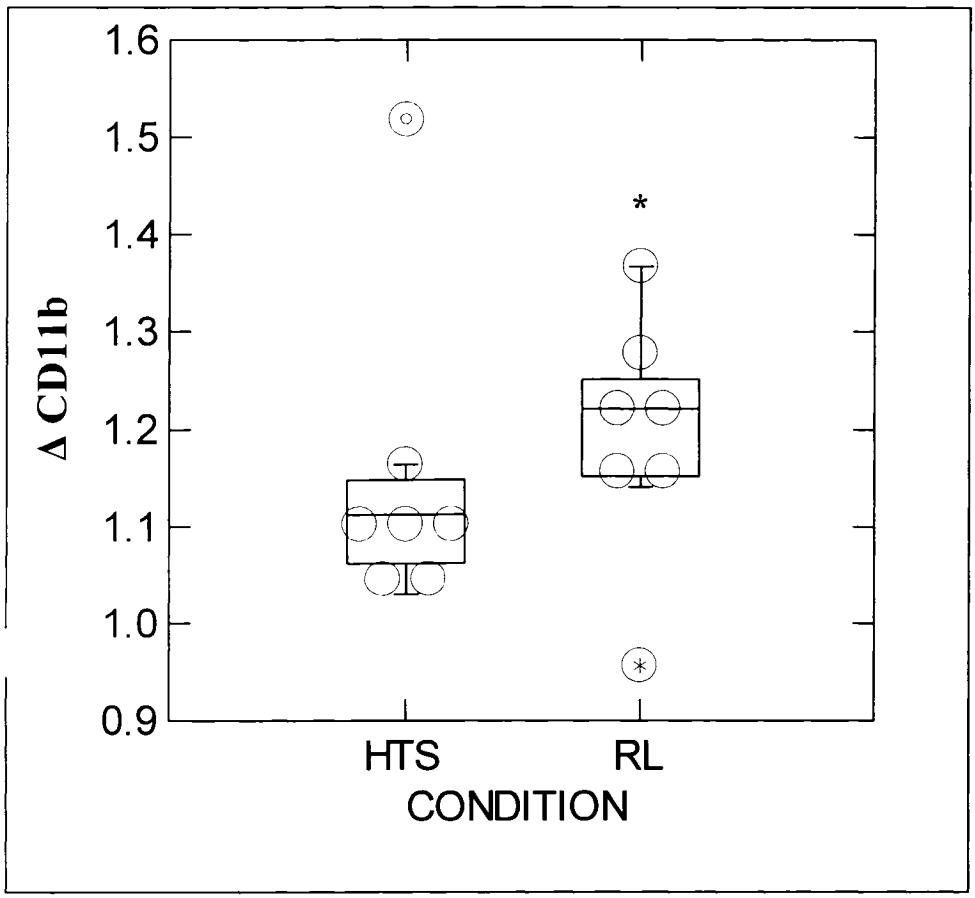

FIGURE 2: Following LPS activation in vitro, upregulation of neutrophil CD11b was attenuated if HTS instead of RL had been used for resuscitation of hemorrhagic shock. (Corrected for outliers (indicated), *p=0.01 vs HTS)

Histological Cremaster Injury Analysis

Prior to sternotomy, anaesthetized phase III animals had their cremaster dissected as for intravital microscopy, but then transected and placed in 3% glutaraldehyde for overnight fixation. As with lung tissue, cremaster samples were embedded, sliced and stained with H & E. The slides were subsequently graded by one of the authors (R.P.M.) using a histological injury score without prior knowledge of treatment groups. The following histological parameters were graded in cremaster sections: marginated intravascular neutrophils, interstitial marginated neutrophils, interstitial edema, and interstitial hemorrhage. For a given sample, each parameter received a score of zero (none), one (scant), two (moderate) or three (extensive) points. Total cremaster histology injury scores were calculated as the sum of individual parameter scores for each animal.

Systemic Complete Blood Count and Serum Chemistry:

Immediately prior to cremaster dissection, anesthetized phase III animals underwent cardiac puncture and 1000 µl of central venous blood was removed for determination of complete blood count (CBC), serum sodium [Na] and chloride [Cl]. The McGill University Health Center hematology laboratory determined the CBC using an Advia Hematology System (model 120, Bayer Systems: Hialeah, FL) while the chemistry laboratory evaluated serum electrolytes using a Synchron Clinical System (model LX20PRO, Beckman Coulter: Fullerton, CA).

Table 1. VENULAR RHEOLOGIC MECHANICS

	HTSb	HTSn	RLb	RLn
Diameter (µm)	30.5 ± 0.9	30.5 ± 1.2	30.5 ± 0.6	33.4 ± 1.3
V_{MEAN} (mm/s)	1.59 ± 0.13*	2.10 ± 0.16	1.80 ± 0.19	1.56 ± 0.10
Shear Stress (sec^{-1})	0.11 ± 0.01	0.14 ± 0.02	0.12 ± 0.01	0.10 ± 0.01

Rheological parameters in venules from all phase II animals did not differ between groups except for mean flow velocity (V_{MEAN}) in HTSb animals which was significantly lower than that in HTSn animals. Other values for V_{MEAN}, as well as all values for venular diameter, shear rate and shear stress and did not differ between groups. *$p<0.05$ vs HTSn V_{MEAN}.

Statistical Analysis:

All data are presented as mean ± standard error of the mean (SEM). Differences between groups were compared using analysis of variance (ANOVA) with Bonferroni correction using Systat 10 data analysis software (SPSS, Chicago, Illinois). Groups that did not have a normal distribution or had a small sample size were evaluated with the Kruskal-Wallis One-Way Analysis of Variance (Mann-Whitney U test statistic). Differences between groups were deemed significant at a probability ≤ 0.05.

Results

Phase I

Neutrophils from HTS-resuscitated animals display less CD11b upregulation by LPS activation in vitro.

There were no differences in withdrawn blood volumes and blood pressure between phase I groups, but total fluid administered (blood + crystalloid) was significantly greater in RL compared to HTS-resuscitated animals (data not shown). In vitro LPS activation of PMNs from animals previously resuscitated with RL caused shedding of L-selectin to a greater degree than in PMN from HTS resuscitated animals, but this difference was not significant (Figure 1). Opposite results occurred with surface PMN CD11b expression, as data analysis corrected for outliers showed a significant blunting of CD11b upregulation in PMN from HTS animals compared to those from RL animals following LPS stimulation (109.4 ± 2.2% vs 123.2 ± 3.3%, respectively, p=0.01) (Figure 2). Thus, hypertonic saline resuscitation attenuated the upregulation of CD11b by activated neutrophils while tending to prevent L-selectin

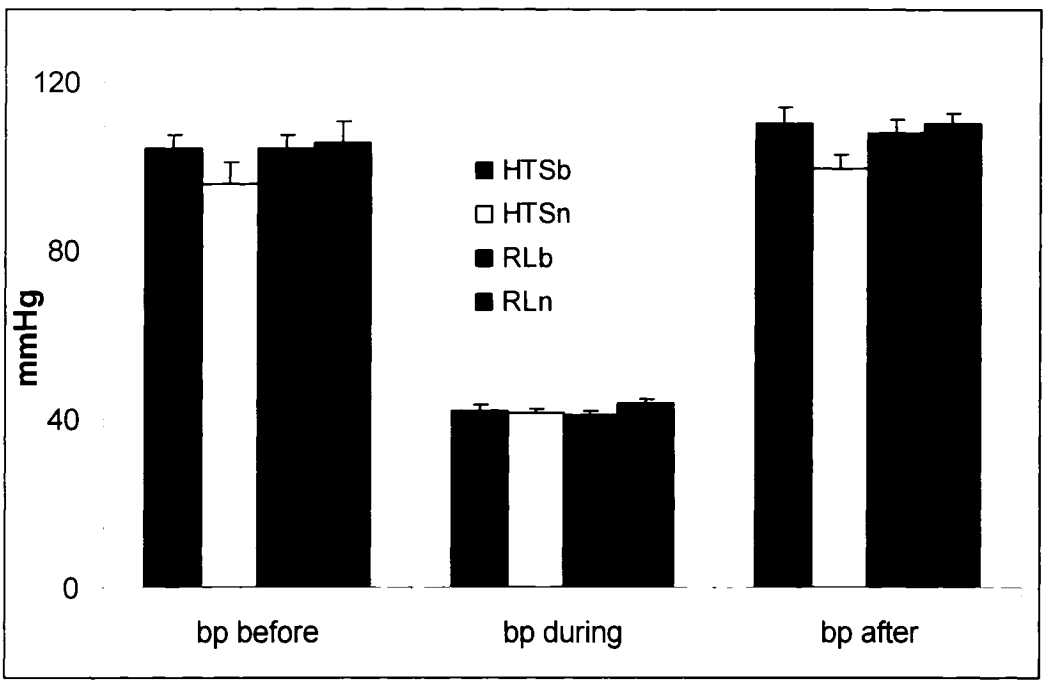

FIGURE 3: Blood pressure in phases II and III did not differ between groups before, during hypotension and after resuscitation (p>0.05 for all comparisons between groups)

shedding. Consequently, monoclonal blockade against PMN adhesion (anti-CD11b and anti-ICAM-1) was used in phase II and III experiments.

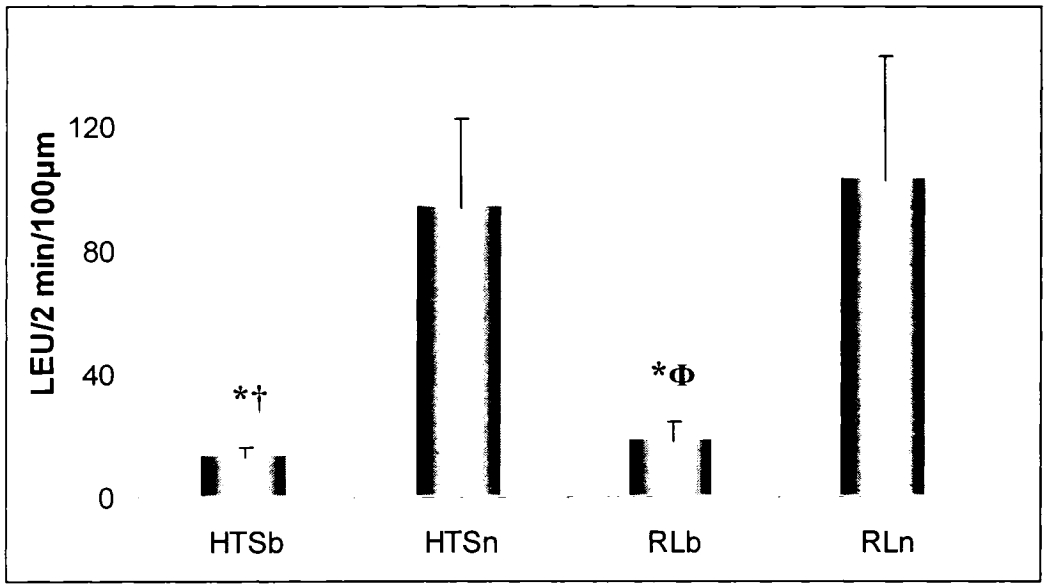

FIGURE 4: Leukocyte rolling flux was fivefold greater in animals without adhesion blockade compared to either resuscitation group having received adhesion blockade.(*p<0.05 vs HTSn, †p<0.05 vs RLn, Φp=0.05 vs RLn).

Phase II and III

Hemodynamic and vascular mechanics were similar in all groups

Blood volume withdrawn (mean 30.0 ± 0.42 cc/kg) in phase II and III animals did not differ between any resuscitation group but total fluid administered (blood + crystalloid) was significantly greater in all RL-resuscitated animals compared to all HTS-resuscitated animals (89.6 ± 1.9 vs 34.2 ±

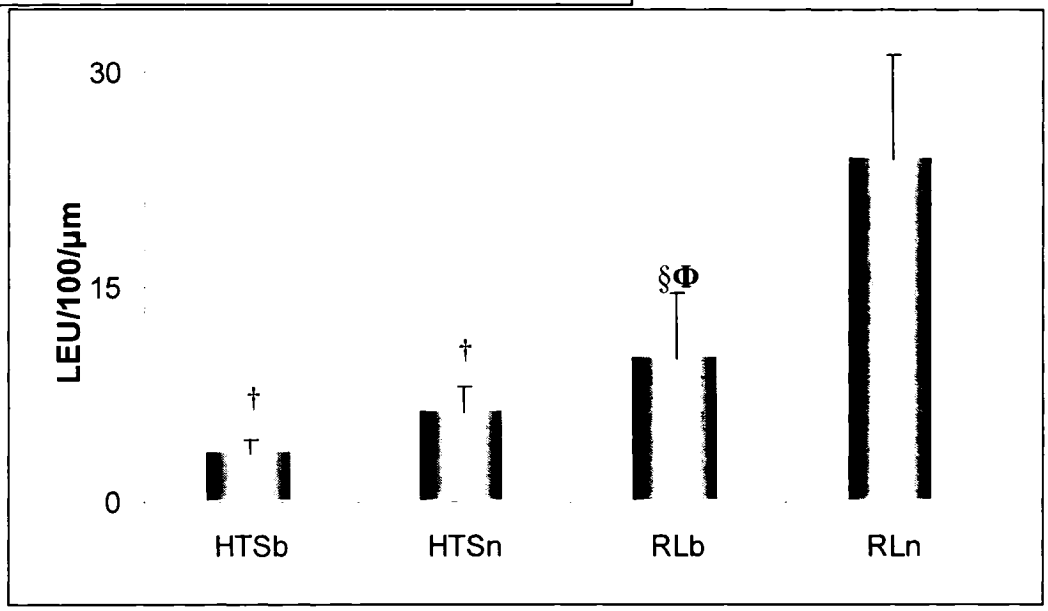

FIGURE 5: Total leukocyte adhesion was reduced with HTS resuscitation and by adhesion blockade. Total adhesion in HTSn and HTSb did not differ. (†p<0.05 vs RLn, Φp=0.05 vs RLn, §p=0.078 vs HTSb)

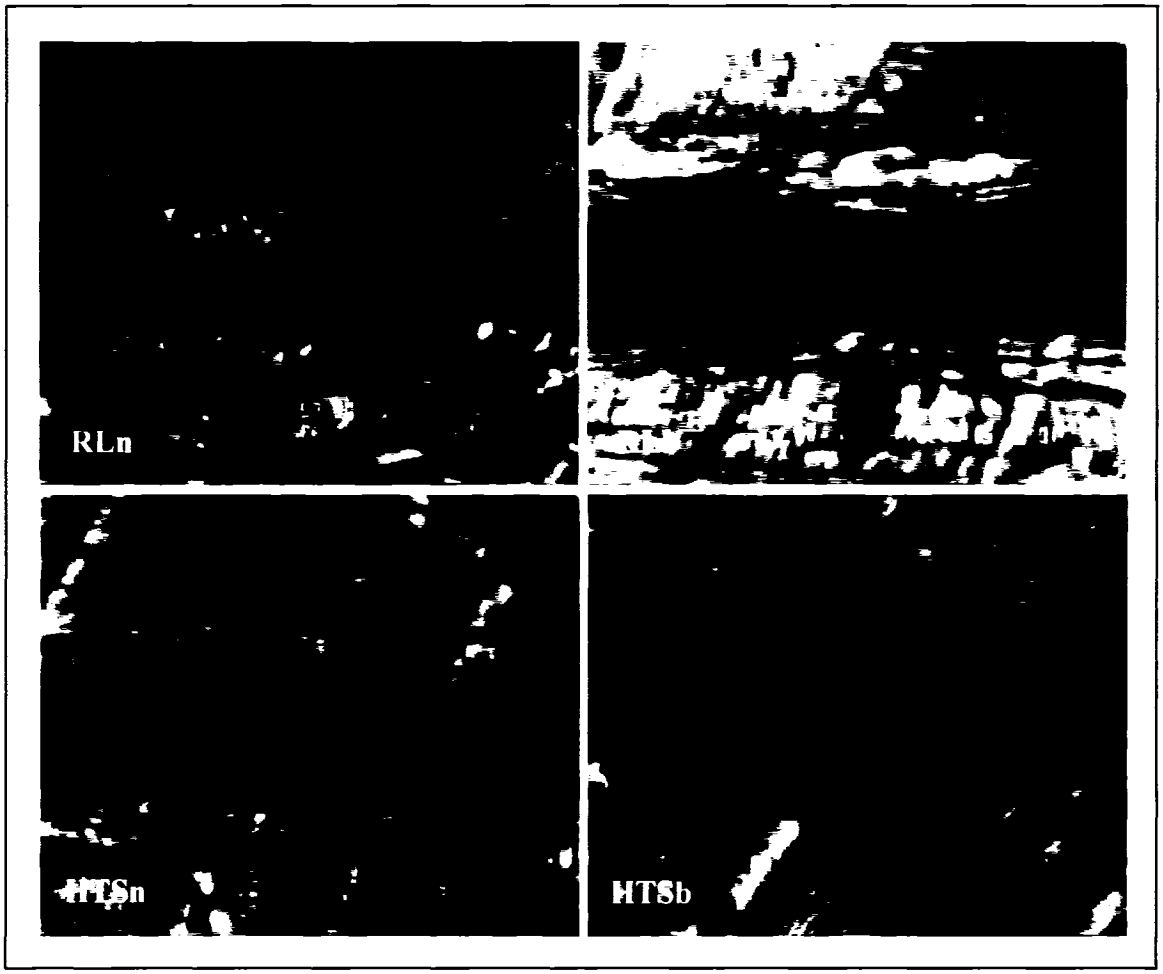

FIGURE 6: Representative images of cremaster PCVs taken from IVM footage 3 hours after resuscitation from each phase II group (clockwise from upper left: RLn, RLb, HTSb, HTSn). Note the paucity of adherent leukocytes in the HTSn and both blockade groups as compared to the RLn group.

1.9 vs 34.2 ± 0.6 cc/kg, respectively, $p<0.001$). Systolic blood pressure before (mean 102.6 ± 2.1 mmHg), during hypotension (mean 41.9 ± 0.6 mmHg) and after resuscitation (mean 107.2 ± 1.7 mmHg) was not different between any of the groups in phases II and III (Figure 3). During intravital microscopy analysis, venular diameter, mean red cell velocity V_{MEAN} and shear stress did not differ significantly between all phase II groups except for V_{MEAN}, which only differed between **HTSb** and **HTSn** groups (Table 1).

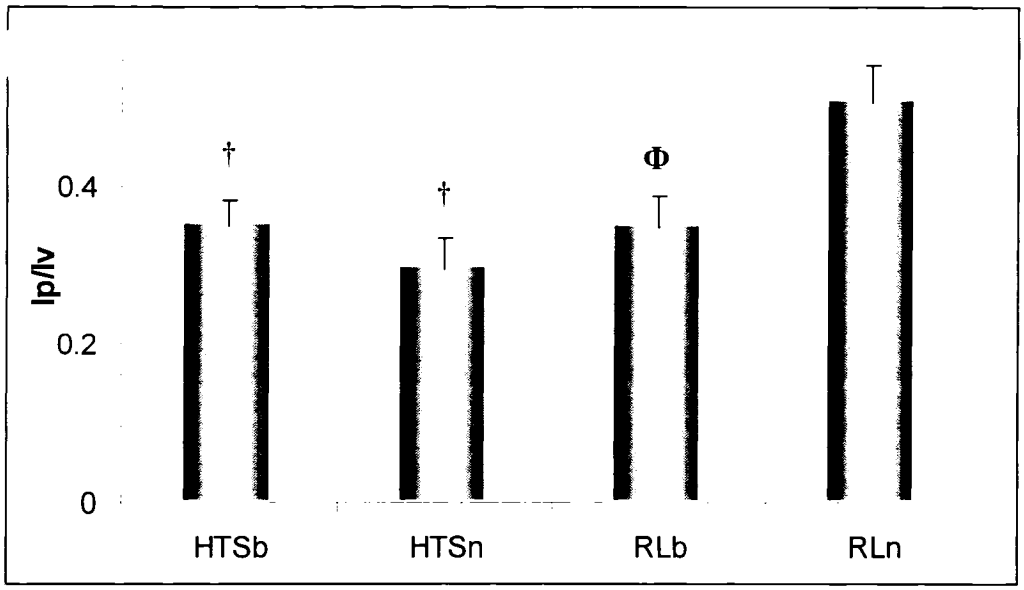

FIGURE 7: Cremaster venular permeability 3.5 hours after resuscitation was reduced if HTS was used for resuscitation. Monoclonal blockade tended to reduce RL associated microvascular leakage but not significantly. (†p<0.05 vs RLn, Φp=0.07 vs RLn)

Phase II

Both HTS and adhesion molecule blockade attenuate leukocyte adhesion

Three hours after resuscitation, leukocyte (LEU) rolling flux was highest in groups receiving inactive isotype monoclonal antibodies (HTSn: 93.3 ± 29.0 vs RLn: 102.5 ± 40.4 LEU /2min/100μm, p>0.05) and lowest in animals having received adhesion molecule blockade (HTSb: 12.7 ± 3.3 vs RLb: 18.2 ± 6.3 LEU/2min/100μm, p>0.05) (Figure 4). Both blockade groups displayed significantly lower leukocyte rolling flux than groups having received isotype controls regardless of resuscitation fluid administered. Total leukocyte adhesion to EC was greatest in RLn animals (23.8 ± 7.3 LEU/100μm), significantly greater than either HTS-resuscitated groups (HTSb: 3.3 ± 1.0 and HTSn: 6.2 ± 1.7 LEU/100μm, p<0.05 either group vs RLn) but also greater than that of RL animals having received adhesion blockade (RLb:10.0 ± 4.5 LEU/100μm, p=0.05 vs RLn) (Figure 5). Leukocyte adhesion differences between either blockade group and HTSn animals were not significant. There were no statistically significant differences in leukocyte rolling velocity between groups (data not shown). Figure 6 contrasts levels of adherent leukocytes in representative cremaster PCVs from each group of phase II animals taken from live footage 2 hours following LPS administration. Thus, even though monoclonal blockade

was against adhesion molecules known to be responsible for neutrophil adherence (not

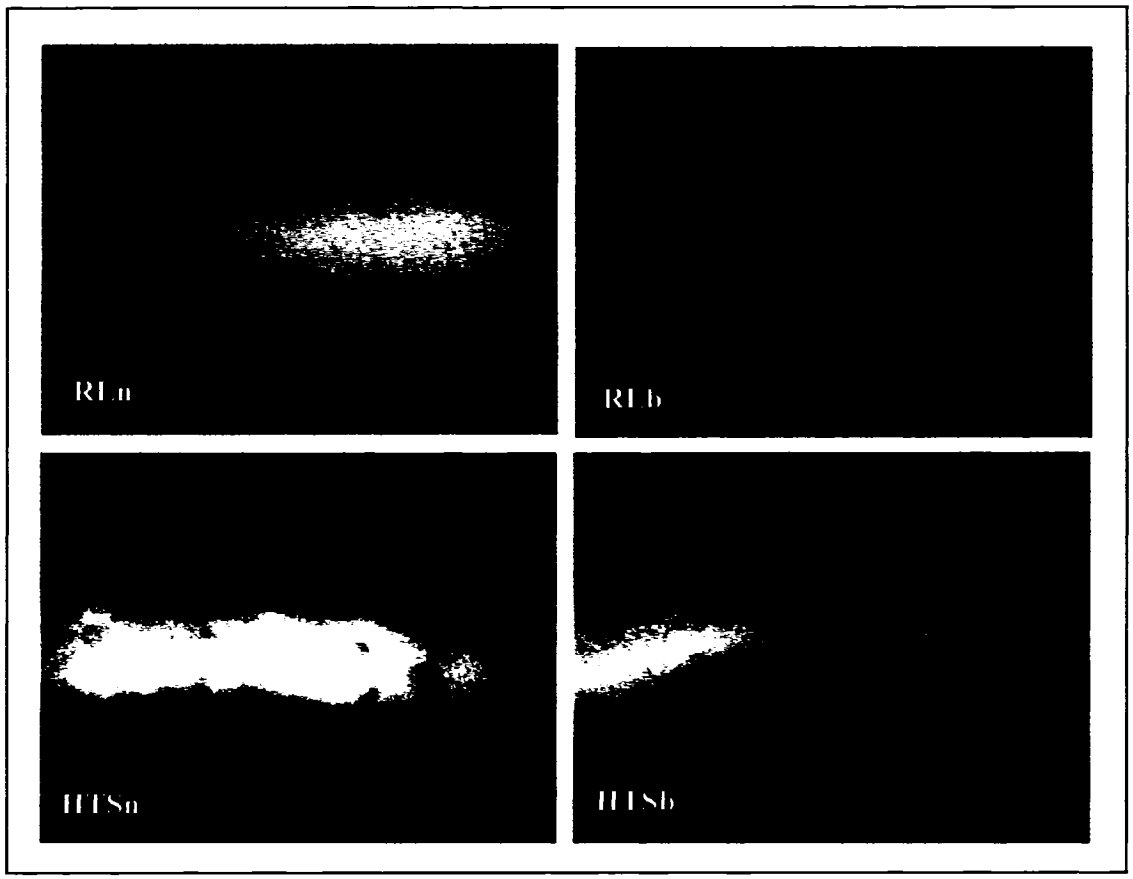

FIGURE 8: Representative images of cremaster PCVs taken 3.5 hours after resuscitation and 5 minutes after administration of FITC-albumin intravenously. Note the significant leakage of fluorescent macromolecules in the immediate perivenular area of the RLn animal.

rolling), it resulted in a significant blunting of leukocyte rolling regardless of fluid used. Total leukocyte adhesion was significantly reduced by HTS resuscitation or by antiadhesion blockade added to Ringers' lactate.

HTS attenuates in vivo macromolecular leakage better than adhesion molecule blockade

Venular permeability (PI) was highest in RLn animals (0.50 ± 0.05) and superior to that found in either HTS groups (HTSb: 0.35 ± 0.03 and HTSn: 0.29 ± 0.04, $p<0.05$ either vs RLn) (Figures 7 and 8). RLb animals also tended to have lower PI levels (0.35

± 0.04) but this was not significant. Hence, hypertonic resuscitation reduced microvascular permeability while adhesion blockade failed to reduce levels significantly.

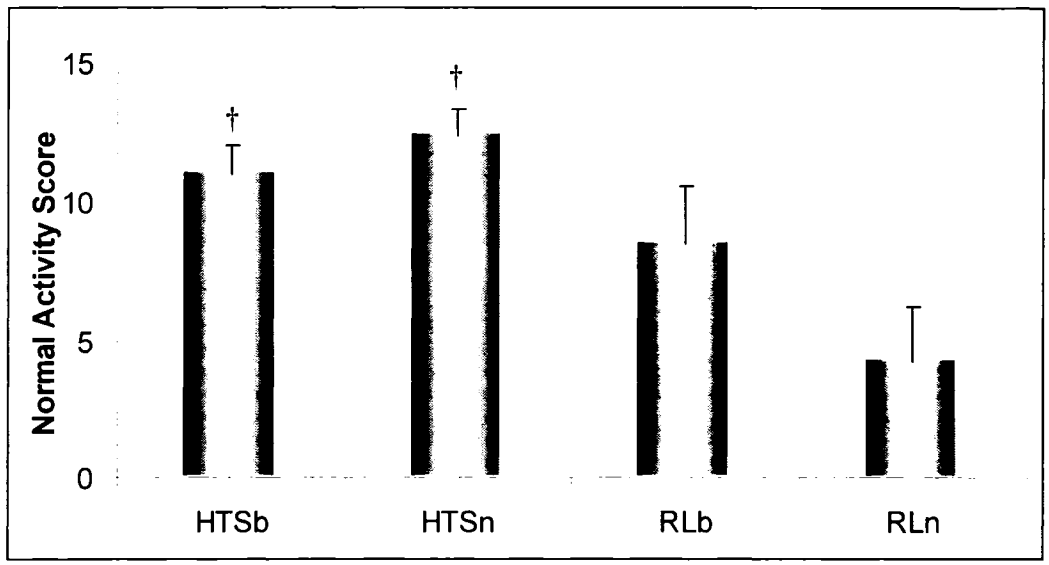

FIGURE 9: Subjective murine activity was evaluated in phase III animals 24 hours after resuscitation and was found greatest in HTS-resuscitated animals regardless of blockade status. Anti-adhesion blockade in RL-resuscitated animals tended to improve performance slightly (†p<0.05 vs RLn).

Phase III Better murine function activity with HTS-resuscitation

Prior to sacrifice, phase III animals were evaluated for performance of normal murine activity in their cages. Twenty-four hours after resuscitation both HTS groups (HTSb: 11.0 ± 1.1 vs HTSn: 12.4 ± 0.9, p>0.05) had higher scores than either RL groups in particular the RLn animals (4.3 ± 2.1, p<0.05 vs either HTSb or HTSn) (Figure 9). While blockade in RL animals tended to slightly improve performance this was not significant (RLb: 8.5 ± 2.1, p>0.05 vs RLn). Thus, 24 hours after resuscitation return to normal activities was better achieved by resuscitation with HTS than with anti-adhesion blockade.

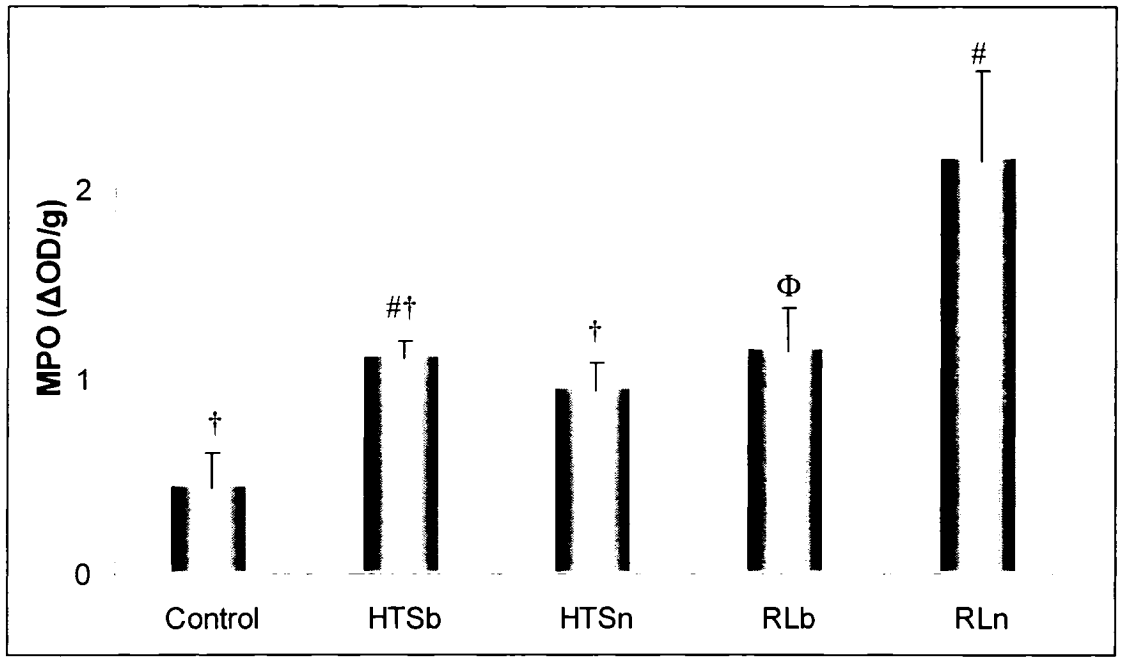

FIGURE10: Lung neutrophil sequestration as evaluated by myeloperoxidase assay. HTS resuscitation or anti-adhesion blockade reduced lung PMN accumulation. All but HTSn animals showed greater sequestration than Controls. (†p<0.05 vs RLn, #p<0.05 vs control, Φp=0.05 vs RLn or control)

Lung neutrophil sequestration is similarly attenuated by either adhesion blockade or HTS

At 24 hours, lung MPO levels in both HTS groups and the RLb group were similar (HTSb: 1.11 ± 0.09, HTSn: 0.94 ± 0.1, RLb: 1.14 ± 0.2 ΔOD/g, P>0.05 for all comparisons) (Figure 10). Both HTS groups had lung MPO levels significantly lower than that of RLn animals (2.13 ± 0.5 ΔOD/g, p<0.05 vs. either HTSb or HTSn). RLb animals also had lower lung MPO levels than RLn counterparts (p=0.05). All groups except for HTSn animals had significantly higher levels of lung MPO than control animals (0.43 ± 0.2 ΔOD/g, p<0.05 vs. either HTSb or RLn; p=0.05 vs. RLb). Thus, while HTS clearly reduced lung PMN accumulation at 24 hours, anti-adhesion blockade significantly attenuated sequestration in RL resuscitated animals but added nothing to HTS animals.

Histologic lung injury scores are only reduced by HTS resuscitation, not anti-adhesion blockade

Twenty-four hours after resuscitation, lung histologic scores were much higher in all RL animals combined (3.8 ± 0.73) than combined HTS animals (all HTS: 1.3 ± 0.31, p=0.001 vs all RL) or controls (Control: 0.9 ± 0.43, p=0.008 vs all RL) (combined data not shown). Anti-adhesion blockade did not attenuate injury regardless which resuscitation fluid was used (RLb: 4.17 ± 0.99 vs RLn: 3.25 ± 1.16, p>0.05; HTSb: 1.58 ± 0.40 vs HTSn: 0.90 ± 0.48, p>0.05) (Figure 11). In fact, monoclonal blockade tended to worsen lung injury for any given fluid group but not significantly.

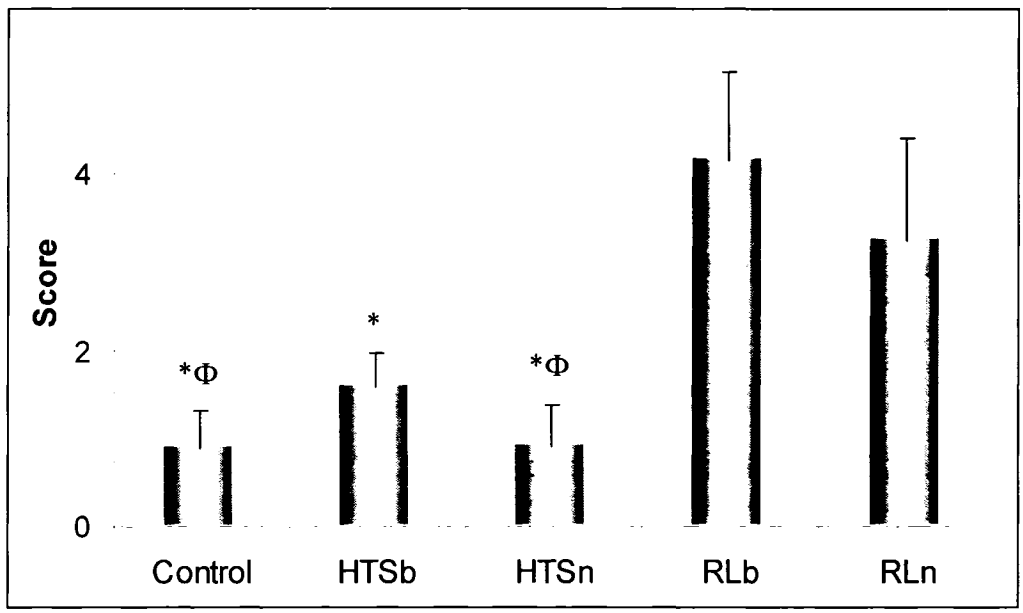

FIGURE 11: Lung injury score as evaluated on H&E stained lung sections from animals 24 hours after resuscitation. The following parameters were scored by a blinded observer: marginated intravascular neutrophils, interstitial transmigrated neutrophils, alveolar transmigrated neutrophils, interstitial edema, septal hyperplasia and alveolar edema/hemorrhage. While blockade caused no decrease in histologic lung injury score, HTS resuscitation reduced scores significantly, down to control levels. (*p<0.05 vs RLb, Φp=0.06 vs RLn).

Both RL groups demonstrated higher scores than controls (0.88 ± 0.43, p=0.01 vs RLb and p=0.06 vs RLn) while both HTS groups showed scores that did not differ with controls. Both HTS groups had significantly lower lung injury scores than RLb animals (p=0.01 either vs RLb) while only HTSn animals showed lower scores than RLn counterparts (p=0.06). The animals resuscitated with RL displayed interstitial edema, hemorrhage and debris with numerous transmigrated neutrophils in the alveoli and

interstitium regardless whether blockade was used or not (Figure 12). Hypertonic resuscitation reversed these changes revealing lungs resembling those of controls. Thus, attenuation of lung tissue injury 24 hours after resuscitation was not present with adhesion blockade, it occured solely if animals had been resuscitated with HTS.

Cremaster tissue injury tends to improve with HTS

There were no significant differences between cremaster injury scores between groups, though combined HTS animals tended to have lower cremaster injury scores than combined RL animals (All HTS: 0.54 ± 0.29 vs all RL: 0.79 ± 0.32, $p>0.05$) (data not shown).

Circulating leukocyte levels were attenuated by adhesion blockade.

There were no significant differences in any of the phase III animal groups with respect to serum chloride (mean 106.3 ± 1.6 mmol/L), sodium (mean 142.7 ± 1.1 mmol/L) or hemoglobin (mean 89.7 ± 4.2 g/L) levels. Circulating leukocyte levels were significantly lowger in animals with adhesion molecule blockade regardless of fluid used (Figure 13). HTSn animals had the highest level of circulating leukocytes (HTSn: 2.25 ± 0.40 X 10^9/L vs. HTSb: 1.21 ± 0.13 X 10^9/L, $p<0.05$, RLb: 1.26 ± 0.23X 10^9/L $p=0.06$ vs HTSn).

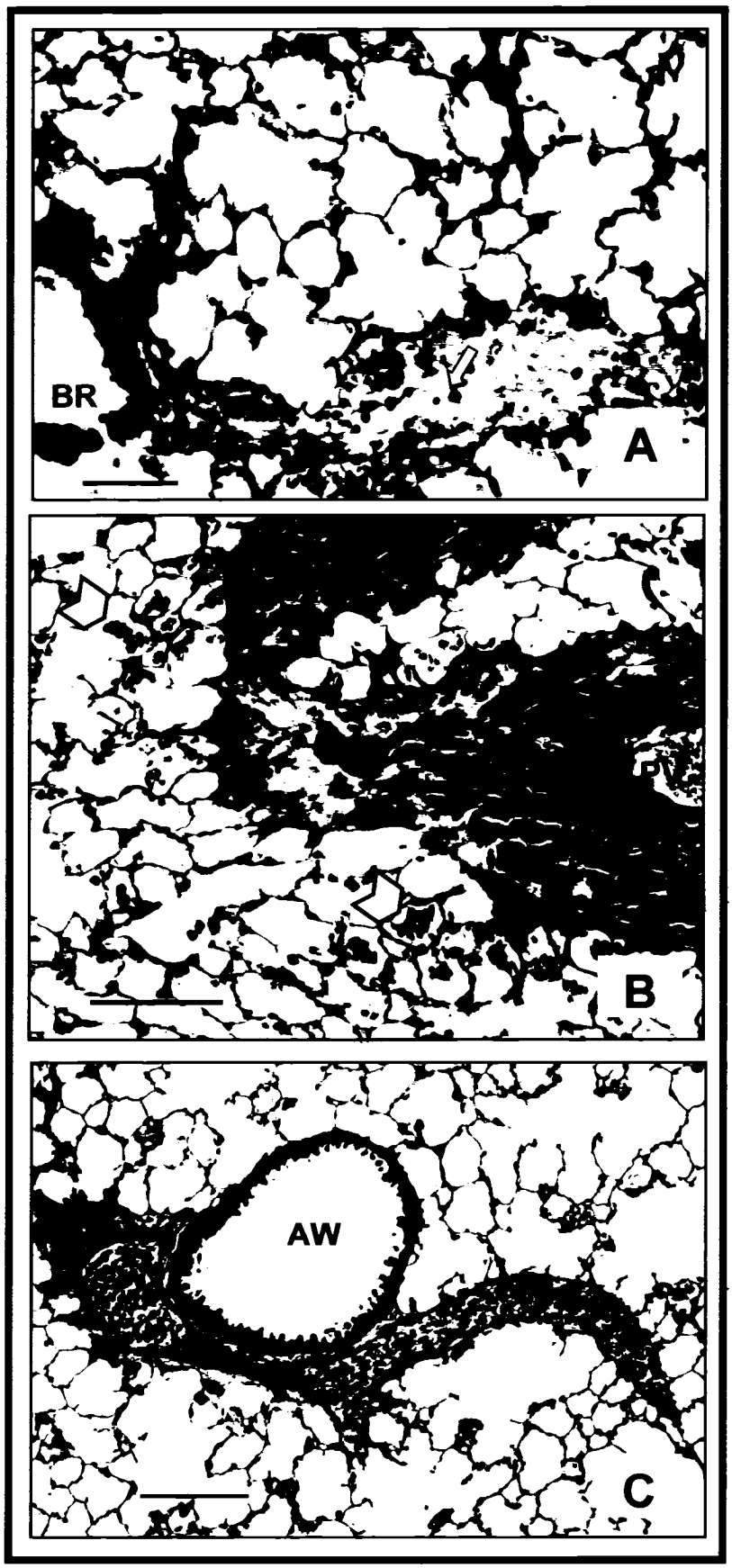

FIGURE 12: [A] Medium power photomicrographs of murine lungs 24 hours after resuscitation. [A] Detail from RLn animal showing interstitial space containing several neutrophils and edema (arrow) adjacent to a small bronchiole (BR). [B] Detail from an RLb mouse displaying edema and numerous neutrophils in the interstitial space around the medium-sized vein (PV). Several alveoli contain transmigrated neutrophils (arrowheads). [C] Detail of a lung tissue from an HTSn mouse showing an airway (AW) flanked by pulmonary arterial (PA) branch. There is no edema or neutrophilic infiltrate in the interstitial or alveolar spaces. Hematoxylin & eosin stains. Bars indicate 100 μm.

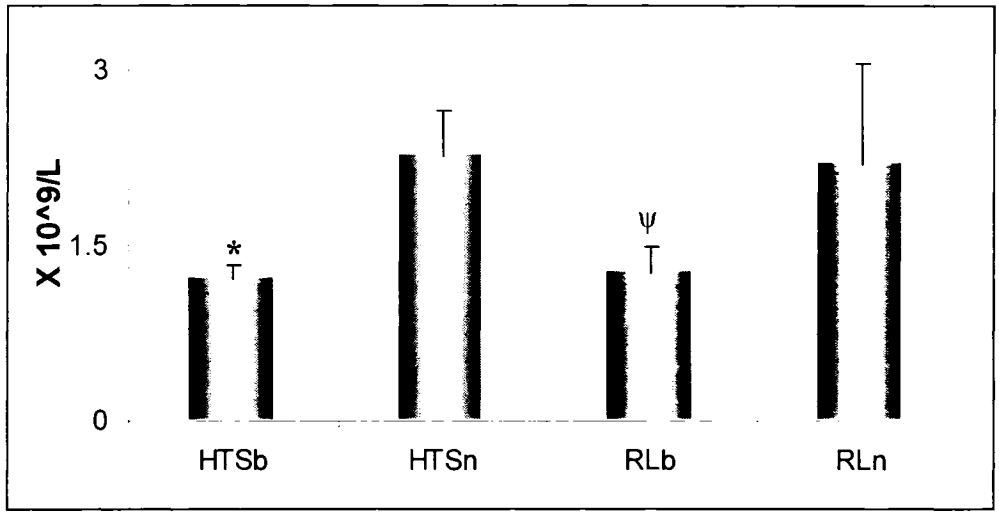

FIGURE 13: Circulating serum leukocyte levels were greatly reduced in both blockade groups irrespective of which resuscitation fluid was used. (*$p<0.05$ vs HTSn, $^{\Psi}p=0.06$ vs HTSn).

Discussion

Resuscitation with hypertonic saline has been shown to have extensive immune benefits, most importantly in reducing the neutrophil mediated tissue injury that ensues after hemorrhagic shock. The present study demonstrates that beneficial effects of HTS resuscitation may be independent of its ability to functionally blunt neutrophil adhesion to endothelium. Monoclonal blockade against CD11b and its endothelial ligand (ICAM-1) with standard resuscitation resulted in attenuations of leukocyte adhesion to endothelium and lung neutrophil sequestration that were similar to that found with hypertonic saline. Nevertheless, while HTS alone reduced vascular permeability and virtually eliminated lung histologic tissue injury, standard RL resuscitation, even supplemented with anti-adhesion blockade clearly worsened lung injury.

Neutrophils taken from animals resuscitated with HTS that were subsequently activated in vitro demonstrated a reduced level of CD11b expression while tending to express greater surface L-selectin than those from RL-resuscitated counterparts. This mild blunting of L-selectin shedding is consistent with other in vitro[37] and in vivo animal[39]

and human [40,41] studies. On the other hand, other investigators report augmented L-selectin shedding with HTS resuscitation in rodent hemorrhagic shock models[17,38]. Neutrophil activation is known to potentiate shedding[78-81] and shedding is likely a requirement for neutrophil rolling[82-84]. Intravital microscopy has shown that hypertonic resuscitation reduces PMN rolling flux in thermal injury[54], ischemia/reperfusion[51]and hemorrhagic shock models[55,56]. Consequently, since HTS reduces PMN rolling, and rolling requires surface L-selectin shedding, one could accept that HTS would cause less L-selectin shedding as this and other studies indicate. Nonetheless, HTS effects on L-selectin remain ambiguous and were not further elucidated in the present study.

As our findings demonstrate, hypertonic saline blunted upregulation of neutrophil surface CD11b; a phenomenon described in both animals and humans[17,40,41,45,48]. While the current study did not evaluate HTS effects on endothelial adhesion molecules, a relatively small number of other studies have. Whereas HTS appears to have a minor effect on EC receptors responsible for neutrophil rolling (E and P-selectin)[38,42,44] it shows important effects on endothelial receptors responsible for neutrophil adhesion. Rodent hepatic ischemia/reperfusion[49] and hemorrhage/resuscitation models[17,50] reveal HTS-mediated attenuation in endothelial ICAM-1 and vascular cell adhesion molecule -1 (VCAM-1), both promoters of neutrophil adhesion. Hence, the available literature and the current phase I results indicate a predominant and unambiguous effect of HTS on adhesion molecules responsible for neutrophil adhesion to endothelium. We thus used monoclonal antibody blockade against CD11b and its endothelial counterreceptor ICAM-1 in phase II and III experiments.

Evaluation of hemodynamic parameters in phase II and III revealed blood pressures that did not differ between groups prior, during and after hypotensive shock. These findngs crudely reflect the appropriateness of resuscitation regimens employed, which have been previously validated by other studies[39,69,73]. The volume of withdrawn

blood in all groups produced a class III hemorrhagic shock yet total fluid volume administered to RL animals was more than double that given to HTS counterparts. This is consistent with clinical administration of much larger doses of isotonic crystalloid in addition to blood (for class III and IV hemorrhagic shock) compared to the small (4cc/kg) doses of HTS resuscitation commonly administered. Phase II rheological factors demonstrated that venular shear stress, the most important microvascular flow parameter, did not differ between groups. This finding is essential if comparisons of leukocyte-endothelial interactions between groups are to follow. While HTSb animals had a slower mean flow velocity than the HTSn group, this difference was not sufficient to cause a significant effect on shear stresses found in these two groups. Nonetheless this may have contributed to the trend of fewer EC-LEU interactions in HTSn as compared to HTSb animals.

Three hours after resuscitation, leukocyte rolling flux was reduced by more than 80% in animals having received monoclonal blockade. This finding was surprising as we did not block receptors generally considered relevant to neutrophil rolling[35,85-87]. Nonetheless, other investigators have reported noteworthy effects of CD11b[88-90] and ICAM-1[91,92] expression on neutrophil rolling. We did not find differences in leukocyte rolling between HTSn and RLn groups. Despite confirming a previous study using the same model[13], these results do not reflect those of other IVM studies using animal IVM in burn[54], ischemia/reperfusion[51] and single-hit hemorrhage and resuscitation[55,56]. Unlike these latter studies, the present animal model involved two distinct host insults (resuscitated hemorrhage followed by mimicked infection) which may have overwhelmed rolling differences at the time of IVM evaluation. Furthermore, the current study may not have had a sufficiently large sample number to appreciate leukocyte rolling differences between resuscitation regimens.

As expected, HTS reduced total leukocyte adhesion to ⅓ that observed with RL. This was true with and without adhesion blockade. While previous IVM models have demonstrated live reductions in leukocyte adherence by HTS resuscitation, the present findings introduce evidence that this effect is similarly recreated in RL resuscitation supplemented by combined monoclonal blockade against CD11b and ICAM-1. Yet, it is clear that neutrophil adhesion to endothelium is not only governed by these two adhesion molecules. Other adhesion molecules (CD11a, VCAM-1, PECAM-1 and others) have been shown to participate in PMN adhesion to endothelium, but little is known about the effect of HTS on them[44,50,84,93,94].

Three and a half hours following resuscitation, HTS animals demonstrated 60% the microvascular leakage seen with RL counterparts receiving isotype antibodies. With adhesion molecule blockade, RL animals also tended to have a reduced venular permeability, but this was not significant. Adhesion blockade in animals resuscitated with hypertonic saline did not cause further reductions in permeability. HTS mediated reductions in permeability were found in parallel to decreases in leukocyte adhesion in the same tissue 30 minutes earlier. Others studies using intravital microscopy have previously reported reduced in vivo venular permeability with HTS administration in ischemia/reperfusion[51], sepsis[71], burns[70] and hemorrhagic shock[55]. Additionally, several studies report HTS mediated reductions in tissue edema and macromolecular leakage using ex vivo histology, bronchoalveolar lavage fluid (BALF) protein content, wet to dry ratios and other techniques[17,64,67,95-98]. Other studies evaluating different injury models have reported diminutions of vascular permeability or tissue edema with blockade against ICAM-1[99-102], CD11b[99,103,104] and other adhesion molecules[105-107] but none have evaluated blockade of the present combination of receptors in hemorrhagic shock. The current findings suggest an important relation,

albeit not necessarily causative, between neutrophil mediated injury and loss of vascular integrity, both attenuated by HTS.

Lung PMN sequestration 24 hours after resuscitation followed the same pattern seen with leukocyte adhesion to endothelium. Myeloperoxidase enzyme levels, a marker of neutrophil accumulation in tissue, was reduced by more than 55% if HTS was used instead of RL in non-blocked animals. Adhesion blockade supplementation in RL animals also reduced sequestration by half. As with neutrophil adhesion, anti-adhesion blockade did not further reduce the lung neutrophil sequestration seen in HTS resuscitated animals. Multiple other studies have demonstrated HTS-related reductions in tissue MPO levels in acute pancreatitis[67], organ ischemia/reperfusion[68] and hemorrhagic shock[13,17,47,64]. As well, monoclonal antibodies against either CD11b[34,108-112] or ICAM-1[32,34,101,111-114] have shown diminished tissue MPO following different forms of organ injury. To our knowledge, no other study has previously reported using complete blockade against the adhesion step of EC/LEU interactions (dual blockade of both CD11b and ICAM-1) in hemorrhagic shock. The present results confirm that HTS resuscitation reduces 24 hour lung neutrophil sequestration to a similar degree as standard RL resuscitation supplemented with anti-adhesion blockade.

Twenty-four hours after HTS resuscitation, serum sodium concentrations resembled those found in RL animals. Thus, differences in leukocyte sequestration and tissue injury were still present despite normalization of sodium plasma concentrations in HTS animals. The lower circulating leukocyte counts found with monoclonal blockade irrespective of type of fluid administered may appear paradoxical. If blockade reduces leukocyte recruitment, one would expect a greater circulating leukocyte pool in these animals. Yet, while reduced circulating leukocyte levels were seen in HTSb animals, this group did not demonstrate greater leukocyte recruitment than HTSn counterparts. Thus, in the current model, fewer circulating leukocytes may not reflect differences in leukocyte

recruitment. We cannot offer a plausible explanation for these findings except that the automated counter system may have failed to discriminate cellular identity when evaluating leukocyte samples bound to different antibodies. To our knowledge, no other study has evaluated effects on circulating leukocytes after double anti-adhesion blockade.

Animal activity one day after resuscitation was more than twice as good with HTS than with standard RL resuscitation. Anti-adhesion blockade added to RL tended to improve activity but not as greatly nor to a significant degree. This followed histologic lung tissue injury, which was also better prevented by HTS than by anti-adhesion blockade. Lung injury found in the combined group of all HTS animals was uniformly better, with injury scores less than 35% those found in combined RL-resuscitated counterparts. Contrarily to earlier leukocyte adherence to endothelium and subsequent lung neutrophil sequestration, monoclonal blockade in either HTS or RL resuscitated animals did not result in reductions of histologic injury scores, tending, instead, to worsen them. This was surprising, as we expected that the reduced neutrophil recruitment following adhesion blockade would necessarily result in attenuations of tissue injury. Both HTS resuscitated groups had histological injury scores that were similar to controls. Direct evaluation of tissue injury by histopathologic evaluation has shown the benefits of HTS resuscitation in trauma[64], pancreatitis[67] and ischemia/reperfusion[68] models. One hit hemorrhagic shock models have also demonstrated diminished lung parenchymal injury following HTS resuscitation in rodents[39,42,47,50,65]. As in the current study, two hit models involving hemorrhagic shock followed by mimicked infection (intratracheal LPS, cecal ligation and puncture) also concur, demonstrating HTS-related reductions in lung and liver histological injury up to 48 hours after resuscitation[13,17,69]. On the other hand, previous studies using adhesion molecule blockade have shown variable effects on tissue lung injury. For example, while in some local lung inflammation models reductions

of neutrophil recruitment and tissue water were noted with monoclonal antibody blockade against the CD18/CD11 complex[103,115] no clear effect on tissue injury was confirmed by histology or other means. In other studies, comparable monoclonal blockade failed to reduce both neutrophil recruitment and tissue injury in lung[116,117] and liver[118]. Moreover, others found that the outcome of blockade was dependent on the type of insult, as anti-CD18/CD11b would reduce lung leukocyte recruitment with certain injury models, but not others[104,119-122]. Anti-ICAM-1 therapy in different local lung and liver insult models has also shown diminished tissue neutrophil recruitment and tissue water content, but again, few studies have evaluated actual tissue injury[114,115,123]. In fact, a study evaluating anti-ICAM-1 treatment in a rat model subjected to local cerebral trauma found a tendency to *worsened* histological tissue injury as compared to controls treated with isotype antibodies[124]. With systemic insults, the beneficial effect of anti-adhesion blockade also remains unclear. Models simulating systemic sepsis have demonstrated that anti-CD18 or anti-ICAM-1 reduced PMN sequestration, having variable effects on tissue water changes and organ function[100,125-127]. Yet, again, none of these studies evaluated tissue injury directly. In a murine model using ceacal ligation and puncture (CLP), CD18 monoclonal antibody prevented peritoneal neutrophil recruitment while increasing lung and liver neutrophil sequestration and worsening liver function[128]. In hemorrhagic shock resuscitation models, while one study reports attenuated histological injury with anti-CD18 blockade[129], others do not evaluate injury by histologic means[28,130]. However, in a two-hit hemorrhagic shock and infection model, investigators found no difference in histological lung injury scores with a tendency to worse scores with monoclonal antibodies against CD18[131]. Furthermore, in the same study, a dramatic *increase* in neutrophil oxidative burst and TNF release was seen by circulating neutrophils from animals treated with anti-CD18 monoclonal antibodies. Few studies have evaluated tissue injury in the context of simultaneous blockade against CD18 and ICAM-1. In a

model of muscular ischemia/reperfusion one such study evaluating this combined blockade demonstrated reduced tissue MPO but failed to show improved tissue viability[34].

Thus, we have demonstrated how, similarly to HTS resuscitation, anti-adhesion blockade with standard resuscitation decreases leukocyte interactions with endothelium (leukocyte rolling, adhesion) and diminishes lung PMN sequestration (MPO). Nonetheless, unlike HTS resuscitation, this treatment did not translate into diminished lung parenchymal injury 24 hours after resuscitation. This remarkable finding could be explained by a variety of mechanisms. First, the antibody clones may have been inadequate, conferring less than complete functional block of adhesion molecules or simply administered in insufficient quantities. Yet, both clones used have been employed widely in the literature. Both the M1/70 anti-CD11b[29,132-135] and the YN1/1.7 anti-ICAM-1[100,136-138] clones have been used extensively to block murine adhesion molecules in vivo. These and other studies also demonstrate that the intravenous dosages of monoclonal antibodies that were given (2.83 and 2.16 mg/kg IV, respectively) represent in vivo saturating concentrations in mice. Furthermore, our phase II experiments demonstrate in vivo that leukocyte adhesion was significantly attenuated by the chosen blockade regimen in RL-resuscitated animals. A second explanation for unabated tissue injury by monoclonal blockade is the fact that adhesion blockade may not affect other injurious functions of neutrophils. Clearly the tissue injury mediated by neutrophil and endothelial activation not only requires neutrophil adhesion to endothelium, but also inappropriate cellular degranulation and release of ROS, matrix metalloproteinases (MMPs) and proteases that directly injure the vessel wall and adjacent tissue parenchyma. As described previously, hypertonic saline administration, blunts the capacity of neutrophils to release ROS and elastases[39,139-144]. Neither the

oxidative burst nor the extent of proteinase release was evaluated in the present study. The phagocytic capacity of neutrophils may also be diminished with hypertonic fluid administration[145,146] independent of its effects on adhesion molecules. Furthermore, the effect of HTS on inflammatory mediators and cytokines is known not to be negligible but has not been addressed by the current study. Finally, our histological results could further be explained by the fact that lung tissue injury resulting from hemorrhagic shock resuscitated with lactated Ringer's is partly CD18/ICAM-1-independent. CD18-dependent PMN transmigration is believed to occur with inflammation in systemic post-capillary venules[147] while in lung, PMN extravasation may occur through CD18-independent mechanisms[120,148]. While hemorrhagic shock resuscitation itself has not been evaluated, it appears that the nature and duration of the stimulus is an important determinant of which type of PMN transmigration occurs after the given insult[149-152]. The present data and that of other studies suggest that the PMN-mediated lung injury following RL-resuscitation in a two-hit model may involve CD18-independent mechanisms. HTS resuscitation alone, on the other hand, not only blocked neutrophil adhesion to endothelium (CD18 dependent mechanism), but also reduced lung injury (CD18 independent mechanisms?) as compared to RL supplemented with anti-adhesion blockade.

In summary, although the present study has confirmed reductions in PMN CD11b expression with HTS-resuscitation, it has shown novel evidence of a more profound effect by HTS, independent of adhesion receptor blockade to maintain microvascular integrity and more particularly to reduce histologic injury in hemorrhagic shock followed by mimicked infection. Further comparisons between PMN blockade and hypertonic resuscitation using monoclonal antibodies or animal genetic knockout models will be needed to qualify the predominant mechanisms behind beneficial tissue effects of hypertonic saline resuscitation in hemorrhagic shock.

References to Manuscript #5

1. Holcroft, J.W., Vassar, M.J., Perry, C.A., Gannaway, W.L. & Kramer, G.C. Perspectives on clinical trials for hypertonic saline/dextran solutions for the treatment of traumatic shock. *Braz J Med Biol Res* **22**, 291-3 (1989).
2. Vassar, M.J., Perry, C.A., Gannaway, W.L. & Holcroft, J.W. 7.5% sodium chloride/dextran for resuscitation of trauma patients undergoing helicopter transport. *Arch Surg* **126**, 1065-72. (1991).
3. Mattox, K.L. et al. Prehospital hypertonic saline/dextran infusion for post-traumatic hypotension. The U.S.A. Multicenter Trial. *Ann Surg* **213**, 482-91 (1991).
4. Younes, R.N. et al. Prognostic factors to predict outcome following the administration of hypertonic/hyperoncotic solution in hypovolemic patients. *Shock* **7**, 79-83. (1997).
5. Vassar, M.J. et al. A multicenter trial for resuscitation of injured patients with 7.5% sodium chloride. The effect of added dextran 70. The Multicenter Group for the Study of Hypertonic Saline in Trauma Patients. *Arch Surg* **128**, 1003-11; discussion 1011-3 (1993).
6. Patrick, D.A., Moore, F.A., Moore, E.E., Barnett, C.B. & Silliman, C.C. Neutrophil priming and activation in the pathogenesis of postinjury multiple organ failure. *New Horiz* **4**, 194-210 (1996).
7. Moore, F.A. & Moore, E.E. Evolving concepts in the pathogenesis of postinjury multiple organ failure. *Surg Clin North Am* **75**, 257-77. (1995).
8. Sauaia, A., Moore, F.A., Moore, E.E. & Lezotte, D.C. Early risk factors for postinjury multiple organ failure. *World J Surg* **20**, 392-400. (1996).
9. Bevilacqua, M.P. & Nelson, R.M. Selectins. *J Clin Invest* **91**, 379-87 (1993).
10. Etzioni, A. Integrins--the glue of life. *Lancet* **353**, 341-3 (1999).
11. Ahmed, N. & Christou, N. Systemic inflammatory response syndrome: interactions between immune cells and the endothelium. *Shock* **6**, S39-42 (1996).
12. Wyman, T.H. et al. A two-insult in vitro model of PMN-mediated pulmonary endothelial damage: requirements for adherence and chemokine release. *Am J Physiol Cell Physiol* **283**, C1592-603. (2002).
13. Pascual, J.L. et al. Hypertonic saline resuscitation attenuates neutrophil lung sequestration and transmigration by diminishing leukocyte-endothelial interactions in a two-hit model of hemorrhagic shock and infection. *J Trauma* **54**, 121-30; discussion 130-2 (2003).
14. Aiboshi, J., Moore, E.E., Ciesla, D.J. & Silliman, C.C. Blood transfusion and the two-insult model of post-injury multiple organ failure. *Shock* **15**, 302-6. (2001).
15. Eissner, B., Matz, K., Smorodchenko, A., Roschmann, A. & v Specht, B.U. Chronic porcine two-hit model with hemorrhagic shock and Pseudomonas aeruginosa sepsis. *Eur Surg Res* **34**, 61-7. (2002).
16. Hoffmann, J.N., Vollmar, B., Inthorn, D., Schildberg, F.W. & Menger, M.D. A chronic model for intravital microscopic study of microcirculatory disorders and leukocyte/endothelial cell interaction during normotensive endotoxemia. *Shock* **12**, 355-64. (1999).
17. Rizoli, S.B. et al. Immunomodulatory effects of hypertonic resuscitation on the development of lung inflammation following hemorrhagic shock. *J Immunol* **161**, 6288-96 (1998).
18. Rivera-Chavez, F.A. et al. L-selectin blockade and liver function in rats after uncontrolled hemorrhagic shock. *J Invest Surg* **14**, 7-12. (2001).
19. Ramos-Kelly, J.R. et al. Multiple selectin blockade with a small molecule inhibitor downregulates liver chemokine expression and neutrophil infiltration after hemorrhagic shock. *J Trauma* **49**, 92-100. (2000).
20. Ramos-Kelly, J.R. et al. Upregulation of lung chemokines associated with hemorrhage is reversed with a small molecule multiple selectin inhibitor. *J Am Coll Surg* **189**, 546-53. (1999).
21. Rivera-Chavez, F. et al. P-selectin blockade is beneficial after uncontrolled hemorrhagic shock. *J Trauma* **45**, 440-5. (1998).
22. Ramamoorthy, C., Sharar, S.R., Harlan, J.M., Tedder, T.F. & Winn, R.K. Blocking L-selectin function attenuates reperfusion injury following hemorrhagic shock in rabbits. *Am J Physiol* **271**, H1871-7. (1996).
23. Schlag, G. et al. Anti-L-selectin antibody treatment of hemorrhagic-traumatic shock in baboons. *Crit Care Med* **27**, 1900-7. (1999).
24. Scalia, R., Armstead, V.E., Minchenko, A.G. & Lefer, A.M. Essential role of P-selectin in the initiation of the inflammatory response induced by hemorrhage and reinfusion. *J Exp Med* **189**, 931-8. (1999).
25. Childs, E.W., Smalley, D.M., Moncure, M., Miller, J.L. & Cheung, L.Y. Effect of LFA-1beta antibody on leukocyte adherence in response to hemorrhagic shock in rats. *Shock* **14**, 49-52. (2000).

26. Bauer, C., Siaplaouras, S., Soule, H.R., Moyle, M. & Marzi, I. A natural glycoprotein inhibitor (NIF) of CD11b/CD18 reduces leukocyte adhesion in the liver after hemorrhagic shock. *Shock* **4**, 187-92. (1995).
27. Mileski, W.J. et al. Inhibition of CD18-dependent neutrophil adherence reduces organ injury after hemorrhagic shock in primates. *Surgery* **108**, 206-12. (1990).
28. Boyd, A.J. et al. A CD18 monoclonal antibody reduces multiple organ injury in a model of ruptured abdominal aortic aneurysm. *Am J Physiol* **277**, H172-82 (1999).
29. Morisaki, T., Goya, T., Toh, H., Nishihara, K. & Torisu, M. The anti Mac-1 monoclonal antibody inhibits neutrophil sequestration in lung and liver in a septic murine model. *Clin Immunol Immunopathol* **61**, 365-75. (1991).
30. Kumasaka, T. et al. Role of the intercellular adhesion molecule-1(ICAM-1) in endotoxin- induced pneumonia evaluated using ICAM-1 antisense oligonucleotides, anti-ICAM-1 monoclonal antibodies, and ICAM-1 mutant mice. *J Clin Invest* **97**, 2362-9. (1996).
31. Liu, P. et al. Activation of Kupffer cells and neutrophils for reactive oxygen formation is responsible for endotoxin-enhanced liver injury after hepatic ischemia. *Shock* **3**, 56-62. (1995).
32. Beyer, A.J., Smalley, D.M., Shyr, Y.M., Wood, J.G. & Cheung, L.Y. PAF and CD18 mediate neutrophil infiltration in upper gastrointestinal tract during intra-abdominal sepsis. *Am J Physiol* **275**, G467-72. (1998).
33. Clark, R.S. et al. Antibodies against Mac-1 attenuate neutrophil accumulation after traumatic brain injury in rats. *J Neurotrauma* **13**, 333-41. (1996).
34. Breidahl, A.F., Hickey, M.J., Stewart, A.G., Hayward, P.G. & Morrison, W.A. Effects of low dose intra-arterial monoclonal antibodies to ICAM-1 and CD11/CD18 on local and systemic consequences of ischaemia-reperfusion injury in skeletal muscle. *Br J Plast Surg* **49**, 202-9. (1996).
35. Nolte, D. et al. Role of Mac-1 and ICAM-1 in ischemia-reperfusion injury in a microcirculation model of BALB/C mice. *Am J Physiol* **267**, H1320-8. (1994).
36. Bucky, L.P. et al. Reduction of burn injury by inhibiting CD18-mediated leukocyte adherence in rabbits. *Plast Reconstr Surg* **93**, 1473-80. (1994).
37. Thiel, M. et al. Effects of hypertonic saline on expression of human polymorphonuclear leukocyte adhesion molecules. *J Leukoc Biol* **70**, 261-73. (2001).
38. Angle, N. et al. Hypertonic saline resuscitation reduces neutrophil margination by suppressing neutrophil L selectin expression. *J Trauma* **45**, 7-12; discussion 12-3 (1998).
39. Murao, Y. et al. Does the timing of hypertonic saline resuscitation affect its potential to prevent lung damage? *Shock* **14**, 18-23. (2000).
40. Angle, N. et al. Hypertonic saline infusion: can it regulate human neutrophil function? *Shock* **14**, 503-8. (2000).
41. Khwaja, K. et al. Hypertonic saline infusion in humans alters the inflammatory response of activated neutrophils. in *Owen H. Wangensteen Surgical Forum, American College of Surgeons* Vol. 195 S87 (San Francisco, California, 2002).
42. Alam, H.B. et al. E- and P-selectin expression depends on the resuscitation fluid used in hemorrhaged rats. *J Surg Res* **94**, 145-52. (2000).
43. Setiadi, H., Sedgewick, G., Erlandsen, S.L. & McEver, R.P. Interactions of the cytoplasmic domain of P-selectin with clathrin- coated pits enhance leukocyte adhesion under flow. *J Cell Biol* **142**, 859-71. (1998).
44. Bradley, J.R., Johnson, D.R. & Pober, J.S. Four different classes of inhibitors of receptor-mediated endocytosis decrease tumor necrosis factor-induced gene expression in human endothelial cells. *J Immunol* **150**, 5544-55. (1993).
45. Ciesla, D.J., Moore, E.E., Gonzalez, R.J., Biffl, W.L. & Silliman, C.C. Hypertonic saline inhibits neutrophil (PMN) priming via attenuation of p38 MAPK signaling. *Shock* **14**, 265-9; discussion 269-70. (2000).
46. Rizoli, S.B., Kapus, A., Parodo, J. & Rotstein, O.D. Hypertonicity prevents lipopolysaccharide-stimulated CD11b/CD18 expression in human neutrophils in vitro: role for p38 inhibition. *J Trauma* **46**, 794-8; discussion 798-9 (1999).
47. Angle, N. et al. Hypertonic saline resuscitation diminishes lung injury by suppressing neutrophil activation after hemorrhagic shock. *Shock* **9**, 164-70 (1998).
48. Rizoli, S.B., Kapus, A., Parodo, J., Fan, J. & Rotstein, O.D. Hypertonic immunomodulation is reversible and accompanied by changes in CD11b expression. *J Surg Res* **83**, 130-5 (1999).
49. Oreopoulos, G.D. et al. In vivo and in vitro modulation of intercellular adhesion molecule (ICAM)-1 expression by hypertonicity. *Shock* **14**, 409-14; discussion 414-5. (2000).
50. Sun, L.L. et al. Early up-regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in rats with hemorrhagic shock and resuscitation. *Shock* **11**, 416-22 (1999).
51. Nolte, D. et al. Attenuation of postischemic microvascular disturbances in striated muscle by hyperosmolar saline dextran. *Am J Physiol* **263**, H1411-6 (1992).

52. Spera, P.A., Arfors, K.E., Vasthare, U.S., Tuma, R.F. & Young, W.F. Effect of hypertonic saline on leukocyte activity after spinal cord injury. *Spine* **23**, 2444-8; discussion 2448-9 (1998).
53. Hartl, R. et al. Hypertonic/hyperoncotic saline attenuates microcirculatory disturbances after traumatic brain injury. *J Trauma* **42**, S41-7 (1997).
54. Barone, M., Jimenez, F., Huxley, V.H. & Yang, X.F. Morphologic analysis of the cerebral microcirculation after thermal injury and the response to fluid resuscitation. *Acta Neurochir Suppl* **70**, 267-8 (1997).
55. Pascual, J.L. et al. Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces in vivo vascular leakage. *Ann Surg* **236**, 634-42. (2002).
56. Saetzler RK, B.M., Buckman Jr RF, Eynon AC, Tuma RF. Hypertonic saline attenuates leukocyte/endothelium and leukocyte/platelet interactions following hemorrhagic shock. *Surgical Forum* **47**, 41-3 (1996).
57. Corso, C.O., Okamoto, S., Ruttinger, D. & Messmer, K. Hypertonic saline dextran attenuates leukocyte accumulation in the liver after hemorrhagic shock and resuscitation. *J Trauma* **46**, 417-23 (1999).
58. Vollmar, B., Lang, G., Menger, M.D. & Messmer, K. Hypertonic hydroxyethyl starch restores hepatic microvascular perfusion in hemorrhagic shock. *Am J Physiol* **266**, H1927-34 (1994).
59. Hirsh, M., Dyugovskaya, L., Bashenko, Y. & Krausz, M.M. Reduced rate of bacterial translocation and improved variables of natural killer cell and T-cell activity in rats surviving controlled hemorrhagic shock and treated with hypertonic saline. *Crit Care Med* **30**, 861-7. (2002).
60. Erbil, Y. et al. Effect of resuscitation on bacterial translocation in haemorrhagic shocked rats. *East Afr Med J* **73**, 699-702. (1996).
61. Tokyay, R. et al. Effects of hypertonic saline dextran resuscitation on oxygen delivery, oxygen consumption, and lipid peroxidation after burn injury. *J Trauma* **32**, 704-12; discussion 712-3. (1992).
62. Reed, L.L. et al. The effect of hypertonic saline resuscitation on bacterial translocation after hemorrhagic shock in rats. *Surgery* **110**, 685-8; discussion 688-90. (1991).
63. Assalia, A., Bitterman, H., Hirsh, T.M. & Krausz, M.M. Influence of hypertonic saline on bacterial translocation in controlled hemorrhagic shock. *Shock* **15**, 307-11. (2001).
64. Shi, H.P., Deitch, E.A., Da Xu, Z., Lu, Q. & Hauser, C.J. Hypertonic saline improves intestinal mucosa barrier function and lung injury after trauma-hemorrhagic shock. *Shock* **17**, 496-501. (2002).
65. Yada-Langui, M.M. et al. Hypertonic saline and pentoxifylline prevent lung injury and bacterial translocation after hemorrhagic shock. *Shock* **14**, 594-8 (2000).
66. Wang, J., Yang, Z. & He, B. [The effects of resuscitation with hypertonic saline solution on the postburn pulmonary injury in scalded dogs]. *Zhonghua Shao Shang Za Zhi* **17**, 276-8. (2001).
67. Shields, C.J. et al. Hypertonic saline attenuates end-organ damage in an experimental model of acute pancreatitis. *Br J Surg* **87**, 1336-40. (2000).
68. Shields, C.J. et al. Hypertonic saline infusion for pulmonary injury due to ischemia- reperfusion. *Arch Surg* **138**, 9-14. (2003).
69. Coimbra, R. et al. Hypertonic saline resuscitation decreases susceptibility to sepsis after hemorrhagic shock. *J Trauma* **42**, 602-6; discussion 606-7 (1997).
70. Barone, M., Jimenez, D.F., Huxley, V.H. & Yang, X.F. Cerebral vascular response to hypertonic fluid resuscitation in thermal injury. *Acta Neurochir Suppl* **70**, 265-6 (1997).
71. de Carvalho, H., Matos, J.A., Bouskela, E. & Svensjo, E. Vascular permeability increase and plasma volume loss induced by endotoxin was attenuated by hypertonic saline with or without dextran. *Shock* **12**, 75-80 (1999).
72. Nolte, D., Lehr, H.A. & Messmer, K. Adenosine inhibits postischemic leukocyte-endothelium interaction in postcapillary venules of the hamster. *Am J Physiol* **261**, H651-5 (1991).
73. Coimbra, R. et al. Hypertonic saline resuscitation restores hemorrhage-induced immunosuppression by decreasing prostaglandin E2 and interleukin-4 production. *J Surg Res* **64**, 203-9 (1996).
74. Baez, S. An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc Res* **5**, 384-94 (1973).
75. House, S.D. & Lipowsky, H.H. Leukocyte-endothelium adhesion: microhemodynamics in mesentery of the cat. *Microvasc Res* **34**, 363-79. (1987).
76. Tangelder, G.J., Janssens, C.J., Slaaf, D.W., oude Egbrink, M.G. & Reneman, R.S. In vivo differentiation of leukocytes rolling in mesenteric postcapillary venules. *Am J Physiol* **268**, H909-15. (1995).
77. Bachofen, M. & Weibel, E.R. Structural alterations of lung parenchyma in the adult respiratory distress syndrome. *Clin Chest Med* **3**, 35-56. (1982).

78. Kishimoto, T.K., Jutila, M.A., Berg, E.L. & Butcher, E.C. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* **245**, 1238-41. (1989).
79. Jutila, M.A., Rott, L., Berg, E.L. & Butcher, E.C. Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1. *J Immunol* **143**, 3318-24. (1989).
80. Maekawa, K. et al. Effects of trauma and sepsis on soluble L-selectin and cell surface expression of L-selectin and CD11b. *J Trauma* **44**, 460-8. (1998).
81. McGill, S.N., Ahmed, N.A., Hu, F., Michel, R.P. & Christou, N.V. Shedding of L-selectin as a mechanism for reduced polymorphonuclear neutrophil exudation in patients with the systemic inflammatory response syndrome. *Arch Surg* **131**, 1141-6; discussion 1147. (1996).
82. Walcheck, B. et al. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. *Nature* **380**, 720-3. (1996).
83. Hafezi-Moghadam, A. & Ley, K. Relevance of L-selectin shedding for leukocyte rolling in vivo. *J Exp Med* **189**, 939-48. (1999).
84. Hafezi-Moghadam, A., Thomas, K.L., Prorock, A.J., Huo, Y. & Ley, K. L-selectin shedding regulates leukocyte recruitment. *J Exp Med* **193**, 863-72. (2001).
85. Becker, M.D., Garman, K., Whitcup, S.M., Planck, S.R. & Rosenbaum, J.T. Inhibition of leukocyte sticking and infiltration, but not rolling, by antibodies to ICAM-1 and LFA-1 in murine endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci* **42**, 2563-6. (2001).
86. Mansson, P., Zhang, X.W., Jeppsson, B., Johnell, O. & Thorlacius, H. Critical role of P-selectin-dependent rolling in tumor necrosis factor- alpha-induced leukocyte adhesion and extravascular recruitment in vivo. *Naunyn Schmiedebergs Arch Pharmacol* **362**, 190-6. (2000).
87. Henderson, R.B. et al. The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and alpha4 integrin in the inflammatory response of neutrophils. *J Exp Med* **194**, 219-26. (2001).
88. Krieglstein, C.F. et al. Effect of anti-CD11b (alphaM-MAC-1) and anti-CD54 (ICAM-1) monoclonal antibodies on indomethacin induced chronic ileitis in rats. *Int J Colorectal Dis* **14**, 219-23. (1999).
89. Kunkel, E.J., Dunne, J.L. & Ley, K. Leukocyte arrest during cytokine-dependent inflammation in vivo. *J Immunol* **164**, 3301-8. (2000).
90. Jung, U., Norman, K.E., Scharffetter-Kochanek, K., Beaudet, A.L. & Ley, K. Transit time of leukocytes rolling through venules controls cytokine- induced inflammatory cell recruitment in vivo. *J Clin Invest* **102**, 1526-33. (1998).
91. Rijcken, E. et al. ICAM-1 and VCAM-1 antisense oligonucleotides attenuate in vivo leucocyte adherence and inflammation in rat inflammatory bowel disease. *Gut* **51**, 529-35. (2002).
92. Foitzik, T., Eibl, G. & Buhr, H.J. Therapy for microcirculatory disorders in severe acute pancreatitis: comparison of delayed therapy with ICAM-1 antibodies and a specific endothelin A receptor antagonist. *J Gastrointest Surg* **4**, 240-6; discussion 247. (2000).
93. Lozano, D.D., Kahl, E.A., Wong, H.P., Stephenson, L.L. & Zamboni, W.A. L-selectin and leukocyte function in skeletal muscle reperfusion injury. *Arch Surg* **134**, 1079-81. (1999).
94. Yadav, S.S. et al. L-selectin and ICAM-1 mediate reperfusion injury and neutrophil adhesion in the warm ischemic mouse liver. *Am J Physiol* **275**, G1341-52. (1998).
95. Shields, C.J. et al. Pulmonary injury due to ischemia - reperfusion is ameliorated by hypertonic saline infusion. in *First joint meeting Surgical Infection Society Surgical Infection Society - Europe* 151 (Westin Palace Hotel, Madrid, Spain, 2002).
96. Kinsky, M.P., Milner, S.M., Button, B., Dubick, M.A. & Kramer, G.C. Resuscitation of severe thermal injury with hypertonic saline dextran: effects on peripheral and visceral edema in sheep. *J Trauma* **49**, 844-53. (2000).
97. Jerome, S.N., Akimitsu, T. & Korthuis, R.J. Leukocyte adhesion, edema, and development of postischemic capillary no- reflow. *Am J Physiol* **267**, H1329-36 (1994).
98. Rabinovici, R., Vernick, J., Hillegas, L. & Neville, L.F. Hypertonic saline treatment of acid aspiration-induced lung injury. *J Surg Res* **60**, 176-80 (1996).
99. Mulligan, M.S. et al. Role of leukocyte adhesion molecules in complement-induced lung injury. *J Immunol* **150**, 2401-6. (1993).
100. Gao, X. et al. Differential role of CD18 integrins in mediating lung neutrophil sequestration and increased microvascular permeability induced by Escherichia coli in mice. *J Immunol* **167**, 2895-901. (2001).
101. Sun, Z. et al. Effects of inhibition of PAF, ICAM-1 and PECAM-1 on gut barrier failure caused by intestinal ischemia and reperfusion. *Scand J Gastroenterol* **36**, 55-65. (2001).
102. Lundberg, A.H. et al. Blocking pulmonary ICAM-1 expression ameliorates lung injury in established diet-induced pancreatitis. *Ann Surg* **233**, 213-20. (2001).
103. Folkesson, H.G. & Matthay, M.A. Inhibition of CD18 or CD11b attenuates acute lung injury after acid instillation in rabbits. *J Appl Physiol* **82**, 1743-50. (1997).

104. Mulligan, M.S. et al. Role of beta 1, beta 2 integrins and ICAM-1 in lung injury after deposition of IgG and IgA immune complexes. *J Immunol* **150**, 2407-17. (1993).
105. Mulligan, M.S., Miyasaka, M., Tamatani, T., Jones, M.L. & Ward, P.A. Requirements for L-selectin in neutrophil-mediated lung injury in rats. *J Immunol* **152**, 832-40. (1994).
106. Mulligan, M.S. et al. Neutrophil-dependent acute lung injury. Requirement for P-selectin (GMP-140). *J Clin Invest* **90**, 1600-7. (1992).
107. Kushimoto, S. et al. Pulmonary vascular injury induced by hemorrhagic shock is mediated by P-selectin in rats. *Thromb Res* **82**, 97-106. (1996).
108. Weaver, K.D., Branch, C.A., Hernandez, L., Miller, C.H. & Quattrocchi, K.B. Effect of leukocyte-endothelial adhesion antagonism on neutrophil migration and neurologic outcome after cortical trauma. *J Trauma* **48**, 1081-90. (2000).
109. Sun, X.M. et al. Role of leukocyte beta 2-integrin in PAF-induced shock and intestinal injury. *Am J Physiol* **270**, G184-90. (1996).
110. Zhang, Z.G., Chopp, M., Tang, W.X., Jiang, N. & Zhang, R.L. Postischemic treatment (2-4 h) with anti-CD11b and anti-CD18 monoclonal antibodies are neuroprotective after transient (2 h) focal cerebral ischemia in the rat. *Brain Res* **698**, 79-85. (1995).
111. Yoshida, N. et al. Role of neutrophil-mediated inflammation in aspirin-induced gastric mucosal injury. *Dig Dis Sci* **40**, 2300-4. (1995).
112. Chopp, M., Li, Y., Jiang, N., Zhang, R.L. & Prostak, J. Antibodies against adhesion molecules reduce apoptosis after transient middle cerebral artery occlusion in rat brain. *J Cereb Blood Flow Metab* **16**, 578-84. (1996).
113. Knoblach, S.M. & Faden, A.I. Administration of either anti-intercellular adhesion molecule-1 or a nonspecific control antibody improves recovery after traumatic brain injury in the rat. *J Neurotrauma* **19**, 1039-50. (2002).
114. Colletti, L.M. et al. Tumor necrosis factor up-regulates intercellular adhesion molecule 1, which is important in the neutrophil-dependent lung and liver injury associated with hepatic ischemia and reperfusion in the rat. *Shock* **10**, 182-91. (1998).
115. Moreland, J.G., Fuhrman, R.M., Pruessner, J.A. & Schwartz, D.A. CD11b and intercellular adhesion molecule-1 are involved in pulmonary neutrophil recruitment in lipopolysaccharide-induced airway disease. *Am J Respir Cell Mol Biol* **27**, 474-80. (2002).
116. Guha, S.C. et al. Is the CD18 adhesion complex of polymorphonuclear leukocytes involved in smoke-induced lung damage? A morphometric study. *J Burn Care Rehabil* **14**, 503-11. (1993).
117. Thomas, D.D. et al. CD18-independent mechanism of neutrophil emigration in the rabbit lung after ischemia-reperfusion. *Ann Thorac Surg* **60**, 1360-6. (1995).
118. Langdale, L.A. et al. Neutrophils contribute to hepatic ischemia-reperfusion injury by a CD18-independent mechanism. *J Leukoc Biol* **53**, 511-7. (1993).
119. Motosugi, H., Quinlan, W.M., Bree, M. & Doerschuk, C.M. Role of CD11b in focal acid-induced pneumonia and contralateral lung injury in rats. *Am J Respir Crit Care Med* **157**, 192-8. (1998).
120. Hellewell, P.G., Young, S.K., Henson, P.M. & Worthen, G.S. Disparate role of the beta 2-integrin CD18 in the local accumulation of neutrophils in pulmonary and cutaneous inflammation in the rabbit. *Am J Respir Cell Mol Biol* **10**, 391-8. (1994).
121. Mulligan, M.S., Miyasaka, M. & Ward, P.A. Protective effects of combined adhesion molecule blockade in models of acute lung injury. *Proc Assoc Am Physicians* **108**, 198-208. (1996).
122. Mulligan, M.S. et al. Roles of beta 2 integrins of rat neutrophils in complement- and oxygen radical-mediated acute inflammatory injury. *J Immunol* **148**, 1847-57. (1992).
123. Tang, W.W. et al. Intratracheal injection of endotoxin and cytokines. IX. Contribution of CD11a/ICAM-1 to neutrophil emigration. *Am J Physiol* **269**, L653-9. (1995).
124. Isaksson, J., Hillered, L. & Olsson, Y. Cognitive and histopathological outcome after weight-drop brain injury in the rat: influence of systemic administration of monoclonal antibodies to ICAM-1. *Acta Neuropathol (Berl)* **102**, 246-56. (2001).
125. Walsh, C.J. et al. Anti-CD18 antibody attenuates neutropenia and alveolar capillary- membrane injury during gram-negative sepsis. *Surgery* **110**, 205-11; discussion 211-2. (1991).
126. Eichacker, P.Q. et al. Leukocyte CD18 monoclonal antibody worsens endotoxemia and cardiovascular injury in canines with septic shock. *J Appl Physiol* **74**, 1885-92. (1993).
127. Thomas, J.R., Harlan, J.M., Rice, C.L. & Winn, R.K. Role of leukocyte CD11/CD18 complex in endotoxic and septic shock in rabbits. *J Appl Physiol* **73**, 1510-6. (1992).
128. Mercer-Jones, M.A. et al. Inhibition of neutrophil migration at the site of infection increases remote organ neutrophil sequestration and injury. *Shock* **8**, 193-9. (1997).
129. Vedder, N.B. et al. A monoclonal antibody to the adherence-promoting leukocyte glycoprotein, CD18, reduces organ injury and improves survival from hemorrhagic shock and resuscitation in rabbits. *J Clin Invest* **81**, 939-44. (1988).

130. Fabian, T.C., Croce, M.A., Stewart, R.M., Dockter, M.E. & Proctor, K.G. Neutrophil CD18 expression and blockade after traumatic shock and endotoxin challenge. *Ann Surg* **220**, 552-61; discussion 561-3. (1994).
131. Lyden, S.P. et al. Transient inhibition of CD18-dependent leukocyte functions after hemorrhage and polymicrobial sepsis. *Surgery* **123**, 679-91. (1998).
132. Burch, R.M., Noronha-Blob, L., Bator, J.M., Lowe, V.C. & Sullivan, J.P. Mice treated with a leumedin or antibody to Mac-1 to inhibit leukocyte sequestration survive endotoxin challenge. *J Immunol* **150**, 3397-403. (1993).
133. Gyetko, M.R. et al. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary Pseudomonas aeruginosa infection. *J Immunol* **165**, 1513-9. (2000).
134. Schon, M., Denzer, D., Kubitza, R.C., Ruzicka, T. & Schon, M.P. Critical role of neutrophils for the generation of psoriasiform skin lesions in flaky skin mice. *J Invest Dermatol* **114**, 976-83. (2000).
135. Hakugawa, J., Bae, S.J., Tanaka, Y. & Katayama, I. The inhibitory effect of anti-adhesion molecule antibodies on eosinophil infiltration in cutaneous late phase response in Balb/c mice sensitized with ovalbumin (OVA). *J Dermatol* **24**, 73-9. (1997).
136. Farkas, S. et al. Quantification of mucosal leucocyte endothelial cell interaction by in vivo fluorescence microscopy in experimental colitis in mice. *Clin Exp Immunol* **126**, 250-8. (2001).
137. Essani, N.A. et al. Cytokine-induced upregulation of hepatic intercellular adhesion molecule-1 messenger RNA expression and its role in the pathophysiology of murine endotoxin shock and acute liver failure. *Hepatology* **21**, 1632-9. (1995).
138. Goncalves, A.S. & Appelberg, R. Modulation of neutrophil influx with cell adhesion molecule specific antibodies during nonspecific and immune mediated inflammatory reactions. *Scand J Immunol* **51**, 485-90. (2000).
139. Ciesla, D.J., Moore, E.E., Musters, R.J., Biffl, W.L. & Silliman, C.A. Hypertonic saline alteration of the PMN cytoskeleton: implications for signal transduction and the cytotoxic response. *J Trauma* **50**, 206-12. (2001).
140. Gonzalez, R.J. et al. Hyperosmolarity abrogates neutrophil cytotoxicity provoked by post- shock mesenteric lymph. *Shock* **18**, 29-32. (2002).
141. Junger, W.G. et al. Hypertonicity regulates the function of human neutrophils by modulating chemoattractant receptor signaling and activating mitogen-activated protein kinase p38. *J Clin Invest* **101**, 2768-79 (1998).
142. Rhee, P. et al. Lactated Ringer's solution resuscitation causes neutrophil activation after hemorrhagic shock. *J Trauma* **44**, 313-9 (1998).
143. Ciesla, D.J., Moore, E.E., Biffl, W.L., Gonzalez, R.J. & Silliman, C.C. Hypertonic saline attenuation of the neutrophil cytotoxic response is reversed upon restoration of normotonicity and reestablished by repeated hypertonic challenge. *Surgery* **129**, 567-75. (2001).
144. Ciesla, D.J., Moore, E.E., Zallen, G., Biffl, W.L. & Silliman, C.C. Hypertonic saline attenuation of polymorphonuclear neutrophil cytotoxicity: timing is everything. *J Trauma* **48**, 388-95 (2000).
145. Hampton, M.B., Chambers, S.T., Vissers, M.C. & Winterbourn, C.C. Bacterial killing by neutrophils in hypertonic environments. *J Infect Dis* **169**, 839-46 (1994).
146. Matsumoto, T. et al. Neutrophil function in hyperosmotic NaCl is preserved by phosphoenol pyruvate. *Urol Res* **19**, 223-7 (1991).
147. Wagner, J.G. & Roth, R.A. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacol Rev* **52**, 349-74. (2000).
148. Doerschuk, C.M. et al. Neutrophil emigration in the lungs. *Nihon Kyobu Shikkan Gakkai Zasshi* **34 Suppl**, 141-5. (1996).
149. Winn, R.K. et al. Role of protein synthesis and CD11/CD18 adhesion complex in neutrophil emigration into the lung. *Exp Lung Res* **19**, 221-35. (1993).
150. Burns, A.R. & Doerschuk, C.M. Quantitation of L-selectin and CD18 expression on rabbit neutrophils during CD18-independent and CD18-dependent emigration in the lung. *J Immunol* **153**, 3177-88. (1994).
151. Issekutz, A.C. Adhesion molecules mediating neutrophil migration to arthritis in vivo and across endothelium and connective tissue barriers in vitro. *Inflamm Res* **47 Suppl 3**, S123-32. (1998).
152. Issekutz, A.C., Chuluyan, H.E. & Lopes, N. CD11/CD18-independent transendothelial migration of human polymorphonuclear leukocytes and monocytes: involvement of distinct and unique mechanisms. *J Leukoc Biol* **57**, 553-61 (1995).

Conclusion

The polymorphonuclear neutrophil is a principal effector of the non specific immune response and is essential for host defence. However, much evidence indicates that the neutrophil is also intimately involved in orchestrating the systemic inflammation that can follow resuscitation of severe hemorrhagic shock. Such inflammation may lead to global tissue destruction and organ dysfunction that may ultimately cause the demise of the host. More particularly, exaggerated neutrophil interactions with activated endothelium appear to be an initial step in the convergence of over-exuberant immune elements leading to systemic inflammation and organ injury.

Isotonic crystalloid solutions most often administered in the form of lactated Ringers' have long been the standard therapy for resuscitating hemorrhagic shock, to restore intravascular volume deficits. While historically, such resuscitation dramatically increased the survival of hemorrhagic shock victims, it is now known that it can result in further activation of the neutrophil and endothelial cell, enhancing their interactions and fostering systemic inflammation. Indeed, one of the major causes of late mortality in resuscitated hemorrhagic shock victims is systemic inflammation leading to multiorgan dysfunction. Hemorrhagic shock resuscitation with hypertonic solutions is not only safe and efficacious for the restoration of macrohemodynamic parameters, but also appears to confer certain immune benefits which may minimize host progression through systemic inflammation and organ dysfunction.

In particular, HTS reduces neutrophil interactions with endothelium for hours to days following resuscitation while maintaining vascular integrity and blunting capillary leakiness. Furthermore, it reduces pulmonary accumulation of activated neutrophils attenuating the resultant histologic injury. These effects of HTS may also translate in improved long term survival. Interestingly, the downregulatory influence of HTS on neutrophil and endothelial adhesion molecules is not the predominant mechanism by

which hypertonicity confers reduced pulmonary histopathology and greater host survival. Other elements such as the capacity of HTS to reduce PMN release of cytotoxic substances may be more critical mechanisms behind the clinical immune benefits of hypertonic resuscitation. Much works remains in the search to characterize the precise nature of effects of hypertonicity in the resuscitation of hemorrhagic shock. Perhaps this work and that of others will help elucidate the future role that HTS will have in reducing trauma-related mortality in intensive care units.

Original Scholarship

We believe the presented work displays original scholarship contributing to the advancement of knowledge.

The novel murine hemorrhagic model which was employed allowed for live observation of the microcirculation never evaluated before in so called 'two hit conditions'. This intravital microscopy model confirmed that hemorrhagic shock resuscitation with hypertonic saline reduces systemic in vivo interactions between neutrophils and endothelium. This concept, though never demonstrated before, had been presupposed from the known HTS-mediated downregulation of PMN and EC adhesion molecules in such conditions. Furthermore, attenuations of live EC/PMN interactions by hypertonic resuscitation were found to be associated with a simultaneous reduction in microcirculatory leakiness of the same vessels evaluated at the same time interval following hypertonic resuscitation; another process often implied but never concomitantly demonstrated in vivo before.

The persistence of HTS effects on EC/PMN interactions well beyond the early post resuscitative phase is also a novel finding never demonstrated before in vivo. While in vitro receptor evaluations and ex vivo tissue histology assessment had previously shown contradictory results unable to establish the precise duration of immune HTS effects, the current findings confirm, both in vivo and ex vivo, that these effects are still ongoing a day after resuscitation.

While some previous reports had shown reductions in parenchymal sequestration of neutrophils by HTS resuscitation, this work is the first to concurrently confirm post mortem reduced accumulation of neutrophils in the lungs of the same animals having previously demonstrated attenuation of EC/PMN interactions in vivo. Additional post-mortem histologic assessment of the lungs of the same HTS resuscitated animals also confirmed attenuated microvascular neutrophil-endothelial interactions.

The last set of experiments shed new light on predominant mechanisms of reduced tissue injury found in the lungs of HTS resuscitated animals. While much of the literature suggests that functional anti-adhesive effects of hypertonic saline resuscitation are at the basis of improved tissue histopathology, the current findings place this widespread belief in doubt. An innovative comparison between standard RL resuscitation supplemented with an artificial blockade of neutrophil adhesion failed to recreate attenuations in histologic injury found with HTS resuscitation alone. This occurred despite validation that the model was adequately working as both groups displayed similar reductions in live EC/PMN interactions and tissue neutrophil sequestration. Thus, more experiments need to be conducted to establish the principal mechanism responsible for beneficial effects of hypertonic saline on tissue histopathology and pulmonary injury.

As all research conducted on animals, this work cannot prove or disprove the clinical usefulness of HTS resuscitation in human patients. Nonetheless, we believe that this work provides valuable advancement knowledge of the microcirculatory effects of hypertonic resuscitation, both in vivo and ex vivo, providing a novel insight into the mechanism by which they occur. This research provides a base on which additional experiments can be designed to better delineate immunemediated effects of hypertonic saline resuscitation of hemorrhagic shock in trauma patients.

Acknowledgements

The work presented could not have been completed without the tireless work, guidance, assistance, patience and mentorship of key individuals. I would like to acknowledge the Surgical Infection Society, the McGill Surgeon Scientist Program and the McGill University Health Sciences Center Research Institute for scholarships that funded over five years of research. As well several colleagues, in particular Andrew Seely, for the long discussions and feedback sessions and for taking the time to thoroughly and honestly criticise ideas, experiments and manuscripts. A special thanks to Lorenzo Ferri, Kosar Khwaja and Giuseppina Campisi for their support, their help with protocol design and experiments in addition to the correction of manuscripts. Much appreciated was the technical assistance of David Hori, Carmen Loiselle, Renée Bernatchez and Lucia Fallavolita for experimental procedures they agreed to help with despite their complete lack of time in their overburdened schedules. I am also grateful for the guidance and assistance of Dr. René Michel; for his patience with my never ending requests. Very importantly, this work would not have been possible without the expertise, skill, guidance and instruction of Betty Giannias who taught me numerous procedures and protocols, who sat with me to learn new ones and to figure out the newer technologies and instruments we acquired over the years. Mary Bouldadakis will also be remembered for always being ready to help with manuscripts and laboratory meetings. My greatest gratitude is for Dr. Nicolas Christou, who accepted me under his supervision despite not having any more available space for research residents; who believed in me and in my work, encouraging me through his patience, support and guidance. He is a mentor that I admire and can only strive to emulate in my future career. My most important role model remains my father, a PhD himself, who taught me the basics of life; honesty, integrity, hard work, discipline and an ever-constant search for truth. He taught me to be suspicious of the glitters of titles, acclamations and awards, which time and

again only reflect superficial facets of the human person. Lastly, I cannot forget my loved ones: my two sons Mateo and Charles but most importantly the endless love, patience and true friendship of Dominique Charette who has made all the work worthwhile, providing me with the best reason to struggle and stay the course with those sometimes difficult endeavours.

rch Personnel and Qualifications (See Instructions - Section 4)

mes of all individuals who will work with animals in this study (including the Principal Investigator) and their employment on (Investigator, technician/research assistant, under graduate or graduate student, fellow or student supervisor). Tho Investigator certifies that all personnel listed in this section will be provided with the specific training and/or experience which s them to perform the procedures described in the protocol. Each person listed in this section must initial to indicate s/he have this Application.

AME	CLASSIFICATION	INITIAL
Nicolas V. Christou MD PhD	Principal Investigator	
Jose L Pascuel MD	Research Fellow	
Lorenzo Ferri MD	Research Fellow	
Betty Giannias	Research Assistant	

5. Reviewer's Modification(s): The following modification(s) have been made to this Application during the review process. Please make these changes to your original documentation. You must comply with the recommended changes as a condition of approval for this Application.

This is a Blend since it is a non-survival experiment

6. Summary (See Instructions - Section 6)

BACKGROUND INFORMATION: (Include here the potential benefit to human or animal health or to the advancement of scientific knowledge) – LAY TERMINOLOGY

> Polymorphonuclear neutrophils (PMNs) are the first cells to be attracted to sites of injury when an inflammatory stimulus affects the host. In health this involves an ordered sequence of steps in which PMNs and vessel endothelial cells (ECs) interact to eventually allow passage of the neutrophil to the injurious agent. When a pathologic stimulus occurs, such as hemorrhagic shock, this orderly process is disturbed leading to inappropriate PMN – endothelial interactions which eventually leads to direct organ damage by PMNs. Hypertonic Saline resuscitation of such shock has demonstrated preliminary evidence that it may reduce such inappropriate PMN interactions and hence lead to less organ damage. Several studies indicate that this protective effect is secondary to alteration in adhesion molecules governing these interactions. However no *in vivo* model exists to date that has actually permitted direct visualization of these altered interactions

GLOBAL AIM OF THE STUDY: (Include here the principal direction of the study) - LAY TERMINOLOGY

>This study plans to quantify alterations in PMN – endothelial interactions in mice after resuscitation of hemorrhagic shock with either hypertonic saline (HTS) or ringer's lactate (RL). Those interactions will be observed and quantified using intravital microscopy.

SPECIFIC OBJECTIVE(S) OF THE STUDY: (Distinct from procedures) - LAY TERMINOLOGY

⇒To determine if mice resuscitated from hemorrhagic shock with hypertonic saline demonstrate less PMN rolling, adhesion and transmigration on endothelium and an increased rolling velocity.

⇒ To determine if the vascular permeability of hypertonic saline resuscitated mice as determined by FITC-labeled albumin leakage is decreased as compared to RL resuscitated counterparts

⇒To determine if Hypertonic saline-resuscitated mice demonstrate diminished PMN expression of CD18/CD11b and L-selectin as well as diminished endothelial expression of ICAM-1 expression as compared to their RL counterparts

4. Research Personnel and Qualifications (See Instructions - Section 4)

Give the names of all individuals who will work with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician/research assistant, under graduate or graduate student, fellow or student supervisor). The Principal Investigator certifies that all personnel listed in this section will be provided with the specific training and/or experience which qualifies them to perform the procedures described in the protocol. Each person listed in this section must initial to indicate s/he have read this Application.

NAME	CLASSIFICATION	INITIAL
Nicolas V. Christou MD PhD	Principal Investigator	
Jose L Pascual MD	Research Fellow	
Lorenzo Ferri MD .	Research Fellow	
Betty Giannias	Research Assistant	

5. Reviewer's Modification(s): The following modification(s) have been made to this Application during the review process. Please make these changes to your original documentation. You must comply with the recommended changes as a condition of approval for this Application.

6. Summary (See Instructions - Section 6)

> Polymorphonuclear neutrophils (PMNs) are the first cells to be attracted to sites of injury when an inflammatory stimulus affects the host. In health this involves an ordered sequence of steps in which PMNs and vessel endothelial cells (ECs) interact to eventually allow passage of the neutrophil to the injurious agent. When a pathologic stimulus occurs, such as hemorrhagic shock, this orderly process Is disturbed leading to inappropriate PMN – endothelial interactions which eventually leads to direct organ damage by PMNs. Hypertonic Saline resuscitation of such shock has demonstrated preliminary evidence that it may reduce such inappropriate PMN interactions and hence lead to less organ damage. Several studies indicate that this protective effect is secondary to alteration in adhesion molecules governing these interactions. However no *in vivo* model exists to date that has actually permitted direct visualization of these altered interactions

>This study plans to quantify alterations in PMN – endothelial interactions in mice after resuscitation of hemorrhagic shock with either hypertonic saline (HTS) or ringer's lactate (RL). These interactions will be observed and quantified using intravital microscopy.

⇒To determine if mice resuscitated from hemorrhagic shock with hypertonic saline demonstrate less PMN rolling, adhesion and transmigration on endothelium and an increased rolling velocity.
⇒To determine if the vascular permeability of hypertonic saline resuscitated mice as determined by FITC-labeled albumin leakage is decreased as compared to RL resuscitated counterparts
⇒To determine if Hypertonic saline-resuscitated mice demonstrate diminished PMN expression of CD18/CD11b and L-selectin as well as diminished endothelial expression of ICAM-1 expression as compared to their RL counterparts

[redacted]

>The following will be performed on the mice under general anesthesia: internal jugular and carotid artery catheterization, cremasteric muscle dissection, induction of hypotension, hypertonic saline or Ringer's Lactate infusion, Fluorescent Albumin Intravenous administration, anesthetic overdose and cervical dislocation

7.a) Animal Subject Description:

Animal Species	Strain	Age	Sex	Weight	# needed at one time	Total per year	#/cage
Mice	Swiss Webster		Male	25-30	10-20	150	4

Animals used for genetic alterations:

Category	Total per year
Oocyte donors	
Pseudo-pregnant females	
Stud males	
Other (specify):	
Other (specify):	

[redacted]

Altered PMN-endothelial interactions: Sham (20), HTS (20), RL (20) Albumin leakage from vasculature: Sham (20), HTS (20), RL (20) Additional mice for losses due to technical complications (as the surgical technique is being learned) requiring premature sacrifice prior to the end of the experiment. (30)

[redacted]

The study requires the characterization of PMN endothelial interactions in vivo and the analysis of fresh blood from recently resuscitated mice.

[redacted]

Our laboratory has significant experience with this strain of mouse especially using intravital microscopy

Purpose of Animal Use (See Instructions - Section 7) 1 [ ] 2 [X] 3 [ ] 4 [ ] 5 [ ]

7.b) Animal Husbandry & Care

Special cages No [X] Yes [ ] Specify

Special diet No [X] Yes [ ] Specify

Special handling No [X] Yes [ ] Specify

Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function, (e.g. stress, radiation, steroids, chemotherapeutics)?

No [X] Yes [ ] Specify

Multiply Facility Housing? Yes [ ] No [X]

Indicate all facilities where animals will be housed: RVH H3.74

☐ the laboratory under supervision of laboratory personnel

The cages will be labeled with biohazard or radiation labels Yes ☐ No ☐

Describe measures that will be used to reduce risk to the environment and animal facility personnel:

---

11. Method of Euthanasia:

SPECIES (specify)

Mice	anaesthetic overdose, agent	Ketamine/xylozine	Route	Intravenously
	exsanguination with anaesthesia, agent		Route	
Mice	Cervical dislocation			
	Decapitation: with anaesthesia ☐	specify	Without anaesthesia ☐	
	CO_2 chamber			
	other (specify)			
	not applicable (explain)			

4. Research Personnel and Qualifications (See Instructions - Section 4)

Give the names of all individuals who will work with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician/research assistant, under graduate or graduate student, fellow or student supervisor). The Principal Investigator certifies that all personnel listed in this section will be provided with the specific training and/or experience which qualifies them to perform the procedures described in the protocol. Each person listed in this section must initial to indicate s/he have read this Application.

NAME	CLASSIFICATION	INITIAL
Nicolas V. Christou MD PhD	Principal Investigator	
Jose L Pascual MD	Research Fellow	
Prosanto Chaudhury MD	Research Fellow	
Betty Giannias	Research Assistant	

5. Reviewer's Modification(s):

The following modification(s) have been made to this Application during the review process. Please make these changes to your original documentation. You must comply with the recommended changes as a condition of approval for this Application.

6. Summary (See Instructions - Section 6)

BACKGROUND INFORMATION (indicate here the [illegible] health or to the advancement of [illegible] knowledge) - LAY TERMINOLOGY

> Polymorphonuclear neutrophils (PMNs) are the first cells to be attracted to sites of injury when an inflammatory stimulus affects the host. In health this involves an ordered sequence of steps in which PMNs and vessel endothelial cells (ECs) interact to eventually allow passage of the neutrophil to the injurious agent. Hemorrhagic shock disrupts this orderly process leading to inappropriate PMN – endothelial interactions eventually leading to direct organ damage by PMNs. We have already demonstrated diminished EC/PMN interactions and vessel albumin leakage when hemorrhagic shock is resuscitated with 7.5% hypertonic saline (HTS) instead of the traditional resuscitation with ringer's lactate (RL). However, it remains unclear whether hypertonicity is protective or whether traditional RL resuscitation is actually deleterious.

GLOBAL AIM OF THE STUDY (indicate here the principal direction of the study) - LAY TERMINOLOGY

>This study will evaluate PMN rolling, adherence, rolling velocity and transmigration across post capillary venule endothelium in animals having undergone hemorrhagic shock followed by resuscitation using different fluid preparations. These interactions will be observed and quantified with intravital microscopy and epifluorescence using FITC-labeled albumin.

SPECIFIC OBJECTIVE(S) OF THE STUDY ([illegible]) - LAY TERMINOLOGY

⇒To determine if mice resuscitated from hemorrhagic shock with 0.9% normal saline (NS), 10% pentastarch, 5% dextrose solution (D5W), D5½NS (half D5W and half NS) and dextran demonstrate diminished PMN rolling, rolling velocity, adherence and transmigration through EC as compared to RL.

⇒To determine if vascular permeability (as determined by FITC-labeled albumin leakage) of differently resuscitated mice (as above) is altered as compared to that using traditional RL resuscitation.

⇒To determine which and how different resuscitation fluids alter surface PMN expression of adhesion molecules CD18/CD11b and L-selectin, both essential for PMN-EC interactions.

SUMMARY OF PROCEDURES REQUIRED FOR [illegible] ANIMAL USE DATA FORM (please give a [illegible] summary [illegible] words or less) that indicates [illegible] the procedures used) - LAY TERMINOLOGY

>The following will be performed on the mice under general anesthesia: internal jugular and carotid artery catheterization, cremasteric muscle dissection, induction of hypotension, hypertonic saline, normal saline, pentastarch, D5W, D5½NS, dextran or ringer's lactate infusion, observation with intravital microscopy, IV fluorescent albumin administration, anesthetic overdose and cervical dislocation

7.a) Animal Subject Description:

Animal Species	Strain	Age	Sex	Weight	# needed at one time	Total per year	#/cage
/lice	Swiss Webster (CD1)		Male	25-30	10-20	165	4

Animals used for genetic alterations:

Category	Total per year
Oocyte donors	
Pseudo-pregnant females	
Stud males	
Other (specify):	
Other (specify):	

Please justify the number of animals to be used (See instructions - section 7)

Altered PMN-endothelial interactions: Sham (10), HTS (10), NS (10), Pentastarch (10), D5W (10), D5½NS (10), Dextran (10), RL (10).
Albumin leakage from vasculature: Sham (10), HTS (10), NS (10), Pentastarch (10), D5W (10), D5½NS (10), Dextran (10), RL (10).
Additional mice for losses from complications of hemorrhage, dying prior to the end of the experiment. (5)

Please justify the usage of live animals versus alternate methods (e.g. tissue culture)

The study requires the characterization of PMN endothelial interactions and vessel leakage *in vivo* and the analysis of fresh blood from recently resuscitated mice.

Describe the characteristics of the animal species selected that justify its use in the proposed study.

Our laboratory has significant experience with this strain of mouse especially using multiple intravital microscopy protocols

Purpose of Animal Use (See Instructions - Section 7) 1 [ ] 2 [X] 3 [ ] 4 [ ] 5 [ ]

7.b) Animal Husbandry & Care

Special cages No [X] Yes [ ] Specify ____________

Special diet No [X] Yes [ ] Specify ____________

Special handling No [X] Yes [ ] Specify ____________

Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function, (e.g. stress, radiation, steroids, chemotherapeutics)?

No [X] Yes [ ] Specify ____________

Multiply Facility Housing? Yes [ ] No [X]

Indicate all facilities where animals will be housed: RVH H3.74

Building: RVH Hershey Pavilion Room #: Surgery #1

If animal housing location and animal use procedure location are in different buildings, briefly describe procedures for transporting animals:

11. Method of Euthanasia:

SPECIES (specify)					
Mice	anaesthetic overdose, agent	Ketamine/xylazine	Route	Intravenously	
	exsanguination with anaesthesia, agent		Route		
Mice	Cervical dislocation				
	Decapitation: with anaesthesia ☐	specify		Without anaesthesia ☐	
	CO_2 chamber				
	other (specify)				
	not applicable (explain)				

4. Research Personnel and Qualifications: List the names of all individuals who will be in contact with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). Indicate any training received (e.g workshops, lectures, etc.). The PI certifies that all personnel listed here have suitable training and/or experience, or will be provided with the specific training which qualifies them to perform the procedures described in the protocol. Each person listed in this section must sign to indicate that s/he has read this protocol. (Space will expand as needed.)

Name	Classification	Training Information	Signature
Nicolas V. Christou	PI	MD, PhD, Laboratory Director	
Jose L. Pascual	PhD student	MD, Research Fellow (workshop)	
Betty Giannias	Technician	Senior Research Technician	

* Enter the first name, press 'enter', then the 2nd name... complete the first column, then the 2nd, then the 3rd
** If an undergraduate student is involved , the role of the student and the supervision received must be described.

5. Summary (In language that will be understood by members of the general public)

a) RATIONALE: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

Polymorphonuclear neutrophils (PMNs) are the first cells to be attracted to sites of injury when an inflammatory stimulus affects the host. In health this involves an ordered sequence of steps in which PMNs and vessel endothelial cells (ECs) interact to eventually allow passage of the neutrophil to the injurious agent. In the systemic inflammation that follows severe hemorrhage and infection these interactions become exaggerated causing the release of toxic substances which result in tissue and organ injury. This study will evaluate PMN rolling, PMN adhesion to endothelium, PMN rolling velocity and PMN transmigration in the microcirculation of mouse cremaster after hemorrhagic shock is resuscitated by either Ringer's lactate (RL) or hypertonic saline (HTS) and is followed by a pulmonary infection. These interactions will be observed and quantified using intravital microscopy and epifluorescence using FITC-labeled albumin. Identified HTS-associated benefits in reducing inflammation will contribute to bringing this fluid to more widespread use in clinical resuscitation both in the ICU and the emergency room.

b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

-To determine if HTS-resuscitated mice with a tracheal infection demonstrate less PMN rolling, adhesion and transmigration on endothelium and increased rolling velocity as compaerd to RL.
-To determine if the vascular permeability of HTS-resuscitated mice (as determined by FITC-labeled albumin leakage) is decreased as compared to that with traditional RL resuscitation when infection follows resuscitation
-To determine if HTS-resuscitated mice having undergone a subsequent infection demonstrate diminished PMN expression of CD18/CD11b and L-selectin as well as diminished endothelial expression of ICAM-1 expression as compared to their RL counterparts

c) PROGRESS REPORT: If this is a renewal of an ongoing project, BRIEFLY summarize what was accomplished during the prior approval period and indicate if and how the current goals differ from those in the original application.

We have already demonstrated that immediately after resuscitation from hemorrhagic shock, HTS results in fewer rolling and adhering neutrophils in post capillary venules. In the ICU, patients having undergone resuscitated hemorrhagic shock are particularly susceptible to a subsequent, seemingly unimportant, infection which, when it occurs, may precipitate patient deterioration to mutiple organ dysfunction (MODS), and death. This more clinically significant scenario will be investigated with the proposed model of murine hemorrhagic shock resuscitated by one of the above mentioned regimens, instillation of LPS (83.3micrograms/kg) into the trachea 1 hour later and evaluation of cremaster intravital microscopy (including fluorescence) (2 hours later) with subsequent post-mortem serum evaluation of PMN adhesion receptors.

d) SUMMARY OF PROCEDURES FOR ANIMAL USE REPORT TO THE CCAC: Using KEY WORDS ONLY, list the procedures used (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). Refer to Appendix 1of the Guidelines for a more complete list of suggested key words.

Vascular catheterization, induction of hypotension, HTS or RL administration in addition to shed blood, LPS instillation into the trachea by direct vision, cremasteric muscle dissection, intravenous fluorescent albumin administration, anesthetic overdose and cervical dislocation

6. Animals To Be Used

a) **Purpose of Animal Use (Check one):**

1. ☐ **Studies of a fundamental nature/basic research**
2. ☒ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**

b) **Will the project involve breeding animals?** NO☒ YES ☐
Will the project involve the generation of genetically altered animals? NO ☒ YES ☐
Will field studies be conducted? NO ☒ YES ☐

c) Description of Animals

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Mice					
Supplier/Source	Charles River St-Constant, Qc					
Strain	Swiss Webster (CD1)					
Sex	male					
Age/Wt	25-30g					
# To be purchased						
# Produced by in-house breeding	0					
# Other (e.g.field studies)	0					
#needed at one time	5-10					
# per cage	4					
TOTAL# /YEAR	130					

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

7. Justification of Animal Usage

a) Please justify the number of animals requested for each species described in the table 6c above, BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT. Include information on experimental and control groups, # per group, and failure rates. Also justify in terms of statistical requirements, product yield, etc. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The numbers of animals are for one year only, not the length of funding. Use the table below when applicable (space will expand as needed).

Altered PMN-endothelial interactions: Sham (20), HTS (20), RL (20) Albumin leakage from vasculature: Sham (20), HTS (20), RL (20). Twenty mice per group will allow to detect 10-20% differences between groups. Additional mice for losses due to technical complications (10).

Test Agents or Procedures e.g. 2 Drugs	# of Animals and Species Per Group e.g. 6 rats	Dosage and/or Route of Administration e.g. .03, .05 mg/kg – IM, IP (4 variables)	# of endpoints e.g. 1, 7, 10 days (3 variables)	Other variables (i.e. sex, weight, genotypes,etc.) e.g. Male, Female groups (2 variables)	Total number of animals per year e.g. 2 x 6 x 4 x 3 x 2 = 288

* For the above table, enter the first agent/procedure, press 'enter', then the 2nd agent... complete the first column, then the 2nd, then the 3rd...

b) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).
The study requires the characterization of PMN endothelial interactions in vivo and the analysis of fresh blood from recently resuscitated mice.

c) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)
Our laboratory has significant experience with this strain of mouse especially using intravital microscopy

8. Animal Husbandry and Care

a) **Special cages** NO ☒ YES ☐ **Specify:**

Special diet NO ☒ YES ☐ **Specify:**

Special handling NO ☒ YES ☐ **Specify:**

b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ **Specify:**

c)
Multiple institution facility housing: NO ☒ YES ☐

Indicate all facilities where animals will be housed: **Building:** RVH H3.74 **Room No:** H3.74

Indicate area(s) where animal use procedures will be conducted: **Building:** RVH Hershey Pavillion **Room No:** H6.40

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:
Transfer 3 floors by elevator, the cages being on a cart and completely covered.

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection (UACC#1)	☐	Production of Monoclonal Antibodies (UACC#7)	☐
Anaesthesia (rodents) (UACC#2)	☒	Production of Polyclonal Antibodies(UACC#8)	☐
Analgesia (rodents/larger species) (UACC#3)	☐	Collection of Amphibian Oocytes (UACC#9)	☐
Breeding (transgenics/knockouts) (UACC#4)	☐	Rodent Surgery (UACC#10)	☐
Transgenic Generation (UACC#5)	☐	Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)	☐
Knockout/in Generation (UACC#6)	☐		☐

10. Description of Procedures

a) **FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES IN THE ORDER IN WHICH THEY WILL BE PERFORMED - surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS**

PER SOP", NO FURTHER DETAIL IS REQUIRED. Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.

>All procedures are performed under general anaesthesia induced by intraperitoneal injection and maintained by IV infusion of Ketamine/Xylazine on CD1 male mice weighing 25-30 gm: The first experiment involves observation and quantification of endothelial - PMN interactions after intratracheal LPS instillation and preceeded by hemorrhagic shock resuscitated with either Ringers lactate (twice the shaed blood volume of RL, 67.7cc/kg), or hypertonic saline (4cc/kg of 7.5% NaCl). After intraperitoneal anaesthesia, a longitudinal neck incision is made and the jugular vein and carotid artery are dissected, isolated, and cannulated. Continuous blood pressure monitoring is recorded via a pressure transducer connected to the carotid artery catheter. Intravenous drugs and anaesthesia, are administered via the jugular vein catheter. Through the carotid catheter, 30-40% of the animal's blood volume is slowly withdrawn (to a BP of 40mmHg) and placed in a warmed, shaken plastic syringe. One hour later the blood is returned to the animal after the administration of one of the above two fluid regimens. The mouse is then placed in the prone position and maintained warmed by a heat lamp for one hour. At this time the trachea is exposed by blunt dissection of pretracheal muscles. A 31G syringe is used to directly instill into the trachea a volume of 25 microliters of LPS (83.3micrograms/kg). The neck inscision is reaproximated. 2 hours later, the cremaster muscle is dissected and splayed open in a 5 point preparation with 4-0 silk. The mouse and cremaster dissection is placed on the plexiglas stage of an inverted microscope. Anaesthesia is continually maintained with the infusion of ketamine/xylazine mix (as above) via the jugular catheter. The cremaster is continually superfused with warmed Bicarbonate Buffer Solution (BBS). PMN – endothelial interactions are observed, recorded and quantified at a later time using video playback. The mice are then sacrificed and blood is collected via cardiac puncture for white cell, PMN counts and receptor (CD18, L-selectin) quantification. To characterize and quantify the effect of the two resuscitation regimens (RL vs HTS) on vascular leakage, mice will be randomized to the different resuscitation fluids prior to receiving LPS. Mice will be prepared in the same manner as above (carotid jugular cannulation, hypotension and resuscitation, LPS instillation, cremaster dissection), but will then receive fluorescent bovine albumin (50mg/kg) intravenously via the jugular catheter. Fluorescent microscopy will follow 15 minutes later to determine how much of the fuorescent macromolecules have leaked out of the microcirculation, in vivo. Once the fluorescent leakage computer analysis is complete (6 minutes), the mice will be sacrificed as above.

b) Field Studies – Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) should be described above.

Method of capture/restraint, duration of captivity, potential injury/mortality, monitoring frequency:

Transportation and /or housing of animals in the field:

Special handling required:

Capture of non-target species, potential injury/mortality:

Will captured animals be released at or near the capture site YES ☐ NO ☐
If not, specify if they will be relocated to other locations and/or populations.

Describe any potential ecological disruption this study may cause:

It is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits must be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.

chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals. **According to University policy, E level studies are not permitted.**

12. Potential Hazards to Personnel and Animals: It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☐

a) Indicate which of the following will be used in animals:

☐ **Toxic chemicals** ☐ **Radioisotopes** ☐ **Carcinogens** ☒ **Infectious agents** ☐ **Transplantable tumours**

b) Complete the following table for each agent to be used (use additional page as required).

Agent	LPS		
Dosage	83.3 micrograms/kg		
Route of administration	intratracheal		
Frequency of administration	once		
Duration of administration	sigle administration of 20 microliters		
Number of animals involved	each animal of the study		
Survival time after administration	2hrs		

c) After administration the animals will be housed in:

☐ **the animal care facility**

☒ **laboratory under supervision of laboratory personnel**

Please note that cages must be appropriately labeled at all times.

d) Describe potential health risk (s) to humans or animals:

none short of universal precautions

e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.