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The Microcirculation in Trauma and Sepsis

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The Microcirculation

in Trauma and Sepsis

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LUND UNIVERSITY
Faculty of Medicine

DOCTORAL DISSERTATION

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Abstract: The microcirculation plays a vital part for fluid-, gas- and solute-exchange; changes in permeability that occur during trauma or sepsis, are in part necessary for the natural healing process, but may also cause hypovolemia and edema formation and lead to disturbances in microvascular exchange. This thesis discusses changes in microvascular flow, permeability and plasma volume (PV) loss after experimental or surgical trauma and experimental sepsis. We evaluated the effect of blunt skeletal muscle trauma itself and thereafter treatment with prostacyclin (PGI_2) on PV-loss, transcapillary escape rate (TER) of ^{125}I-albumin and cytokine release. In experimental sepsis, we studied the importance of charge for microvascular permeability and observed the effectiveness of albumin versus Ringer's acetate compared to a hemorrhage model. Peri-operatively, we evaluated changes in the sublingual microcirculation in patients undergoing major abdominal surgery, using Sidestream Darkfield-imaging (SDF) in relation to the outcome.

Skeletal muscle trauma caused PV-loss, increase in permeability and cytokine release and these changes were attenuated by treatment with PGI_2.

Sepsis led to a breakdown of the negatively charged glycocalyx, which is likely to be important for the normally low permeability for albumin.

The plasma volume-expanding effect of albumin as compared to Ringer's acetate was independent of the state of permeability.

Peri-operative changes in the sublingual microcirculation during major abdominal surgery are minor and had no correlation to outcome or parameters which reflect global oxygen delivery.

microcirculation, prostacyclin, plasma volume, trauma, sepsis, permeability, sidestream darkfield imaging, albumin, volume expansion, transcapillary escape rate, glycocalix, charge

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Faculty of Medicine

2013

Das Wissen hat Grenzen, das Denken nicht

(Albert Schweitzer, 1875-1965)

To Renate, Dirk, Jenny and Jonathan

CONTENTS

Original studies

This doctoral thesis is based on the following papers:

Paper I   **A model for evaluating the effects of blunt skeletal muscle trauma on microvascular permeability and plasma volume in the rat**

Bansch P, Lundblad C, Gränd P-O, Bentzer P. *Shock* 2010

Paper II   **Prostacyclin reduces plasma volume loss after skeletal muscle trauma in the rat**

Bansch P, Lundblad C, Gränd P-O, Bentzer P. *Journal of Trauma and Akute Care Surgery* 2012

Paper III   **Effect of charge on microvascular permeability in early experimental sepsis in the rat**

Bansch P, Nelson A, Ohlsson T, Bentzer P. *Microvascular Research* 2011

Paper IV   **Perioperative changes in the sublingual microcirculation during major surgery and postoperative morbidity: An observational study**

Bansch P, Flisberg P, Bentzer P. Submitted for publication

Paper V   **Plasma volume expansion of Albumin relative to Ringer's Acetate during normal and increased microvascular permeability. A randomized trial in the rat**

Bansch P, Statkevicius S, Bentzer P. Manuscript

Abbreviations

A	Area
ABG	Arterial blood gases
AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
ANP	Atrial natriuretic peptide
ARDS	Adult respiratory distress syndrome
ATP	Adenosin triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosin monophosphate
c-BSA	charge modified BSA
cGMP	Cyclic guanosin monophosphate
CLI	Cecal ligation and incision
D	Diffusion coefficient
DV	Distribution volume
ECV	Extracellular volume
EDHF	Endothelium-derived hyperpolarizing factor
GAG	Glycosaminoglycans
GFR	Glomerular filtration rate
Gs	Stimulating G-protein
GTP	Guanosin triphosphate
HES	Hydroxyetyl starch
HI	Heterogeneity index
HMGB1	High mobility group box 1
IFN-γ	Interferon gamma
IL	Interleukin
ISV	Interstitial space
ISV	Interstitial volume

JAM	Junctional adhesion molecule
J_s	Diffusion of a solute per unit time
J_v	Net fluid movement between the compartments
LED	Light emitting diode
L_p	Hydraulic conductance of the vessel wall
MAP	Mean arterial pressure
MFI	Microvascular flow index
MHC	Major histocompatibility complex
MODS	Multiple organ dysfunction syndrome
NF-κB	Nuclear transcription factor-κB
NFP	Net-filtration pressure
NNT	Numbers needed to treat
NO	Nitric oxide
OPS-imaging	Orthogonal polarized spectral imaging
P_a	Arterial pressure
P_c	Hydrostatic capillary pressure
PECAM	Platelet endothelial cell adhesion molecule
PGI_2	Prostacyclin
pI	Isoelectric point
P_i	Interstitial pressure
P-POSSUM	Portsmouth Physiological and Operative Severity Score for the enUmeration of Mortality and Morbidity
PV	Plasma volume
P_v	Venous pressure
PVD	Perfused vessel density
R_a	Pre-capillary resistance
RBC	Red blood cells
R_v	Post-capillary resistance
S	Surface area

ScvO$_2$	Central venous oxygenation
SDF-imaging	Sidestream Darkfield-imaging
SIRS	Systemic inflammatory response syndrome
TER	Transcapillary escape rate
TNF-α	Tumor necrosis factor alpha
VE	Vascular endothelial
vWF	von Willebrand factor
WHO	World health organization
ZO	Zonula occludens
σ	Reflection coefficient
ΔC	Concentration gradient
Δx	Diffusion distance
π_i	Interstitial oncotic pressure
π_p	Plasma oncotic pressure

Introduction

Trauma and Sepsis

Trauma is the 4th leading cause of death in Europe and the most common cause of death before the age of 40 (1), creating immense suffering and costs. In western countries, traffic accidents, fall accidents or violence are the most common reasons for traumatic injuries. Trauma can be isolated or involve multiple parts of the body, with central nervous system injuries as the leading cause of death (2). A coarse differentiation can be made between penetrating and blunt trauma, but often both types are present. This leads to a local reaction at the site of the injury and, in a more severe trauma, to a generalized response of the body to promote damage control and healing (3, 4). The hormonal response consists of a release of stress hormones such as adrenalin, cortisol, glucagon, growth hormone, aldosterone and anti-diuretic hormone. It is accompanied by an initial reduction in the metabolic rate, followed by hypermetabolism with hyperglycaemia, and catabolism of muscle, fat and bones (5, 6). This increases oxygen demands of the body significantly and may be deleterious in patients with co-morbidities limiting the possibility to increase oxygen delivery. The initial hemodynamic response leads to vasoconstriction and relocation of extra-vascular fluids to the intra-vascular compartment to maintain central organ perfusion. Later, vasodilatation and increase in blood flow follows to meet the increased demands for oxygen and nutrients of the injured tissue. At the site of the injury, capillary damage and thrombosis often develop, leading to a capillary leak with local tissue swelling. Within a week, revascularisation and regress of oedema usually occurs (7-9). As a third response of the body to the injury, inflammation occurs due to the release of local mediators such as kinins, arachnoidonic acid metabolites and histamin, causing an increase in capillary permeability, facilitating the infiltration of immuno-competent cells. Necrotic and injured cells release "high mobility group box 1" protein (HMGB1), which locally attracts macrophages and neutrophiles and also increases the vascular leak. Activation of the complement cascade leads to bacterial lysis, opsonisation of antigens, attraction of neutrophiles and platelet activation (10-14). The coagulation cascade is activated via tissue factor release from damaged endothelium, leading to platelet activation and thrombin release. Monocytes and the damaged endothelium releases pro-inflammatory cytokines such as IL-1, TNF-alpha, IL-6, IL-8 and Interferon-gamma, which is later counteracted by anti-inflammatory substances such as IL-10. In severe injury, the pro-inflammatory reaction may not be self-limiting and can lead to a systemic inflammatory response syndrome (SIRS) with increased risk for infection and multiple organ dysfunction (MODS) (9, 11,

15). Even intentional trauma because of surgery can cause similar reactions in the body and therefore mimic accidental trauma (16).

Sepsis is a generalized inflammation (SIRS) caused by micro-organisms that have entered the usually sterile bloodstream. The incidence is about 0.3% in the western population with a mortality rate of 15-20% and causes millions of deaths each year (17, 18). The body's innate immune system recognizes the foreign organisms, which leads to a SIRS reaction not unlike that in trauma. First, macrophages and neutrophils detect different pathogens like bacterial lipopolisaccharides (LPS), peptidoglycans or flagellin via so-called "toll-like receptors" (TLR). Activation of a nuclear transcription factor (NF-kB) leads to cytokine release and inflammation (19). Different cytokine patterns can be found in different types of sepsis, but usually, an increase in pro-inflammatory TNF-alfa, IL-6 and IL-1beta is observed together with the anti-inflammatory cytokines IL-10, IL1ra and TNF SR I+II. Furthermore, macrophages "present" pathogens on their cell surface for T-cells in form of a major histocompatibility complex (MHC) (20, 21). T- and B-cells then act in part directly toxic on pathogens, in part via production of antibodies and opsonisation of pathogens (adaptive immunity). The immunologic reaction of the body in severe sepsis and bacterial toxins may lead to leukocyte adhesion and endothelial dysfunction, release of tissue factor and activation of the coagulation system, an increased vascular permeability and mitochondrial dysfunction, and eventually lead to multiple organ-failure (22, 23).

Aims of this thesis

To evaluate different aspects of microcirculatory disturbances caused by trauma or sepsis with emphasis on changes in plasma volume and microvascular permeability. We tested the potential of prostacyclin as a treatment against increased permeability and the effectiveness of albumin versus Ringer's acetate as plasma volume expanders in a setting with normal and increased permeability. We also evaluated the importance of negative charges inside the capillary wall for the normally low permeability for albumin and, in human subjects, the correlation between sublingual microcirculatory changes with post-operative morbidity in patients undergoing major abdominal surgery.

The macro- and microcirculation

The macrocirculation basically consists of a high- and a low-pressure-system with the heart at its centre. Oxygen-rich blood is pumped with high pressure (blood pressure) from the left ventricle through the aorta and large arteries to all

organs and tissues, and returns as de-oxygenated blood via the veins to the right ventricle. From here it is pumped via a low-pressure-system through the pulmonary circulation, where oxygen uptake occurs, back to the left ventricle. At organ level, the blood passes through the microcirculation, which consists of arterioles (Ø 100-10μm) and capillaries (Ø 5-8μm), where gas- and solute exchange takes place. Blood flow is regulated via local autoregulation, circulating hormones and autonomic innervation of a smooth muscle layer around the arterioles, controlling vessel diameter and therefore resistance to blood flow. The capillary wall, however, consists of only a single layer of endothelial cells, which minimizes the transport distance for gases and solutes. The smallest arteries and arterioles stand for about 60% of the total resistance to the blood flow and the capillaries for about 20%, making the microcirculation the major contributor to resistance in the body. At the same time, the capillary network of a single human consists of millions of microvessels which, laid out in a row, could span the whole earth. This huge cross-sectional area is needed for gas- and solute exchange, which mainly occurs via diffusion and is only effective if diffusion distances are small. It also slows down the blood flow, leaving sufficient time for diffusion to take place. After passing the microcirculation, blood is collected in venules and veins that contribute to only about 15% of the resistance to blood flow (Fig 1). Pressures are relatively low after the pressure drop over the microcirculation, but sufficient to drive the venous blood back to the right atrium. The venous system contains about 2/3 of the total blood volume and the veins are therefore also called capacitance vessels. Also veins have a smooth muscle layer in their walls and are innervated by sympathetic fibres, making it possible for the body to mobilize blood from this reservoir if needed. In certain situations, arterio-venous shunting can occur, where blood bypasses the capillary network (24, 25).
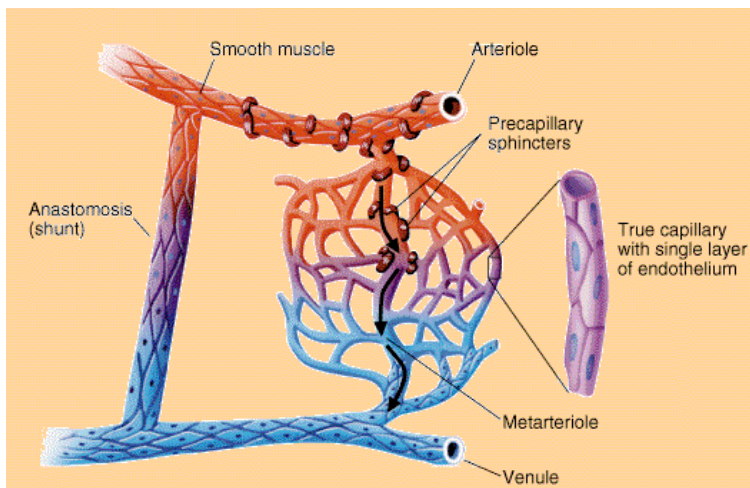

Fig 1. Schematic drawing of the microcirculation

Transcapillary exchange and permeability

Fluid- and solute-exchange over the capillary membrane is dependent on several factors and differs in different types of capillaries. As mentioned earlier, the capillary wall consists of a single layer of interconnected endothelial cells surrounded by a basement membrane. The inside of the capillaries is coated with the glycocalyx, a layer of different, negatively charged glycoproteins and proteoglycans. The cells are connected via gap- and tight junctions with intercellular clefts in between. Size and number of these clefts vary in different tissues, from rather impermeable junctions in brain tissue, forming the so-called blood brain barrier, to wider and more frequent clefts in skeletal muscle. In tissues specialized in fluid exchange, like kidneys, endocrine and exocrine glands, intestinal mucosa and the choriod plexus, capillaries have small perforations in the endothel, called fenestraes. These have a diameter of 50-60 nm, allowing for water and proteins to cross much faster than in continuous capillaries. A third type, discontinuous capillaries with gaps of over 100 nm, can be found in bone marrow, spleen and liver, where erythrocytes and leukocytes need to pass through the capillary wall (24, 26, 27).

Permeability for oxygen (O_2) and carbon dioxide (CO_2) is extremely high in all capillaries due to the high lipid solubility of these gases, allowing them to freely diffuse through the endothelial cell into the surrounding tissues and vice versa along a concentration gradient. Transport of water and small solutes like electrolytes, glucose and urea, for example, across the capillary wall is restricted to the intercellular gaps, leaving a rather small exchange area for convection and diffusion. Water flows passively along a pressure gradient across the gaps, carrying along electrolytes and other solutes (convective transport). For glucose and urea, diffusion is the more important way of transport and depends on the concentration gradient of the substance across the capillary membrane, the area available for diffusion, the membrane thickness and a specific diffusion gradient for each substance (24). This connection is described in **Fick's first law of diffusion**:

$$J_S = -DA\Delta C/\Delta x$$

(J_s=diffusion of a solute per unit time; D=diffusion coefficient; A=area; ΔC=concentration gradient; Δx=diffusion distance)

The diffusion coefficient of a substance is dependent on its size, form and charge. The smaller and more circular the molecule, the faster it diffuses through a gap or pore. Another effect impeding the diffusion is steric exclusion: Large molecules have a relatively smaller area available for diffusion since they are restricted to the centre of the pore. Also, in larger molecules approaching the

diameter of the pore, water "slips past" the molecule less easily, slowing down its passage through the pore, a phenomenon called restricted diffusion. Furthermore, pores are not always the shortest available connection across the capillary wall since they also may pass through it obliquely, thereby prolonging the diffusion distance.

As mentioned earlier, flow of water is governed by a pressure gradient across the capillary wall, as opposed to a concentration gradient for solutes. A second factor influencing the movement of water is the colloidosmotic or oncotic pressure, caused by plasma proteins that exert an osmotic force on smaller molecules and water since they cannot easily pass the capillary wall, which therefore acts as a semi-permeable membrane. In addition, negative charges on the protein-surface attract positively charged ions, increasing its osmotic force (Gibbs-Donnan effect). Since the capillaries are not completely impermeable to plasma proteins responsible for the oncotic pressure, a reflection-coefficient has to be taken into account, with a value of 1 for impermeable substances, and zero for molecules with unimpeded passage. For plasma proteins, the reflection-coefficient is about 0.8-0.95. Furthermore, the hydraulic conductance (L_p) describes how permeable the membrane is to water, with high values indicating high permeability (27-29). **The Starling equation** for fluid filtration summarizes the factors governing water-exchange across the capillaries:

$$J_v = L_pS[P_c - P_i] - \sigma[\pi_p - \pi_i]$$

(J_v = net fluid movement between the compartments; L_p = hydraulic conductance of the wall; S = surface area; P_c and P_i = capillary and interstitial hydraulic pressure; σ = reflection coefficient; π_p and π_i = plasma and interstitial oncotic pressure)

For the majority of capillaries, this leads to a net-filtration of 10-20% of the fluid passing the microcirculation, with a filtration being predominant at the beginning of the capillary, successively turning into a net-absorption towards the venous end of the capillary (Fig 2). Filtrated interstitial fluid is then transported via the lymphatic system back to the intra-vascular compartment. In hypovolemia following haemorrhage for example, sympathetic stimulation raises pre-capillary sphincter tone and reduces filtration, leading to a net-absorption over the capillary passage, which helps to replenish the decreased plasma volume.
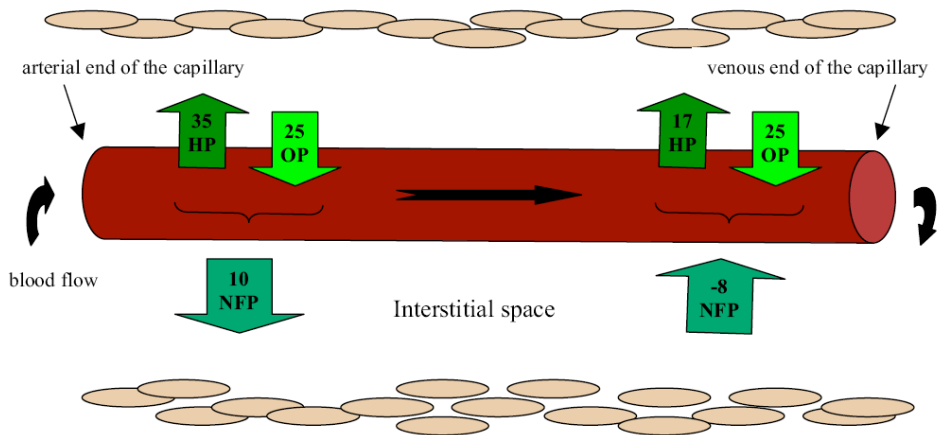
Fig 2: HP = net hydrostatic capillary pressure, OP = net oncotic pressure, NFP = net filtration pressure

The two-pore-model

As mentioned earlier, even plasma proteins can pass the capillary wall, despite their relatively large size. Albumin for example "leaks" from the vascular department into the interstitial space at a rate of ca. 5-15% per hour, depending on the species. With an estimated pore radius of 4-5 nm and an albumin molecule being just slightly smaller than that, it should leak to a much lesser extent than observed. One suggested explanation is a vesicular transport through the endothelial cell, but such a transport is too slow and energy craving and can not explain the amount of plasma-protein leakage: For one, protein permeability is proportional to the hydraulic "driving pressure" across the capillary wall, following Starling's law, an observation which is not compatible with an active vesicular transport. For another, cooling, which should slow down any vesicular transport, does not have any effect on protein transport. Furthermore, caveolin knock-out mice incapable of vesicular transport have basically unchanged permeability for plasma proteins (30, 31). A more likely explanation is therefore the existence of a large pore system, allowing bigger molecules to pass into the interstitial space. Based on mathematical models and observations, the pore size in that system is estimated to be around 20-30 nm, with a ratio of large pores to small pores of about (1:10.000-30.000) (32). Since large pores are so rare, they are very difficult to observe, and the two-pore-model therefore remains a hypothetical model which fits best to explain the current knowledge about the behaviour of plasma-proteins within the circulation. Since the discovery of aquaporins, specific water channels in the endothelial cell, the model is sometimes termed three-pore-model. Aquaporins normally contribute little to

water permeability, but in tissues with narrow tight junctions like the blood brain barrier, these channels may be the main pathways for water transport (33).

Permeability of a membrane is dependent on several factors and varies greatly for different solutes. Expressed in a mathematical term, it can be written as:

$P = J_s/S\Delta C$ [cm/s]

(J_s=diffusion of a solute per unit time; S=surface area; ΔC=concentration gradient)

As mentioned earlier, oxygen and carbon dioxide diffuse freely across the endothelial cell with a large surface area for gas exchange. Solutes on the other hand are mainly restricted to diffusion via inter-endothelial gaps or pores limiting the surface area significantly.

For example, permeability for oxygen is about 100.000 cm/s, for glucose 9-13 cm/s and for albumin about 0.03 cm/s. Glucose and albumin have the same surface area available for diffusion, but due to its much bigger molecular size, approaching the diameter of the small pores, albumin diffuses much slower than glucose (24). Also, albumin is a negatively charged protein, which restricts its permeability through the negatively charged glycocalyx layer on the luminal side of the endothelium (Fig 4), thereby contributing to the semi-permeable membrane properties of the capillary wall. In states of inflammation or ischemia for example, the glycocalyx can be degraded, causing an increase in permeability and protein leakage (34).

The lymphatic system

Since the net-filtration of fluid in the capillaries is usually slightly higher than the net-absorption, the filtrated fluid needs to be transported from the interstitial space in order to avoid tissue swelling. This occurs via the lymphatic system. Lymph is collected in lymphatic microvessels and collecting lymphatics and transported via the afferent lymphatic towards the lymph nodes. Here, connections with nodal blood vessels allow an exchange of lymphocytes. Lymph is then transported further, mainly via the cysterna chyli, where fatty lymph from the intestines (chyle) is added, before it enters the blood stream via the thoracic duct into the left subclavian vein. Lymphatic vessels are surrounded by smooth muscle, pumping the lymph forward, supported by extrinsic propulsion via muscle movement. Semilunar valves permit flow to move in only one direction. Lymphatic flow can manifold, but if lymphatic function is impaired or filtration greatly increased, oedema can develop (24).

Endothelial function

Capillary endothelium consists of a single layer of cells connected via tight- and gap junctions. It has a variety of important functions. The luminal side contains angiotensin-converting-enzyme, responsible for angiotensin II formation, an important regulator of vascular smooth muscle tone, blood pressure and sodium balance (via aldosteron-release). Endothelium releases pro- and anticoagulatory substances like nitric oxide (NO), prostacyclin (PGI_2) and von Willebrand factor (vWF), regulating trombocyte aggregation. It is an important regulator of vascular smooth muscle tone. Secretion of nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) promote smooth muscle relaxation whereas endothelin causes contraction, which in turn can affect pore size and therefore permeability.

In inflammation, endothelial cells promote leukocyte adhesion as part of the immune response. Via formation of large gaps, the endothelium allows circulating immunoglobulins to access the inflamed site more easily, at the same time increasing the permeability for all plasma proteins. Endothelium also promotes new tissue growth via angiogenesis. Smaller amounts of plasma macromolecules, like for example immunoglobulins and lipoproteins, can be transported into or through the endothelial cell via vesicular endo- or transcytosis (Fig 3).
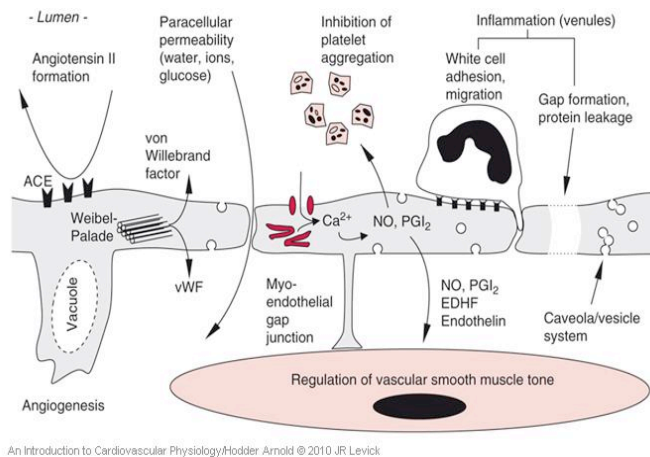

Fig 3. From: Cardiovascular Physiology by J.R. Levick, Hodder Arnold, Copyright (2010). Reproduced by permission of Taylor & Francis Books UK.

An actin-myosin skeleton inside the endothelial cell is responsible for its shape and stability and may change the contractile status of the cell and affect the so called "adherens type junctions" consisting of vascular endothelial (VE) cadherin, thereby changing the size of the intercellular clefts. This in turn may lead to a change in capillary permeability. Other junctions between the cells consist of the platelet endothelial cell adhesion molecule (PECAM) and junctional adhesion molecules (JAM), the so called "occludens type junctions", consisting of claudin and occludin, forming the tight junctions (Fig 4). These intercellular connections are responsible for leukocyte-platelet-cell interactions and cell-emigration in inflammatory states. This junctional complex is not fixed, but a dynamic structure that can be influenced by different mechanisms. Activation of beta-adrenergic receptors with the release of cAMP, for example, leads to an increase of junctional strands, reducing permeability. The cGMP pathway, on the other hand, activated for example through release of atrial natriuretic peptide (ANP), can increase permeability (35-40).
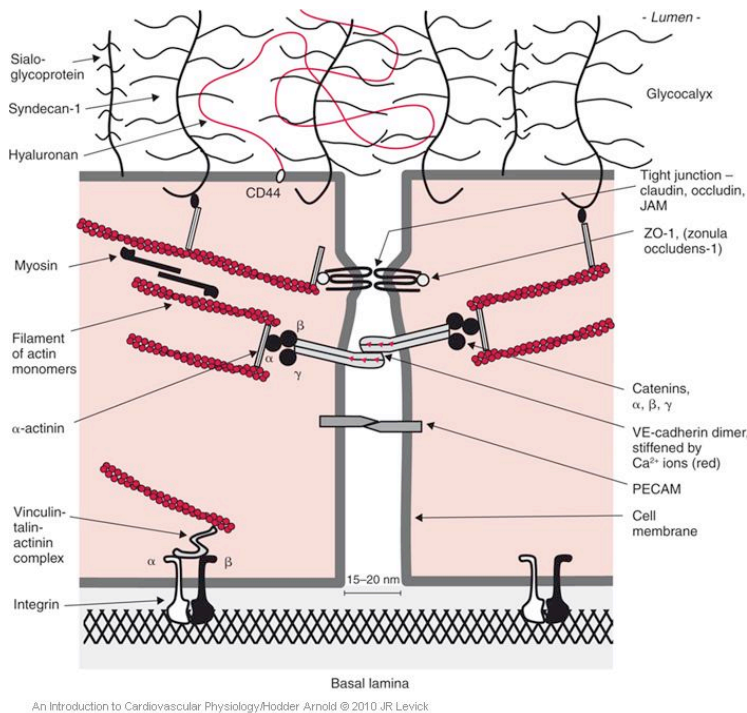

An Introduction to Cardiovascular Physiology/Hodder Arnold © 2010 JR Levick

Fig 4. From: Cardiovascular Physiology by J.R. Levick, Hodder Arnold, Copyright (2010). Reproduced by permission of Taylor & Francis Books UK.

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Prostacyclin is a product of the arachidonic acid metabolism via cyclo-oxygenases. It exerts its vasodilator action mainly via an increase of cyclic adenosine monophosphate (cAMP) via activation of inositol-phosphate receptors in the smooth muscle cell. This leads to G-protein stimulation (Gs) and activation of adenylyl cyclase (AC), which promotes conversion of adenosine trisphosphate (ATP) to cAMP. As mentioned earlier, this leads to a decrease in vascular permeability by enhancing junctional strand formation. PGI_2 also plays an important role as inhibitor of platelet aggregation and leukocyte adhesion and has anti-inflammatory and scavenging effects (41-44).

The vasodilator action of NO is exerted via stimulation of guanylyl cyclase, leading to cyclic guanosine monophosphate (cGMP) production from guanosine trisphosphoate (GTP). This then leads to smooth muscle relaxation. Similar to PGI_2, NO inhibits platelet aggregation, counteracting the pro-coagulatory action during inflammation and therebye reducing the risk for thrombosis. In regards to the effects of NO on vascular permeability there is still some controversy, with some studies suggesting an increase (45, 46) and some a decrease in permeability (47-49). Nagy et al suggested that NO-effects on permeability might be dependent on the underlying pathophysiology, varying in situations with normal, acutely and chronically altered permeability (50).

Crystalloid and colloid solutions

Crystalloids are solutions containing water and small ions like sodium, chloride, potassium, bicarbonate or glucose, which are responsible for the solutions' osmolality. Due to the small molecular size of these ions, they easily permeate the capillary walls together with water in a mainly convective manner and distribute into the whole extracellular fluid volume (ECV).

Colloid solutions contain water and relatively large molecules (>30 kDa), which have a high reflection-coefficient and therefore do not easily pass across the capillary wall (see Starling equation). They exert a colloid-osmotic or oncotic pressure, which is the main force keeping fluid in the intravascular space (Fig 2). They may contain starch (HES), sugar (dextrane), gel (succinylated gelatine) or plasma proteins (albumin, blood-plasma) as the main component. In states with increased vascular permeability like severe trauma or sepsis, colloid solutions may leave the intravascular space more easily through formation of intercellular gaps as mentioned earlier.

About 1/3 of the total body-water lies in the ECV and 2/3 in the intracellular volume (ICV). ECV can be divided into plasma volume (PV) and interstitial volume (ISV). With a plasma volume of about 3 L and a ECV of about 14 L, the ratio between PV and ECV is about 1:4.5 (Guyton and Hall 290-293 12th edition 2011). Of an intravenously administered isotonic crystalloid solution of

1 L, only about 0.22 L remain therefore in the PV after its distribution in the whole ECV (25).

Sidestream darkfield imaging (SDF)

In 1999, a new method for visualization of the microcirculation has been described by Groner et al., called "orthogonal polarized spectral imaging" or OPS-imaging. The method has been validated against conventional capillary microscopy (51) and intravital fluorescence microscopy (52, 53) and showed a good correlation. Later, a similar method called "Sidestream Darkfield-imaging" or SDF-imaging with improved picture quality was developed (54). The method is based on the illumination of the microcirculation through green light emitting diodes (LED) at a wavelength of 530 nm that surround a camera in the centre of the device. The light is absorbed by red blood cells (RBC) that appear dark on the image recorded by the camera. Pulsed or stroboscopic illumination improves visualization of moving structures like RBC (Fig 5+6).
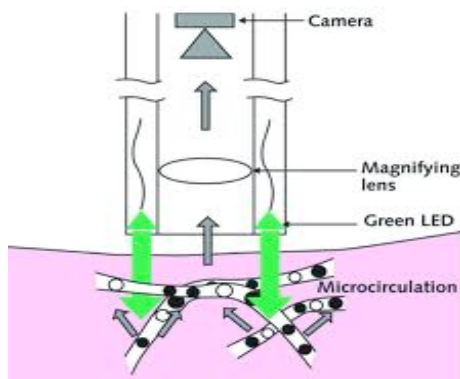
Fig 5. Schematic drawing of the SDF camera filming the underlying microcirculation.
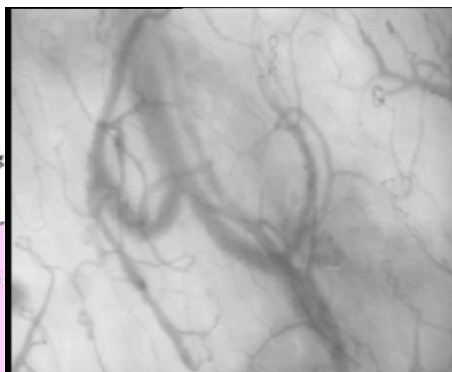
Fig 6. Sublingual microcirculation visualized with help of SDF-imaging in a patien

Aims of the studies

I. To develop an experimental model suitable for studying the effects of a non-hemorrhagic soft tissue trauma on plasma volume (PV) and microvascular permeability

II. To test whether prostacyclin-administration has an effect on the observed plasma volume loss and permeability after soft tissue trauma

III. To study whether charge effects contribute to the increased vascular permeability observed in sepsis

IV. To study whether peri-operative microcirculatory alterations are associated with post-operative morbidity and/or with changes in parameters reflecting oxygen delivery

V. To evaluate whether there is a difference in the plasma volume expanding effect of Albumin as compared to Ringer's acetate in states of normal and increased permeability

Methods

In studies I-III and V, anaesthetized Sprague-Dawley rats were used for the experiments. Study IV is a clinical study.

Materials and anaesthesia (I-III + V)
All studies were approved by the Ethics Committee for Animal Research at Lund University, Sweden. Animals were treated in accordance with the guidelines of the National Institutes of Health for Care and Use of Laboratory animals.
Animals were anaesthetized with an isoflurane/air-mixture in a glass container. After tracheostomy, animals were connected to a ventilator and anaesthesia was maintained with isoflurane and fentanyl after establishing arterial and venous access. Body-heat was maintained via a feedback controlled heating pad. Urine was collected in a glass vial placed at the external meatus of the urethra. At the end of the experiment, animals were killed via an intravenous injection of potassium chloride.

Materials and anaestheisa (IV)
The study was approved by the Human Research Ethics Committee at Lunds University and written consent was obtained prior to surgery. It is an observational study and anaesthesia and peri-operative care was performed in a standardized way according to local guidelines for this type of surgery. Patients did not receive any premedication and anaesthesia was induced by propofol and maintained with iso- or desflurane. Intravenous fentanyl, and in some cases additional epidural mepivacaine, was used for intra-operative analgesia. Suxamethonium or rocuronium was used for intubation and rocuronium thereafter if needed. Basal infusions of Ringer's acetate and 5 % glucose were given, with additional fluids if needed to maintain normovolemia. Patients received blood and plasma transfusions if deemed necessary to maintain oxygen delivery and to preserve normal coagulation capacity. In addition, noradrenalin, dopamine or nitroglycerin were used in some cases to optimize hemodynamics for the respective type of surgery.

Experimental trauma (I + II)
Animals were subjected to a standardized blunt muscle trauma on the abdominal rectus muscle with an anatomical forceps at 12 different locations. Great care was taken to avoid bleeding and to minimize evaporation.

Experimental sepsis (III + V)
Abdominal sepsis was triggered by cecal ligation and inscision (CLI). The cecum was ligated and incised on a length of about 1 cm whereafter the abdomen was closed again.

Experimental hemorrhage (V)
Animals were bled 8 ml/kg within 5 minutes.
Surgical trauma (IV)
Patients underwent elective major abdominal surgery, mainly pancreatic and liver resection and some cases of upper gastrointestinal surgery.

Experimental protocol (I-II)
Three different groups were studied. Preparation and anaesthesia was the same for all groups. In the TER-group, the transcapillary escape rate of albumin was measured during 1 hour, starting 30 min after the experimental trauma. In the PV-group, plasma volumes were measured before and 3 hours after the trauma, and in the cytokine groups, blood was analyzed 1 and 3 hours after the trauma. Arterial blood gases were analyzed before the trauma and at the end of the experiments in the TER- and PV-groups. In paper I, results of the traumatized animals were compared to a sham group not subjected to muscle trauma. In paper II, all animals were subjected to trauma and received either a prostacyclin infusion of 2 ng/kg/min or NaCl 0.9%, with both infusions given at a rate of 0.5 µl/min.

Experimental protocol (III)
The distribution volume and TER of normal bovine albumin (BSA, isoelectric point (pI) about 4.5) and charge-modified albumin (cBSA, pI about 7.1) were measured 3 hours after a CLI procedure or in control animals. To evaluate the shedding of the glycocalyx, concentrations of glycosaminoglycans (GAG) were measured in separate experiments in a CLI- and a control group at baseline and 3 hours after CLI or sham.

Experimental protocol (IV)
Adult patients with an estimated P-POSSUM score (Portsmouth Physiological and Operative Severity Score for the enUmeration of Mortality and Morbidity) of above 30 and an expected operating time of > 3 hours were eligible for inclusion. The sublingual microcirculation was evaluated using Sidestream Darkfield-imaging (SDF-imaging) before and directly after induction of anaesthesia, during the last hour of surgery and within 2 hours of arrival in the recovery room. Perfused vessel density (PVD), microvascular flow index (MFI) and a heterogeneity index (HI) were measured according to the recommendations of a consensus conference (55). Arterial and venous blood gases (ABG, VBG) were analyzed simultaneously except before the start of anaesthesia, when cannulations had not yet been performed. Data about post-operative complications were collected during a 30-day follow up period according to pre-defined criteria.

Experimental protocol (V)
The rats were either subjected to a CLI procedure (high permeability group), or were bled 8ml/kg (normal permeability group). 3 hours after CLI or directly after haemorrhage, animals were resuscitated during a 30-min period with either

5% albumin or Ringer's acetate at a ratio of 1:4.5 between the two solutions with an amount reflecting the calculated or measured PV-loss. Plasma volumes were measured at baseline, 15 min and 2 hours after completed resuscitation and 3 hours after CLI. In additional and otherwise identical experiments, PV was measured after 4 hours instead of 2 hours in the septic animals.

Plasma volume measurement (I-III + V)
Plasma volume was determined by measuring the increase in radioactivity in the blood 5 min after intravenous injection of ^{125}I-albumin with known amount of activity. For subsequent measurements, a blood sample was taken just before the next injection and the measured activity was subtracted from the one taken 5 min after the injection. Remaining activity in the syringe and needles was measured to determine the exact dose given. This technique has been shown to produce reliable and reproducible results (56, 57).

Measurement of transcapillary escape rate - TER (I-III)
TER was determined by measuring the disappearance of ^{125}I-albumin or ^{131}I-albumin (III) from the circulation during a 1 h period by taking plasma samples at 5 (I+II) or 10 min (III), 15, 30, 45 and 60 min after the injection of a known amount of activity. Plotting the results in a diagram gives a sloping line, which presents the decrease in activity and determines TER. This method is well established in experiments with both humans and animals (58-60).

Cytokine measurement
Cytokines were measured in plasma samples with a flow cytometer using cytometric bead array kits specific for the respective cytokines (BD Biosciences, Franklin Lakes, NJ).

Muscle water content
Muscle water was determined with a wet-dry tissue technique, comparing muscle water in sham animals with that in traumatized muscle.

Charge-modified albumin - cBSA (III)
The negative charge of normal albumin is caused by numerous carboxyl-groups. For charge-modification, BSA is activated by carbodiimide, followed by amidation with glycine methyl ester according to a method described by Hoare and Koshland in 1967 and modified by Wiig 2003. The resulting charge-modified was then labeled with ^{131}I to permit differentiation with negatively charged ^{125}I-labeled albumin (61, 62).

Measurement of glycosaminoglycans - GAGs (III)

This method to measure GAG was described by Björnsson in 1998. Measurement was achieved by adding acidulous buffer to the plasma samples or different standard solutions, which are then colour-marked with Alician blue solution. The resulting solutions were then filtered through a membrane where the colour-marked GAG molecules left an imprint with an intensity that correlates to the amount of GAG in the sample (63).

Sidestream Darkfield-imaging - SDF (IV)

A camera with a 5 x lens was used (Microvision Medical, Amsterdam, Netherlands) and on each occasion, a film-sequence lasting 20 seconds was recorded at 5 different sublingual locations. To evaluate perfused vessel density (PVD), 3 equidistant vertical and horizontal lines were laid across the stabilized (AVA version 2.0) films. The number of perfused capillaries crossing a line of the grid pattern was then divided by the total grid length. Microvascular flow index (MFI) was evaluated by dividing the stabilized picture into 4 quadrants, and each quadrant was assigned a number from 0-3, where 0 stands for no flow, 1 for intermittent flow, 2 for sluggish flow and 3 for continuous flow, depending on the predominant flow pattern in that quadrant. MFI is the average flow pattern of all 4 quadrants. The heterogeneity index (HI) is then calculated by subtracting the lowest MFI of any quadrant from the highest MFI, divided by the average MFI of all quadrants.

Main results

Study I

Our model of a skeletal muscle trauma caused a decrease in plasma volume 3 hours after the trauma as compared to baseline or sham animals (Fig 1). This was accompanied by an increase in the transcapillary escape rate of albumin (TER) (Fig 2) and an increase in the plasma concentrations of IL-6 and IL-10 after 1 hour, but not after 3 hours (Fig 3a+b).
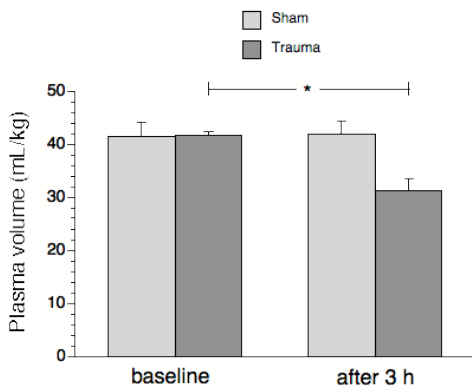

Fig 1. Plasma volume 3 h after the trauma or sham procedure (n = 7 per group). *p < 0.05.
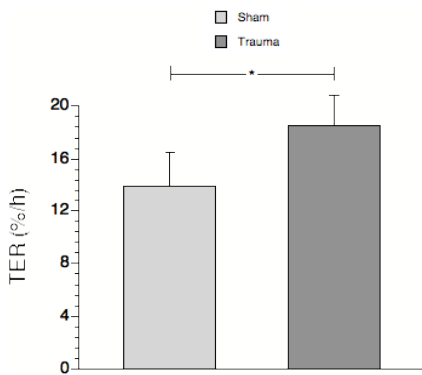
Fig 2. Transcapillary escape rate for albumin after the trauma or sham procedure (n = 7 per group). *p < 0.05.
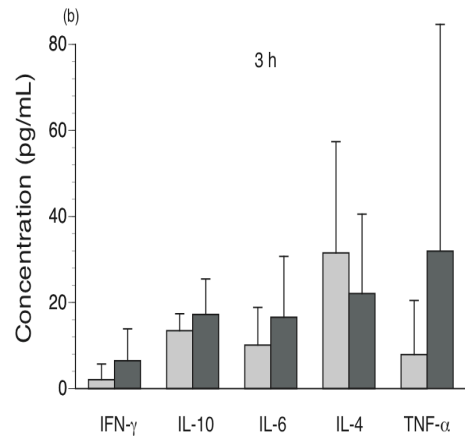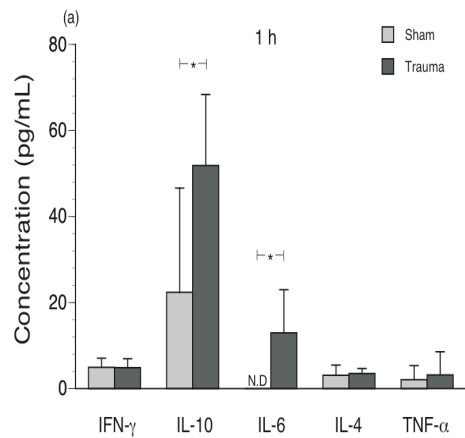
Fig 3a+b. Plasma concentrations of IFN-γ, IL-4, IL-6, IL-10 and TNF-α at 1 h and 3 h after the trauma or sham procedure (n = 8 per group). *p < 0.05

Study II

Infusion of prostacyclin (PGI$_2$) attenuated the loss of plasma volume in this trauma model (Fig 4) and decreased plasma levels of the pro-inflammatory cytokine IL-6 as compared to animals that received NaCl 3 hours after the trauma (Fig 6a). TER showed a tendency towards a decrease in the PGI$_2$-treated animals (Fig 5).
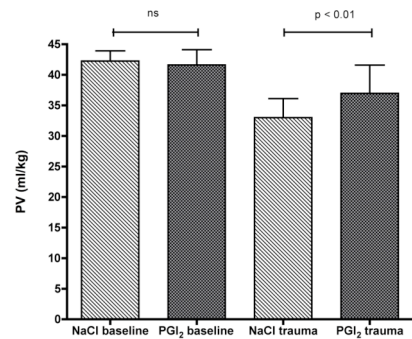
Fig 4. Plasma volumes for the NaCl (n=14) and PGI2-treated animals (n=13) at baseline and 3 hours after trauma.

(a)

(b)
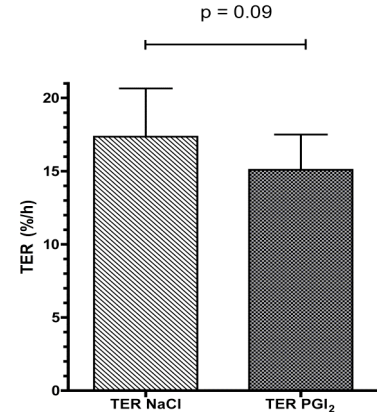
Fig 5. Transcapillary escape rate (TER) for NaCl and PGI2-treated animals during trauma (n=10 per group).
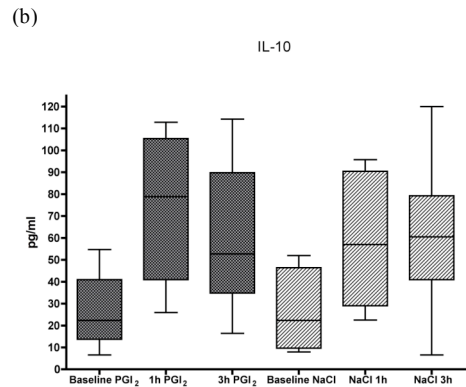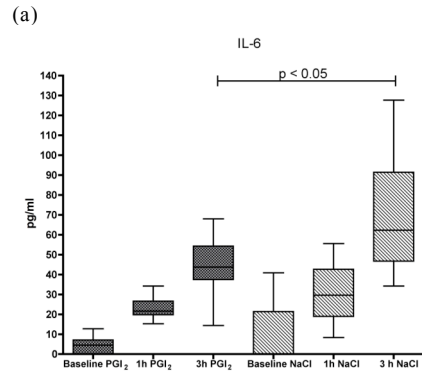
Fig 6a+b. Plasma concentrations of IL-6 and IL-10 at baseline, 1 hour and 3 hours after trauma for the NaCl or PGI2-treated animals (n=11 per group)

Study III

TER for charge-modified albumin (c-BSA) was higher than TER for normal albumin (BSA) in the control and in the sepsis group. TER for BSA, but not for c-BSA increased 3 hours after CLI as compared to control (Fig 7). The ratio of BSA/c-BSA was decreased in sepsis (Fig 8).
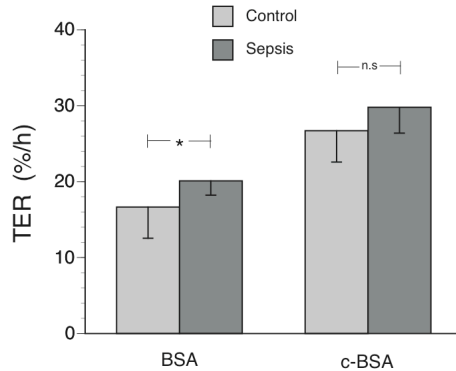
Fig 7. Transcapillary escape rate (TER) for 125I-labeled bovine serum albumin (BSA) and 131I-labeled charge-modified bovine serum albumin (c-BSA) during control conditions (n = 12) and following induction of sepsis (n = 11). *p < 0.05.
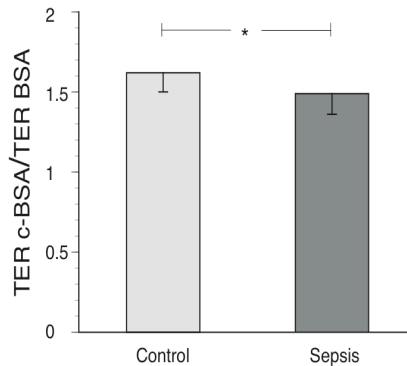
Fig 8. Ratio of 125I-labeled BSA to 131I-labeled c-BSA during control conditions and following induction of sepsis. *p < 0.05.

The distribution volume (DV) for both BSA and c-BSA decreased 3 hours after sepsis. DV was higher for BSA than for c-BSA during both, control and sepsis conditions (Fig 9). Plasma concentrations for glucosaminoglycanes (GAGs) increased in plasma after sepsis, but not in control animals (Fig 10).
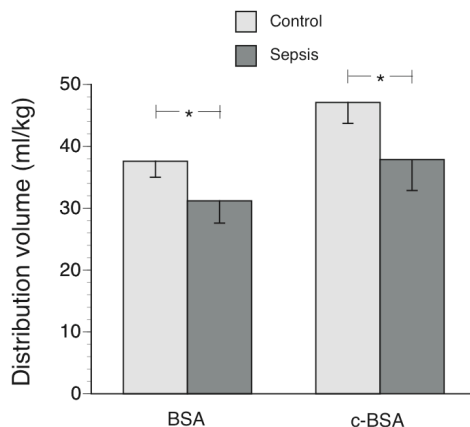
Fig 9. Distribution volumes for BSA and c-BSA during normal conditions (n = 12) and 3 hours after induction of sepsis (n = 11). *p < 0.05.
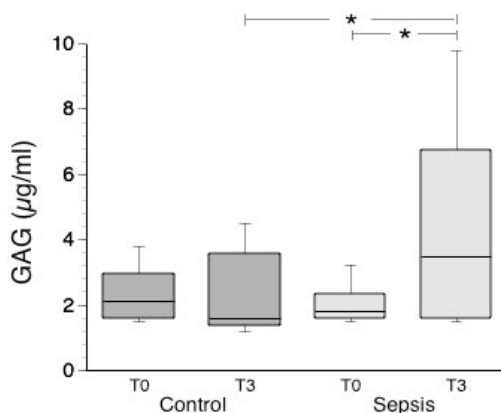
Fig 10. Plasma concentrations of glycosaminoglycans (GAGs) at baseline (T0) and at 3 h (T3) in control animals (n = 14) and in septic animals (n = 14). *p < 0.05.

Study IV

A total of 42 patients with a median age of 66 yrs were included in the analysis. 16 patients (38%) developed a total of 23 complications. In the whole group, $ScvO_2$ increased during surgery and deceased postoperatively, with a further decrease on the next morning after surgery. Lactate concentrations increased during surgery and decreased towards normal values on the first postoperative morning. Of the measured microcirculatory parameters, only the microvascular flow index (MFI) changed perioperatively, with an increase after induction of anaesthesia and a decrease in the early postoperative period (Fig 11).
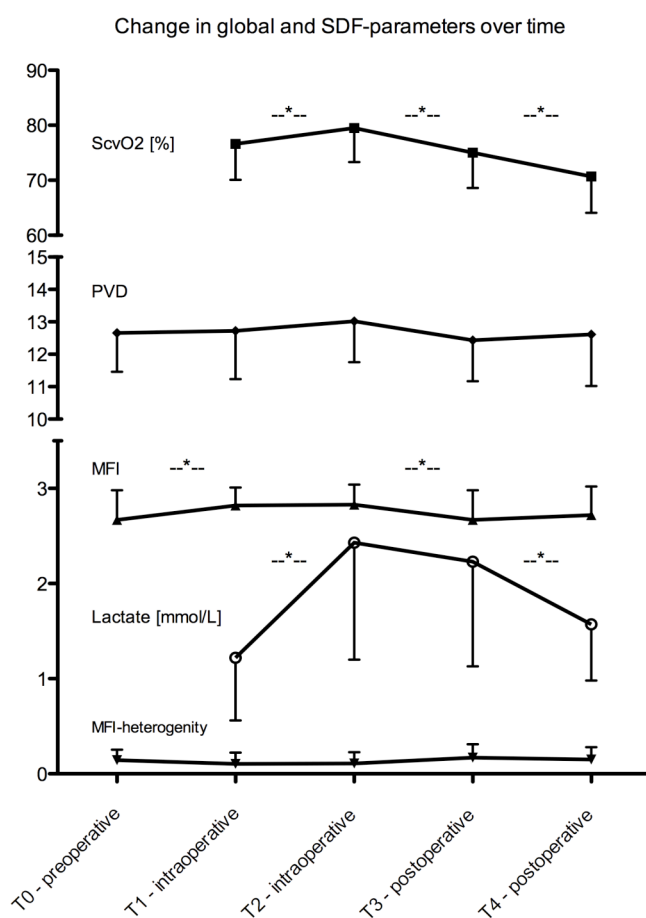
Fig 11. Change of central venous saturation ($ScvO_2$), lactate, perfused vessel density (PVD), microvascular flow index (MFI) and heterogeneity index (HI) throughout the experiments.

There were no differences in the demographic data between patients with and without complications (Tab 1), with no differences in regards to fluid therapy or drug administration either, except that patients who developed complications received more blood products. Hospitals stay was longer in the group with complications (Tab 2).

Table 1.

Demographic data for patients with and without complications. Data are presented as median with interquartile range 1-3.

	Complications (n = 16)	No complications (n=26)	p-value
Age	66 (43-86)	64 (43-86)	0.38
Gender (female/male)	9 / 7	11 / 17	0.39
P-Possum score	33 (27-42)	32 (25-42)	0.70
P-Possum surgical score	15 (9-26)	14,5 (8-26)	0.76
Duration of surgery (h)	7.3 (3.5-13)	6.6 (3.5-10.5)	0.35
Liver surgery	6	14	0.57
Pancreatic surgery	8	11	0.35
Gastrointestinal surgery	2	1	0.30

Table 2.

Perioperative fluid loss, fluid- and drug administration for patients with and without complications. * Statistically significant difference. Data are presented as median with interquartile range 1-3.

	Complications(n=16)	No complications (n=26)	p-value
IV fluids intraoperatively (mL)	4000(1500-5500)	3900 (1500-7500)	0.68
Total IV fluids (mL)	5900(3000-7250)	5600(3000-9000)	0.75
Estimated blood loss (mL)	915(250-4000)	740(50-4500)	0.29
Total blood products (mL)	460(0-2750)	80(0-500)	*<0.01
Hospital stay (days)	16.4	9.5	*<0.05
Vasoactive drugs			
- Norepinephrine	5	11	0.31
- Dopamine	1	5	0.25
- Nitroglycerin	1	7	0.10

No difference in ScvO$_2$ and lactate and microvascular parameters could be detected between the patients with and without complications and there was no correlation between global parameters reflection oxygen delivery like ScvO$_2$ and lactate, and the measured microvascular parameters (Tab 3).

Table 3.

Microvascular flow index (MFI), heterogeneity index and perfused vessel density, central venous saturation (ScvO$_2$) and lactate in groups the groups with and without complications.

	T0	T1	T2	T3	T4
MFI					
Complications	2.7 (2.1-3.0)	2.8 (2.2-3.0)	2.8 (2.4-3.0)	2.7 (1.9-3.0)	2.7(2.0-3.0)
No complications	2.6 (2.0-3.0)	2.8 (2.4-3.0)	2.8 (2.3-3.0)	2.6 (2.1-3.0)	2.7 (2.0-3.0)
Estimated difference	- 0.1 (-0.3 to 0.1)	0.0 (-0.1 to 0.1)	0.1(-0.1 to 0.2)	- 0.1(-0.3 to 0.2)	0.0(-0.20 to 0.2)
Heterogenity Index					
Complications	0.14 (0-0.31)	0.12 (0-0.48)	0.14 (0-0.35)	0.16 (0-0.54)	0.14 (0-0.43)
No complications	0.13 (0-0,32)	0.10 (0-0.25)	0.09 (0-0.45)	0.18 (0-0.49)	0.17 (0-0.42)
Estimated difference	-0.01 (-0.1 to 0.1)	-0.02(-0.1 to 0.1)	-0.05(-0.1 to 0.0)	0.02 (-0.1 to 0.1)	0.03 (0.1 to 0.1)
PVD (n/mm)					
Complications	12.6 (11.4-15.1)	12.6 (10.4-17.0)	12.8 (10.7-14.7)	12.4 (10.2-15.2)	12.7 (8.8-16.6)
No complications	12.7 (9.7-14.9)	12.8 (10.5-14.5)	13.2 (11.3-15.7)	12.4 (9.7-14.6)	12.5 (10.0-14.8)
Estimated difference	0.03 (-0.7 to 0.8)	0.2 (-0.7 to 1.2)	0.3 (-0.5 to 1.1)	0.1 (-0.9 to 1.0)	-0.2 (-1.3 to 0.9)
ScvO$_2$ (%)					
Complications		76 (69-89)	77 (63-86)	78 (67-84)	71 (59-81)
No complications		77 (67-88)	81 (66-89)	74 (64-84)	71 (55-82)
Estimated difference		1 (-3 to 6)	4 (0 to 8)	-4 (-9 to 1)	0.1 (-4 to 5)
Lactate (mmol/L)					
Complications		1.2 (0.5-3.1)	2.6(0.7-5.8)	2.5 (0.9-4.0)	1.7 (0.8-3.2)
No complications		1.2 (0.4-3.6)	2.3(0.8-4.2)	2.1 (0.6-4.6)	1.5 (0.5-2.9)
Estimated difference		0.0 (-0.4 to 0.5)	-0.3(-1.0 to 0.5)	-0.4(-1.2 to 0.4)	- 0.3(-0.7 to 0.1)

Measurements were performed prior to surgery (T0), following induction of anesthesia (T1), during the last hour of surgery (T2), within two hours after arrival at the recovery room (T3) and in the morning of the first postoperative day (T4). Estimated difference is presented as mean ± 95% confidence interval all other values are presented as median and interquartiles.

Study V

In the hemorrhage group (normal permeability group), plasma volumes (PV) decreased after bleeding and increased after resuscitation with both albumin or Ringer's acetate and remained unchanged thereafter, with no difference between the albumin and the Ringer's acetate treated animals (Fig 12a + 13a).

In the sepsis group (high permeability group), PV decreased 3 hours after the CLI maneuver and increased after resuscitation with both, albumin or Ringer's acetate. PV decreased again 2 and 4 hours after resuscitation (Fig 12b). PV-expansion was higher in the albumin treated animals at 15 min after resuscitation, but not after 2 or 4 hours (Fig 13b).
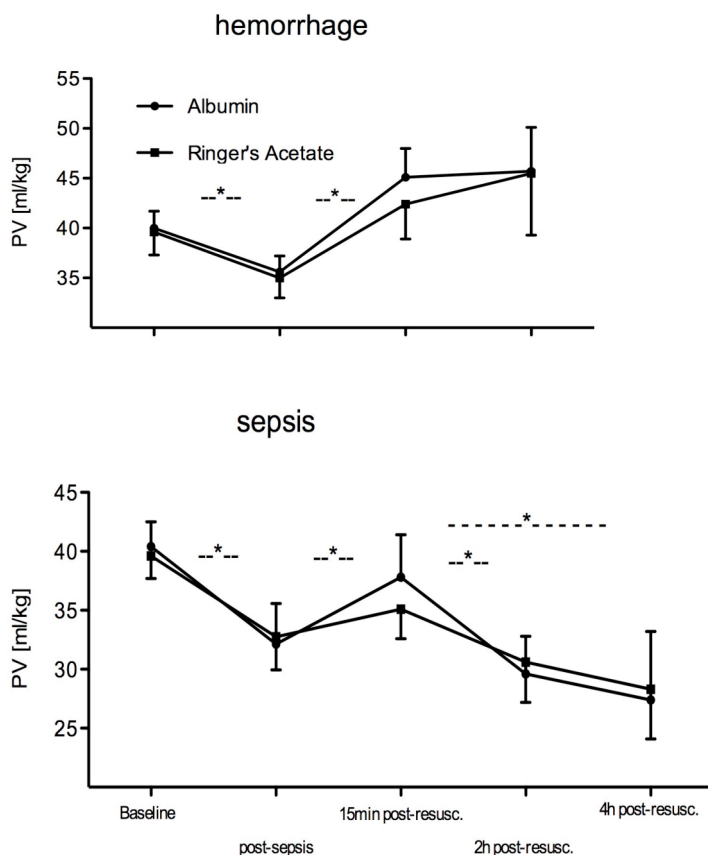

Fig 12a+b. Absolute plasma volumes at baseline and 15 min, 2h or 4h (only sepsis) after resuscitation with either albumin or Ringer's acetate (* = p<0.05).
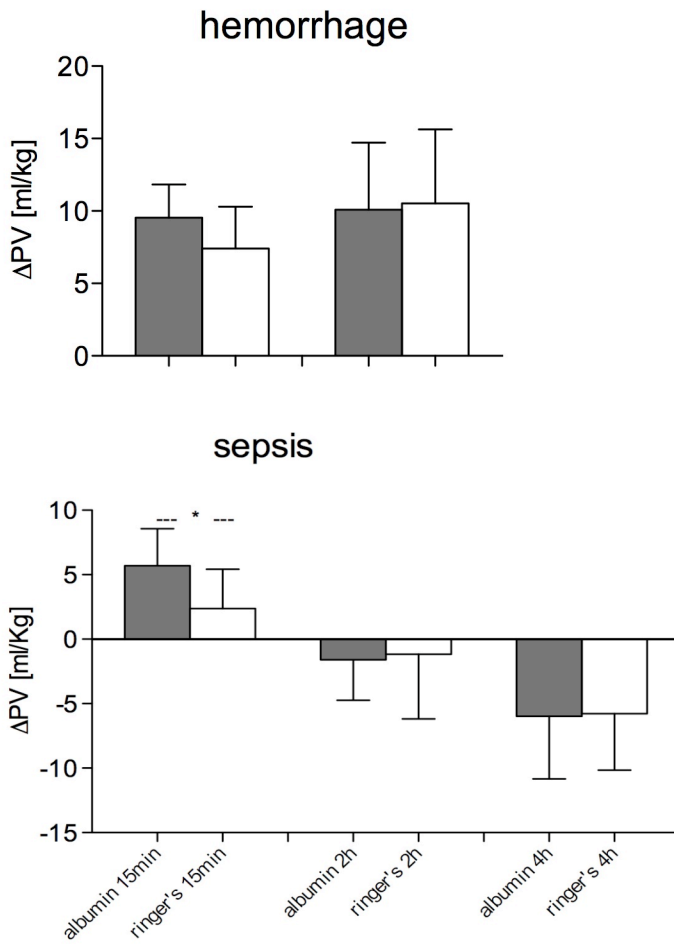
Fig 13a+b. Change in plasma volumes at 15min, 2h or 4h (only sepsis) after resuscitation with either albumin or Ringer's acetate (* = p<0.05).

Discussion

This thesis discusses different aspects of changes in the microcirculation and of transcapillary fluid exchange in states of trauma and sepsis, with a focus on plasma volume and microvascular permeability. The trauma models used were of blunt, non-hemorrhagic or hemorrhagic nature in rats or of mixed nature in the case of a surgical trauma in human subjects. The sepsis model was an abdominal sepsis in rats.

Like in all models using live subjects, there is an expected variation in the host response to trauma or sepsis, necessitating a certain amount of animals to be studied for being able to draw any conclusions, even when using a very standardized kind of experiment. In case of study IV, where human subjects were studied, this variation is even larger due to the different nature of surgery and the underlying disease. For this thesis, 228 rats were studied and put to sleep (killed), not including those used for eventual pilot studies, failed experiments, or those to come for completing experiments - hopefully for a greater good. The number of human subjects put to sleep (anaesthetized), in each case with prior consent given, was 49.

Plasma volumes, microvascular permeability and inflammation after soft tissue trauma

In papers I and II, we first developed a trauma model mimicking blunt skeletal muscle trauma and studied its effect on plasma volume, permeability and the release of inflammatory parameters. We then studied the effect of prostacyclin (PGI_2) on these parameters, a substance that has been shown to have permeability-reducing effects after muscle injury (64-66). The local skeletal muscle trauma caused an increase in microvascular permeability (TER) together with an increase in the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10, leading to a significant PV-loss. This loss could not be explained by the local muscle trauma alone, and we concluded that our trauma model caused similar reactions as other types of a clinical trauma and caused a generalized increase in microvascular permeability. This model was then used to study the effects of PGI_2 as compared to normal saline 0.9% on the observed pathophysiological changes. Our main finding was that PGI_2 attenuated the PV-loss after this soft tissue trauma, probably via a modulation of the vascular permeability and the inflammatory response, and since inflammatory parameters such as IL-6 influence permeability, these reactions are most likely interconnected (67, 68).

It is known that the endothelium can dysfunction during sepsis and that this leads to an imbalance of the release of vasoactive substances like for example NO and PGI_2 (68-71). Whether the permeability-reducing effect of PGI_2 is caused by an endothelial smooth muscle relaxation via an intracellular increase

of cAMP, an enhancement of intercellular junctional strand formation, a modulation of the cytokine release and/or other mechanisms is still not fully understood. Prostacyclins' inhibiting effect on leukocyte and trombocyte adhesion may contribute to these observations by reducing microthrombosis, thereby improving microvascular flow and decreasing fluid extravasation. Chen et al showed that PGI_2 also has an effect on the intracellular peroxisome proliferator-activated receptor-α (PPAR-α), thereby decreasing activation of the nuclear transcription factor-κB (NF-κB) and the release of pro-inflammatory TNF-α (72). An attenuation of the known permeability-increasing effect of TNF-α by PGI_2 has also been shown by Jahr et. al., and the same group showed that PGI_2 decreases the capillary filtration coefficient (CFC) whilst maintaining myogenic reactivity in the microvascular bed (73, 74). In our institution, PGI_2 is frequently used as an anticoagulant during dialysis and occasionally in patients with severe ARDS and capillary leak or pulmonary hypertension with a sometimes dramatic improvement on oxygenation, and we usually do not observe problems with hypotension or increased bleeding tendencies. This is of course a clinical observation and has not been confirmed with a prospective randomized trial. Recently, other therapies like activated protein C, statins and sphingosine 1-phosphate that also target the disturbed endothelial function in SIRS/sepsis have shown promising results and these findings may ultimately lead to a new approach in the treatment of hypovolemia in those patients.

Function of the endothelial glycocalyx
In paper III we studied the importance of charge on vascular permeability in a sepsis model.
The glycocalyx consists of a layer of negatively charged carbohydrate polymers like sialoglycoproteins, syndecan-1 and hyaluronan, coating the luminal side of the vascular endothelium. It has several important functions: It functions as a mechano-sensor, affecting for example NO-release in reaction to changes in blood flow, therebye modulating autoregulation. It lubricates erythrocytes and it functions as a semi-permeable membrane by establishing a size- and charge selectivity of the endothelium (24). This function is of importance for the microvascular permeability for water, small solutes and macromolecules, allowing basically free passage for water and small solutes while impeding passage for larger plasma proteins due to their larger size and the negative charges on the protein surface (75). By comparing TER for normal, negatively charged albumin with charge modified (neutral) albumin under normal and septic conditions, we hypothesized that TER for normal albumin should be affected more by the shedding of the negatively charged glycocalyx during sepsis than neutral albumin, which was supported by our experiments. During control conditions, TER was higher for neutral albumin, confirming the importance of charge for albumins' normally low permeability. During sepsis, TER for neutral albumin had a relatively lesser increase than TER for normal

albumin, and we concluded that the importance of charge for macromolecular permeability is decreased in states causing a breakdown of the glycocalyx. One factor that may have influenced our results is the slightly smaller molecular size of c-BSA, since the loss of negative charge allows the molecule to become more compact. Considering this change in size in a mathematical model provided by the "Rippe-group" (32), it could account for about 30% of the observed difference in TER in this study. Also, an increased glomerular filtration (GFR) of c-BSA may contribute to an overestimation of the importance of charge, with a GFR of about 1.5% for normal BSA and about 13% for c-BSA (76), which could explain up to 40% of the whole observed difference in TER. Vesicular transport of albumin is likely to be of minor importance as discussed in the introduction, but even here, charge appears to be of some importance (77). Also an increased uptake of c-BSA by the reticuloendothelial system could have influenced TER, but probably not to a major extent, since clearence for c-BSA did not change significantly in earlier studies (78, 79). Since negative charges in the glycocalyx would mainly restrict albumin transport through the small-pore-system, an increased number of large pores during sepsis could also have influenced our result of a changed ratio of c-BSA to BSA (80, 81). Recently, Landsverk et al showed that hyaluronidase, an enzyme breaking down parts of the glycocalyx, decreased the functional capillary density, but did not lead to increased vascular leakage (82). Taken together, charge probably plays an important role for the permeability of negatively charged plasma-proteins, but with the possible pitfalls in our study-technique that are discussed above, we can not be certain of our hypothesis that shedding of the glycocalyx is a contributing factor to the increase in permeability for albumin during sepsis and suggest that further research is needed to clarify this.

Sublingual microcirculation measured with SDF
In study IV, we used the Sidestream Darkfield-imaging technique to evaluate peri-operative changes in the microcirculation of patients undergoing major abdominal surgery and found that the observed changes in this setting were small and had no correlation to outcome, which makes it unlikely that this technique will help us to further improve the anesthetic management of these patients. Perfused vessel density (PVD) as a measure for capillary density, and microvascular flow index (MFI) together with a heterogeneity index (HI) as measures for flow were evaluated. The quality of the evaluation is dependent on the quality of the film-sequences taken with the camera. Difficulties can occur from sublingual saliva and fogging of the camera lens, from surgical electrocautherization during the measurement, from pressure artifacts and from moving artifacts when patients were awake. Image recording was repeated if deemed of insufficient quality until most often 5, but at least 3 film sequences of good quality could be recorded at each time point. Moving artifacts may lead

to a smaller image size available for analysis, which can lead to unreliable results for PVD. Therefore, films with a reduction in image-size > 20% after image stabilization were excluded. The sample size with only 42 patients included in this study may appear small and we could not exclude that there may be differences between the groups with and without complications that might have been detected if the sample size had been much larger. If such a method is to be useful for managing the peri-operative management of patients and help reducing postoperative morbidity, it needs to be quite sensitive with a low number of patients needed to treat/to be observed (NNT), which is why we stopped the study after the interim-analysis. The method showed interesting results in some ICU studies (83-85), but patients there were much sicker and microvascular alterations more pronounced than in our cohort. The lack of correlation with lactate and central venous oxygenation (ScvO$_2$) has been described earlier (83, 86) and is an interesting observation, since measurement of lactate, ScvO$_2$ and other macrocirculatory parameters often guide our anesthetic management. Even though optimizing these parameters is an important goal for our therapy, it does not necessarily lead to an improved microcirculation, which may be a similarly important target for our interventions (87).

Colloid versus crystalloid solutions

In paper V we studied the effect of albumin versus Ringer's acetate on plasma volume expansion in states of normal (hemorrhage model) and increased (sepsis model) microvascular permeability and found that the correlation in the distribution between PV and in the interstitial space (ISV) of these 2 solutions appears to be independent of the state of permeability. Our hypothesis was that the normal PV-expanding effect of albumin in relation to Ringer's acetate of about 1:4.5 would change in favor of Ringer's acetate in a state with increased capillary permeability, since mainly the permeability for albumin, but not the already high permeability for Ringer's acetate should increase during sepsis. Our results could not confirm this hypothesis, at least not during the study period, which lasted for up to 4 hours. It would have been interesting to prolong the study-period even further to see whether the PV-expanding effect of albumin becomes less effective due to increased leakage or accumulation in the interstitial space, thereby affecting oncotic pressures in the Starling-equilibrium, but in our experimental setting, with no antibiotic- or additional fluid-treatment, the mortality rate is too high to permit an extension of the experiments. It could be argued that even haemorrhage can lead to an increase in permeability, but we let the animals only bleed 8 ml/kg with minimal trauma (due to tracheostomy and catheterization), which is unlikely to have such an effect. Also, our results of PV increasing above baseline-values and maintaining these values even at 2 hours after resuscitation would be an unlikely observation if permeability had been increased. That permeability is increased in the septic animals is supported

by the facts that hematocrit increased and PV decreased, most likely due to ongoing PV-leakage. In an earlier study using the CLI procedure we showed that also TER increased significantly, suggesting an increase in permeability (88). Since plasma protein leakage mainly occurs via convective transport, a decrease in hydrostatic capillary pressure (Pc) may have led to a diminished loss of albumin whereas some literature suggests that Pc may be increased (89, 90). This relation will be discussed later more extensively.

Plasma volume measurement and transcapillary escape rate (TER)

A main focus in all the animal studies was the measurement of plasma volume (PV), which is why it is discussed here in more detail: The ^{125}I-albumin method (and ^{131}I-albumin in case of study III) is a reliable and reproducible technique, directly measuring PV and making it possible to judge whether animals truly are normo- or hypovolemic, without having to rely on indirect hemodynamic parameters (56, 57). A possible error may occur in case of insufficient distribution of the tracer in the whole blood volume, but with an average normal cardiac output of around 100 ml/min, and not below 30 ml/min even in severe hemorrhage or sepsis, the 5 min allowed for mixing should be more than sufficient (91-93). Another possible error may originate from unbound radioactivity, but this was measured and found to be below 1% in all experiments. The natural or increased transcapillary escape rate of albumin (TER) may lead to a slight overestimation of PV since some of the tracer disappears from the intravascular compartment during the 5-min mixing period, but with TER between 12-19 %/h in study I+II, the 5 min should account for only minor inaccuracies. Even in study III, with a TER up to 30 %/h for c-BSA, the difference to TER for normal BSA was less than 10 %/h and the potential error during the 5 min period therefore less than 1%. It could also be speculated that the coupling of an iodine molecule may change the way the body handles the radioactive albumin, but change in molecular size or charge is negligible and even if tracer distribution should be effected, it would be the same error for all measurements. TER itself is calculated by measuring ^{125}I-albumin concentration in plasma samples taken at 5 time points during a 1-hour period. The plasma disappearance of albumin has earlier been shown to be linear between 10 and 60 min (94) and our regression lines with an R^2 value above 0.9 support this finding (95, 96). Apart from an increase in microvascular permeability, TER is also influenced by hydrostatic capillary pressure, but since there were no differences in arterial or central venous pressures (study I) between the groups, it is unlikely that a difference in hydrostatic pressures can account for the observed differences in TER.

Microvascular permeability and transcapillary fluid exchange

The state of the capillary barrier and the hydrostatic capillary pressures govern the Starling-equilibrium over the microcirculation. Hydrostatic pressure (P_c) depends on arterial pressure (P_a), venous pressure (P_v) and on arteriolar pre-capillary (R_a) and post-capillary (R_v) resistance (97):

$$P_c = P_a + P_v * (R_a/R_v) / (1 + R_a/R_v)$$

The observed PV-loss in our animal studies could therefore also be caused in part by an increased hydrostatic pressure instead of an increased permeability, as we postulated. Most literature suggests that capillary blood flow is decreased in SIRS/Sepsis due to decreased MAP, increased arteriolar vasoconstriction and an increase in capillary shunting (98-101), but there is also evidence that pre-capillary small arteriolar resistance (R_a) decreases in sepsis (99) and that post-capillary resistance (R_v) can be increased (89, 90), which would lead to an increase in P_c. It has also been suggested that a decrease in interstitial pressure (P_i) due to the release of cellular tension exerted on the interstitial collagen and microfibril networks during inflammation, may lead to an increased fluid filtration (102), but when interstitial edema develops, as is often seen during inflammation, P_i is more likely to increase. Altogether, the net-effect of changes in capillary hydrostatic pressure on transcapillary fluid exchange depends on several factors and therefore may not be easy to estimate. Nonetheless, plasma disappearance of macromolecules such as albumin, as seen in our studies, can only increase to a smaller extent due to increased hydrostatic pressures as long as normal capillary permeability is preserved, as opposed to situations with increased permeability (56). The increase in permeability during SIRS and sepsis is part of the natural defense mechanism, allowing macromolecules in the blood stream like leukocytes, macrophages and immunoglobulins to enter the affected tissue through an increased amount of large gaps or pores in the endothelium. This necessary mechanism also leads to loss of plasma volume and to tissue swelling, which, in case of severe damage or sepsis, can have deleterious effects on the micro- and macrocirculation, leading to multiple organ failure and eventually death. Treatment consists of antibiotic therapy in case of sepsis, damage control in case of haemorrhage and in restitution of plasma volume with intravenous fluid therapy. In cases with maintained microvascular permeability, for example in our haemorrhage experiments in study V, fluid therapy is very effective in increasing the diminished PV. In cases of increased permeability on the other hand, like after soft tissue trauma in study I+II or after sepsis in study III+V, plasma volume depletion continues as long as the pathophysiologically increased permeability persists. In practice, this necessitates large volumes of iv-fluids, which in turn can increase the tissue edema and therefore worsen organ function (103). The lymphatic system plays an important role in re-circulating the filtrated fluid back to the intravascular

space, and transport capacity can increase during SIRS/sepsis (104-106) but lymphatic dysfunction has been described in inflammatory states (107-110) and large enough filtration will eventually lead to interstitial fluid overload and edema.

Interleukin measurement
The different cytokines in study I+II were analyzed using flow cytometry, which is a standardized and reliable method (111). Cytokine-release is dependent on the time of analysis and on the type and severity of the underlying condition (112-114). In paper I, we analyzed 5 different cytokines known to be released after trauma of which IL-6 and IL-10 increased significantly. Those 2 interleukins were therefore also analyzed in study II and have earlier been shown to correlate with injury severity and mortality (115).

Main conclusions

Paper I + II

Blunt skeletal muscle trauma leads to a decrease in plasma volume, caused by a generalized inflammatory reaction with an increase in capillary permeability.

Prostacyclin attenuates the loss of plasma volume in this model, probably due to a decrease in permeability and a modulation of the inflammatory reaction.

Paper III

Negative charge of the glycocalyx appears to be important for the normally low permeability for albumin. The CLI maneuver in this model caused an increase in permeability and a breakdown of the glycocalix.

Paper IV

Peri-operative changes in the sublingual microcirculation are small and are not correlated to outcome, lactate or central venous saturation in major abdominal surgery.

Paper V

The plasma volume-expanding effects of albumin and Ringer's acetate appear to be independent of the state of capillary permeability.

Populärwissenschaftliche Zusammenfassung in Deutsch

Diese Doktorarbeit mit dem Titel "Die Mikrozirkulation während Trauma und Sepsis" besteht aus 5 Teilarbeiten. In den ersten zwei Arbeiten haben wir erst ein Muskeltrauma (Muskelquetschung) an der Magenwandmuskulatur bei Ratten verursacht und verschiedene Kreislaufreaktionen untersucht. Hier hat sich gezeigt, dass dieses lokale Trauma zu einer generalisierten Erhöhung der Durchlässigkeit der kleinsten Gefässe "Kapillaren" und zu einer "Leckage" von Fluessigkeit aus dem Kreislaufsystem fuehrt, was unter anderem zu einem Blutdruckabfall führte. Danach haben wir untersucht, welchen Einfluss die Gabe der körpereigenen Substanz "Prostazyklin" auf diese "Leckage" hat und festgestellt, dass der Verlust von Volumen aus den Gefässen damit geringer war. Dies führte uns zu der Vermutung, dass die Behandlung mit Prostazyklin potenziell bei Situationen helfen könnte, bei denen eine erhöhte Gefässleckage (Permeabilitet), wie zum Beispiel bei Unfallopfern, ein Problem darstellt.

Die dritte Arbeit untersucht die Bedeutung von negativen Ladungen in der Auskleidung der Gefässwände, der sogenannten "Glykokalyx". Diese Schicht hilft, die "Leckage" von Proteinen, die ebenfalls negativ geladen sind, gering zu halten. Wir haben bei Ratten eine Blutvergiftung durch Punktion des Darms ausgelöst und festgestellt, dass diese Schicht dadurch zum Teil zerstört wurde. Durch ein Vergleichen der Verteilung von negativ geladenem im Verhältnis zu ungeladenem Albumin im Blut bei Ratten mit normaler und erhöter Permeabilitet konnten wir feststellen, dass die Glykokalix wichtig ist fuer die normalerweise geringe Leckage von Albumin, und dass diese Bedeutung im Falle einer Blutvergiftung abnimmt.

In der vierten Arbeit haben wir untersucht, ob Veränderungen im Blutfluss der kleinsten Gefässe (Kapillaren) während und direkt nach chirurgischen Baucheingriffen mit Komplikationen innerhalb der ersten 30 Tage nach der Operation korrelieren. Die Gefässe unter der Zunge wurden mit Hilfe einer stark vergrössernden Kamera gefilmt und danach auf verschiedene Aspekte des Blutflusses untersucht, in der Hoffnung, dass diese Methode zu einer verbesserten Behandlung von Patienten beitragen könnte. Hier konnten wir keine Übereinstimmung von verschlechtetem Blutfluss mit Komplikationen oder anderen normalerweise untersuchten Blutwerten feststellen, was die Methode in diesem Zusammenhang als nicht hilfreich erscheinen lässt.

In der letzen Arbeit haben wir untersucht, ob die Effektivität von zwei häufig benutzen Infusionslösungen für die Behandlung von niedrigem Blutdruck, Albumin oder Ringer Acetat, von der Gefässdurchlässigkeit (Permeabilität) abhängig ist. Normalerweise ist Albumin ca. 4,5 Mal effektiver als Ringer

Acetat das verlorene Blutvolumen wieder aufzufüllen, da Albumin als grosses Molekül im Vergleich zur Ringer Acetat-Lösung mit kleinen Molekülen die Blutbahn nicht so schnell verlässt. In Situationen mit erhöhter Permeabilität wie bei einer Blutvergiftung könnte dieser Vorteil aber verloren gehen und damit die Effektivität von Albumin als Blutvolumenexpander abnehmen. Unsere Versuche bei Ratten mit normaler im Vergleich zu erhöhter Permeabilität haben doch gezeigt, dass dies zumindest innerhalb der ersten vier Stunden nach Blutvergiftung oder Blutung nicht der Fall ist.

Populärvetenskaplig sammanfattning på Svenska

Denna doktorsavhandling med titeln "mikrocirkulation vid sepsis och trauma" består av 5 delarbeten. I de första två arbeten har vi undersökt effekten av en muskeltrauma (muskel kontusion) av musklerna i bukväggen hos råttor på olika kardiovaskulära reaktioner. Vi har visat att detta lokala trauma leder till en generell ökning av kärlgenomsläppligheten (permeabiliteten) för de minsta kärlen "kapillärer" och till ett "läckage" av vätska från cirkulationssystemet, bland annat med blodtryckssänkning som följd. Därefter undersökte vi påverkan av det kroppsegna ämnet "prostacyklin" på detta "läckage" och har funnit att det kunde minska förlusten av blodvolymen. Vi konkluderade att en behandling med prostacyklin eventuellt skulle kunna hjälpa i situationer där ett ökat läckage från kärlen är ett problem, exempelvis hos olycksoffer.

Den tredje studien undersöker betydelsen av negativa laddningar på insidn av av kärlväggen, I den så kallade "glykokalyx." Detta skikt hjälper till att hålla "läckagen" av proteiner, som också är negativt laddade, lågt. Vi har orsakad en blodförgiftning hos råttor genom en punktering av tarmen och fann att glykokalix bröts ner på grund av detta. Genom att undersöka beteendet av negativt laddad jämfört med neutralt laddat albumin (ett protein) fann vi att glykocalix är viktigt för den normalt låga läckage av albumin, och att denna betydelse minskar vid blodförgiftning.

I den fjärde arbete har vi undersökt om förändringar i blodflödet av de minsta blodkärlen (kapillärerna) innan, under och omedelbart efter bukoperationer korrelerade med komplikationer inom de första 30 dagarna efter operationen. Kärlen filmades med en stark förstorande kamera och undersöktes sedan för olika aspekter av blodflödet i hopp om att denna metod skulle kunna bidra till förbättrad patientvård. Här hittade vi ingen korrelation mellan försämrad blodflöde och komplikationer eller andra normalt undersökta blodvärden, vilket gör att metoden i detta sammanhang inte verkar kunna bidra till att förbättra omhändertagandet av patienterna som genomgår bukkirurgiska ingrepp.

I det sista arbetet vi har undersökt om effektiviteten av två vanlig förekommande infusionslösningar för behandling av lågt blodtryck, albumin eller Ringeracetat, beror på kärlens permeabilitet (genomsläpplighet). Albumin är normalt ca 4,5 gånger mer effektiv än Ringer acetat i att expandera den förlorade blodvolymen, eftersom albumin, som är en stor molekyl, inte lämnar blodbanan lika snabbt som Ringeracetat-lösning som innehåller små molekyler. I situationer med ökad permeabilitet som vid blodförgiftning skulle denna fördel kunna gå förlorad, och därmed minska effektiviteten av albumin som blodvolym-expander. Våra experiment hos råttor med normal kontra förhöjd

permeabilitet har visat att så inte är fallet, åtminstone inom de första fyra timmarna efter sepsis eller blödning.

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Appendix

Original studies I-V

A MODEL FOR EVALUATING THE EFFECTS OF BLUNT SKELETAL MUSCLE TRAUMA ON MICROVASCULAR PERMEABILITY AND PLASMA VOLUME IN THE RAT

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ABSTRACT—The objective of the present study was to develop an experimental model suitable for studying the effects of a nonhemorrhagic soft tissue trauma on plasma volume (PV) and microvascular permeability. Anesthetized Sprague-Dawley rats were exposed to a sham procedure or a laparotomy followed by a standardized trauma to the abdominal rectus muscle. We evaluated the effects of trauma on transcapillary escape rate and on PV (3 h after trauma) using ^{125}I-albumin as tracer and on edema formation in the traumatized muscle with a wet- versus dry-weight method. The effects of the trauma on the cytokines IFN-γ, IL-4, IL-6, IL-10, and TNF-α were investigated 1 and 3 h after trauma in a separate group. Transcapillary escape rate was 13.9% per hour in the sham animals compared with 18.5% per hour in the traumatized animals ($P < 0.05$). Because arterial and venous blood pressures were not altered by the trauma, the change in transcapillary escape rate most likely reflects a change in microvascular permeability. Plasma volume decreased from 42 mL/kg at baseline to 31 mL/kg at the end of the experiments ($P < 0.05$) in the trauma group, whereas PV remained unchanged in the sham group. Only 15% of the PV loss could be referred to edema in the traumatized muscle. Trauma induced a significant increase in IL-6 and IL-10 after 1 h. We conclude that the present nonhemorrhagic trauma induces an increase in microvascular permeability in the traumatized tissue and in other parts of the body, resulting in hypovolemia. The model may be used for the evaluation of different therapeutic interventions aimed at the correction of hypovolemia.

KEYWORDS—Hypovolemia, shock, albumin, cytokines, inflammation

INTRODUCTION

For a long time, it has been recognized that trauma induces a general increase in microvascular permeability, which may cause hypovolemia and shock even in the absence of hemorrhage (1–4). In clinical practice, this condition necessitates treatment with intravenous administration of fluids to restore blood volume and to improve tissue perfusion. However, administration of intravenous fluids has side effects such as edema formation, which can increase oxygen diffusion distances and increase tissue pressure. It is likely that treatments aimed at reducing the trauma-induced loss of fluid from the circulation and/or optimization of fluid therapy can improve outcome. For this purpose, there is a need for experimental models in which a trauma-induced nonhemorrhagic hypovolemia can be produced in a standardized and reproducible fashion.

To the best of our knowledge, there is only 1 model published in which changes in plasma volume (PV) and permeability were analyzed after a nonhemorrhagic trauma (5). However, the trauma in that study was unspecific and included several intra-abdominal organs, and concerns about the standardization of the trauma can be raised. Furthermore, no attempt was made to evaluate if the trauma induced a local or general increase in permeability. Although a clinical trauma often involves several organs, there is an advantage of limiting the experimental trauma to a single organ for best reproducibility to facilitate the interpretation of the observed hemodynamic alterations, and it allows a standardized injury. Skeletal muscle tissue is a suitable organ to study in this respect because it is the largest internal organ of the body and frequently suffers traumatic injuries.

The aim of the present study was to establish a standardized and reproducible nonhemorrhagic trauma model for the analysis of trauma-induced changes in permeability and PV and for the investigation of underlying mechanisms and potential treatment strategies. For this purpose, the abdominal rectus muscle of the rat was traumatized in a standardized fashion, and the effects of the trauma on PV and transcapillary escape rate (TER) of albumin were evaluated. The plasma concentrations of various cytokines were measured to evaluate if the trauma induced a systemic inflammatory response.

METHODS

Material and anesthesia

The study was approved by the Ethics Committee for Animal Research at Lund University, Sweden (application no. M8-08), and the animals were treated in accordance with the guidelines of the National Institutes of Health for Care and Use of Laboratory animals. Adult male Sprague-Dawley rats (n = 60) weighing 355 ± 14 g (mean ± SD) were used. Anesthesia was induced by placing the animals in a covered glass container with a continuous supply of 5% isoflurane in air (Forene, Abbot Stockholm, Sweden). After induction, the animals were removed from the container, and anesthesia was maintained with 1.6% to 1.8% isoflurane in air delivered via a mask. After tracheostomy, the animals were connected to a ventilator (Ugo Basile; Biological Research Apparatus, Comerio, Italy) and ventilated in a volume-controlled mode using a positive end-expiratory pressure of 4 cm water. Body temperature, measured rectally, was kept at 37.1 to 37.3°C via a feedback-controlled heating pad. End-tidal P_{CO_2} was monitored continuously and kept between 4.8 and 5.5 kPa (Capstar-100, CWE, Ardmore, Pa). Left femoral artery was cannulated for measurement of MAP and to obtain blood samples for measurement of
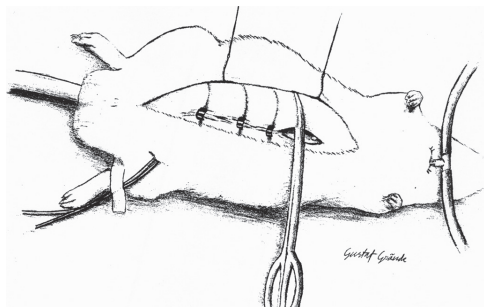
Fig. 1. **A schematic drawing of the muscle trauma procedure.**

arterial blood gases, electrolytes, and hematocrit ([Hct] I-STAT; Abbot, Abbot Park, Ill).

The left femoral vein was cannulated and used for injections and kept open with a continuous saline infusion of 0.5 µL/min. The internal jugular vein was cannulated in some animals for measurement of central venous pressure. Urine was collected in a glass vial from the end of the preparation until the end of the experiment. After the experiment, animals were killed with an intravenous injection of potassium chloride.

Experimental trauma

After a longitudinal midline skin incision over the abdominal wall with diathermia, a laparotomy was performed by an incision along the linea alba. This was followed by the standardized trauma of the rectus muscle at 12 different locations, 6 on each side of the midline, extending approximately 4 cm laterally using a medium-sized anatomic forceps (Fig. 1). The trauma was induced by closing the forceps for 3 to 5 s, 3 times at each of the 12 locations. To reduce evaporation, we kept the time of exposure to the atmosphere of the wound area at a minimum. For this reason, the trauma was performed in 2 steps. First, half of the laparotomy (approximately 4 cm in length) was performed, and the trauma was induced on the corresponding part of the muscle, after which the abdominal opening was closed with surgical clips. After that, the other half of the trauma was performed in the same way. The abdomen was kept open to the atmosphere for 5 min at the most. Careful inspection revealed no signs of hemorrhage after trauma in any of the animals. The skin was closed with clips. Sham trauma animals were not subjected to any surgical trauma but only to anesthesia, cannulation, and tracheostomy.

In additional experiments, all surgical procedures except the muscle trauma itself were performed in an attempt to separate the effects of the skeletal muscle trauma from those of the rest of the surgical procedures (skin dissection and laparotomy).

Measurement of PV

Plasma volume was determined by measurement of the increase in radioactivity per milliliter of plasma after an intravenous injection of a known amount of activity of human ^{125}I-albumin (GE Health Care, Bio-Science,

Kjeller, Norway). The increase in radioactivity was calculated by subtracting the activity in a blood sample taken just before the injection from that taken 5 min after the injection. Through this technique, the PV measurement was independent of the remaining radioactivity from previous radioactive injections. To determine the exact dose injected, we subtracted the remaining radioactivity in the emptied vial, syringe, and needle from the total radioactivity in the prepared dose. As discussed previously, this is a reliable technique, giving reproducible results, and possible sources of error are small (6, 7).

Measurement of skeletal muscle water content

To evaluate to what extent the amount of a PV loss can be referred to edema in the traumatized muscle, we measured and compared with total PV reduction the increase in water content of the muscle after the trauma. Muscle edema in the rectus muscle was estimated by determination of water content 3 h after the trauma or the sham procedure. For this purpose, the traumatized muscle (measuring approximately 6.5×4.0 cm on each side) and the corresponding part of the muscle in the sham animals were resected, weighted, and put in an oven at a temperature of 100°C for 1 week. The water content in the tissue was measured with a wet-dry tissue technique as follows: [(wet tissue weight − dry tissue weight) / wet tissue weight] × 100. By subtracting the increase in tissue water content (mL/kg body weight) in the traumatized muscle from the PV loss (mL/kg body weight) induced by the muscle trauma, the fluid loss in noninjured parts of the body can be calculated (for details of the calculation, see Results).

Measurement of TER for albumin

Transcapillary escape rate for albumin after trauma was determined by measurement of the reduction in the radioactivity per time unit after injection of a bolus dose of ^{125}I-albumin. For this purpose, blood samples of 250 µL were taken in heparinized vials at 5, 15, 30, 45, and 60 min after the ^{125}I-albumin injection. After centrifugation at 8000 rpm, radioactivity in a PV of 100 µL was measured with a gamma counter (Wizard 1480; LKB-Wallace, Turku, Finland). The amount of unbound radioactivity in the injected ^{125}I-albumin in the PV and TER groups was measured regularly after precipitation with trichloroacetic acid and was found to be less than 1% in all cases.

Cytokines

The plasma concentrations of IFN-γ, IL-4, IL-6, IL-10, and TNF-α were measured from arterial blood samples collected 1 and 3 h after the trauma or the sham procedure. Cytokine levels were determined with a flow cytometer using cytometric bead array kits specific for respective cytokines according to the instructions provided by manufacturer (BD Biosciences, Franklin Lakes, NJ).

Experimental protocol

The study consisted of 3 main groups: a PV group, a TER group, and a cytokine group. The experimental protocols for these groups are illustrated in Figure 2. For all 3 groups, preparation including anesthesia, cannulation, and tracheostomy lasted for about 40 min. The animals were undisturbed during the next 15 min to assure hemodynamic stability. This was followed by the experimental trauma or sham trauma, lasting for about 25 min from start of the surgical preparation until the skin was closed (see above).

In the PV group, the PV was measured before the trauma and 3 h after the trauma, and the data were compared with those from sham animals (n = 7 per
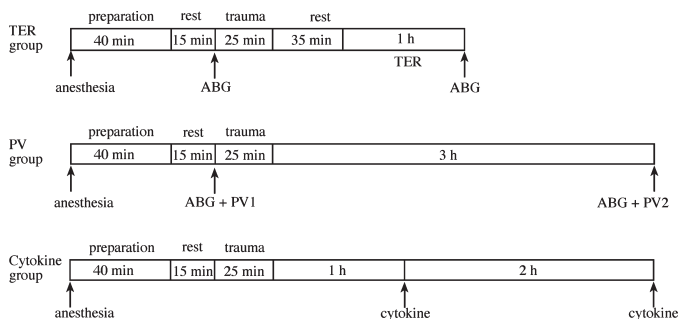

Fig. 2. **Time scale for the experiments in the PV group, the TER group and the cytokine group.** Cytokine concentrations were analyzed at 1 and 3 h after the trauma. ABG indicates arterial blood gas.

TABLE 1. **Na$^+$ concentration, K$^+$ concentration, Hct, pH, Paco$_2$, Pao$_2$, and BE before trauma or sham trauma, 90 min after trauma or sham trauma (end of experiment TER group), and 180 min after trauma or sham trauma (end of experiment PV group)**

	Na$^+$, mmol/L	K$^+$, mmol/L	Hct, %	pH	Paco$_2$, kPa	Pao$_2$, kPa	BE
Baseline trauma, n = 14	136 ± 2	4.8 ± 0.2	41 ± 2	7.51 ± 0.03	4.7 ± 0.3	12.3 ± 0.9	5.6 ± 1.2
Baseline sham, n = 14	135 ± 1	4.8 ± 0.4	42 ± 2	7.50 ± 0.02	5.0 ± 0.4	12.1 ± 0.9	6.1 ± 1.5
90 min after trauma, n = 7	134 ± 2	6.0 ± 0.6*	43 ± 1	7.45 ± 0.02*	5.0 ± 0.2	11.5 ± 0.6	1.3 ± 1.7*
90 min after sham, n = 7	136 ± 1	4.9 ± 0.5	37 ± 1*	7.50 ± 0.02	4.7 ± 0.2	11.0 ± 0.6	4.1 ± 1.1
180 min after trauma, n = 7	130 ± 1*	6.4 ± 0.2*	45 ± 2*	7.45 ± 0.03*	4.8 ± 0.3	12.0 ± 1.0	1.0 ± 1.5*
180 min after sham, n = 7	134 ± 1	5.0 ± 0.9	40 ± 2	7.46 ± 0.03	4.9 ± 0.3	11.3 ± 0.6	3.0 ± 1.4*

*P < 0.05 compared with baseline in respective group.

group). The water content of the rectus muscle in the trauma and the sham group was determined at the end of the experiment as described earlier. On a *post hoc* basis, experiments were performed to investigate to what extent the skin preparation and the laparotomy *per se* contributed to the observed PV decrease after trauma. These experiments followed the same protocol, except that no rectus muscle trauma was performed (n = 4 per group).

In the TER group, the ^{125}I-albumin was injected 30 min after completion of the trauma, followed by the TER measurement lasting for 1 h as described earlier. The TER data were compared with corresponding data from sham animals (n = 7 per group). The TER measurement started 30 min after the trauma because it takes some time for the increase in capillary permeability to develop, and we wanted to make the TER measurement in the middle of the experimental period. Change in central venous pressure was measured via the right internal jugular vein to evaluate if the change in venous pressure could affect the TER measurement via a change in hydrostatic capillary pressure (see Discussion). On a *post hoc* basis, experiments were performed to investigate to what extent the skin preparation and the laparotomy *per se* contributed to the observed increase in TER. These experiments followed the same protocol, except that no rectus muscle trauma was performed (n = 4 per group).

The cytokine concentrations were measured in the cytokine group at 1 and 3 h after the trauma to be compared with the corresponding values in the sham animals (n = 8 per group).

Statistics

The results are presented as mean ± SD. Statistical comparisons between 2 groups were performed with the Student *t* test, when the data were normally distributed, and with the Mann-Whitney rank sum test, when the normality test failed. Physiological data were analyzed with the Kruskal-Wallis test followed by the Dunn multiple comparison test. *P* values below 0.05 were considered significant. Sigma Plot 11 software was used for the analysis.

RESULTS

Physiological data

Data for sodium (Na$^+$) and potassium (K$^+$) concentrations, Hct, pH, Paco$_2$, Pao$_2$, and base excess (BE) for the TER and the PV groups are summarized in Table 1. There were no differences in these parameters at baseline between the groups. There was a trend toward an increase in Hct 1.5 h after trauma, which reached statistical significance 3 h after trauma. Sodium concentration was unchanged during the experiments in the sham group and was slightly decreased 3 h after trauma. Potassium increased in the traumatized animals, whereas no change could be detected in the sham group

animals. Base excess and pH decreased in the traumatized animals, and a decrease in BE compared with baseline could be detected 3 h after the sham procedure in the sham group animals. There was no difference in Pao$_2$ and Paco$_2$ during the experiments between the trauma and the sham groups. In the trauma and the sham groups, the urine production was 0.9 ± 0.5 and 1.0 ± 0.2 mL/kg per hour, respectively, and did not differ between the groups.

A summary of the blood pressure values for the PV group and the TER group at baseline, just after completion of the trauma, 30, 60, 90, 120, and 180 min after the trauma is presented in Table 2. The mean values for the PV group and the TER group are presented together up to 90 min after the trauma. Only the PV group values are presented after 90 min because the TER experiment was terminated at that point of time. There was a significant reduction in blood pressure at the end of the experiments compared with baseline in both groups (*P* < 0.05), but blood pressure did not differ significantly between the trauma and the sham groups.

Plasma volume

In the traumatized animals, PV decreased from 41.8 ± 0.6 mL/kg at baseline to 31.4 ± 2.2 mL/kg at the end of the experiments (n = 7; *P* < 0.05). In the sham animals, PV was 41.4 ± 2.6 mL/kg at baseline and 42.0 ± 2.4 mL/kg at the end of the experiment (n = 7; Fig. 3). The PVs in the animals exposed only to skin incision and laparotomy were 41.0 ± 2.7 mL/kg at baseline and 39.2 ± 3.3 mL/kg at the end of the experiment (n = 4).

Skeletal muscle edema

The relative water content in the traumatized muscle was 79.5% ± 0.6% as compared with 73.4% ± 2.1% in the sham animals, giving a difference of about 6% between the groups (*P* < 0.01; n = 7 per group). With a mean weight of the analyzed rectus muscle per rat of 9.5 ± 0.9 g for nontraumatized tissue, the 6% correspond to a mean increase in water content

TABLE 2. **MAP after trauma or sham trauma for the PV group and the TER group (n = 14 up to 90 min and n = 7 thereafter because the TER experiments terminate at 90 min)**

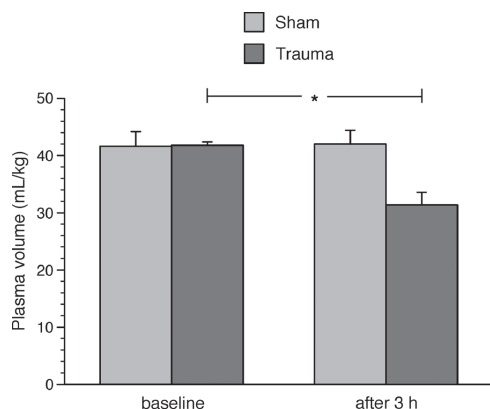| MAP, mmHg	Baseline	After trauma	30 min after trauma	60 min after trauma	90 min after trauma	120 min after trauma	180 min after trauma
Trauma	93 ± 15 (n = 14)	96 ± 11 (n = 14)	81 ± 14 (n = 14)	72 ± 5 (n = 14)	73 ± 8 (n = 14)	77 ± 14 (n = 7)	77 ± 12 (n = 7)
Sham	90 ± 18 (n = 14)	86 ± 13 (n = 14)	77 ± 10 (n = 14)	79 ± 12 (n = 14)	77 ± 11 (n = 14)	80 ± 17 (n = 7)	80 ± 14 (n = 7)

FIG. 3. **Plasma volume 3 h after the trauma or the sham procedure (n = 7 per group; *P < 0.05).**

of the traumatized muscle of 0.6 ± 0.1 mL. This corresponds to a fluid loss from the intravascular compartment of approximately 1.6 mL/kg body weight.

TER for albumin

In the traumatized rats, TER was 18.5% ± 2.3% compared with 13.9% ± 2.5% per hour in the sham group (n = 7 per group; $P < 0.05$; Fig. 4). A regression line with an R^2 value above 0.9 for all measurements confirmed that there was a good agreement between the measured values and the slope of the curve. The corresponding TER in the experiments exposed only to skin incision and laparotomy was 14.2% ± 3.1% per hour (n = 4).

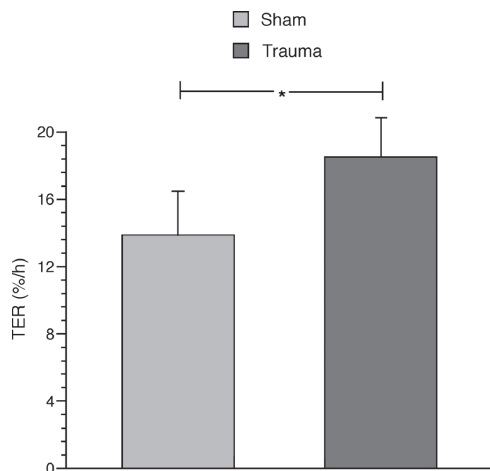For the purpose of evaluating a possible effect of venous pressure for the TER results, central venous pressure in the

FIG. 4. **Transcapillary escape rate for albumin after the trauma or the sham procedure (n = 7 per group; *P < 0.05).**

trauma and the sham animals was measured in the TER group. Mean central venous pressure was 2.8 ± 1.0 and 2.6 ± 0.7 mmHg before start of trauma and sham trauma, respectively; 2.3 ± 0.3 and 2.6 ± 0.2 mmHg 30 min after trauma and sham trauma, respectively; and 2.4 ± 0.4 and 2.7 ± 0.7 mmHg at the end of trauma and sham trauma, respectively. There was no difference in central venous pressure between the trauma group and the sham group at any point in time.

Cytokines

The concentrations of the cytokines IFN-γ, IL-4, IL-6, IL-10, and TNF-α at 1 and 3 h after trauma or sham trauma are presented in Figure 5. A significant increase in IL-6 and IL-10 could be detected 1 h after trauma (n = 8 per group). One 1-h value in the sham group was excluded due to an analytical error.

DISCUSSION

The present study on the rat aimed at designing an experimental trauma model that can be used for the evaluation of changes in PV and microvascular permeability after a non-hemorrhagic trauma. The results showed that a blunt trauma to the abdominal rectus muscle induced a decrease in PV, coinciding with an increase in Hct, and an increase in TER for albumin compared with sham injured animals. The trauma also induced an increase in water content of the traumatized
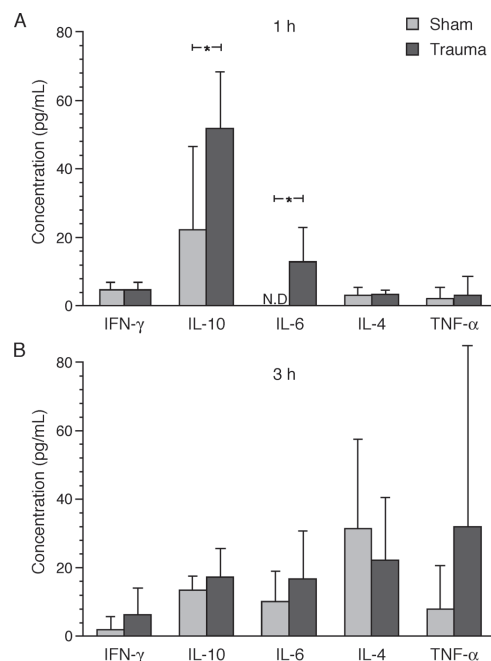

FIG. 5. **Plasma concentrations of IFN-γ, IL-4, IL-6, IL-10, and TNF-α at 1 h (panel a) and 3 h (panel b) after a trauma or a sham procedure (n = 8 per group; *P < 0.05).** N.D. indicates not detectable. Values are given in pg/mL.

muscle and an increase in plasma concentrations for IL-6 and IL-10. The increase in K^+ after trauma was most likely caused by release from the damaged tissue. We have no reasonable explanation for the unexpected decrease in Na^+ concentration at 3 h after the trauma (Table 1). There was no difference between the sham and the trauma groups regarding MAP, urine output, or central venous pressure.

The PV measurement dilution technique using ^{125}I-albumin as tracer is well established for the measurement of PV, both in experimental and in clinical studies, showing reproducible results during normal as well as inflammatory states (6, 7). The fact that measured baseline PVs of 41 to 42 mL/kg were in the same range as those presented in the literature for the rat supports the reliability of the PV-measuring technique (8, 9). Because of the transcapillary escape of albumin during the 5-min period between tracer injection and blood sampling, the albumin-derived radioactivity measured in plasma may have been somewhat decreased, resulting in an overestimation of the PV. The overestimation, however, must be of about the same size for all groups, and it must be small because the blood sample was taken shortly after the tracer injection.

At 3 h after trauma, PV had decreased by about 10 mL/kg, and the increase in muscle water content during the same period was estimated at 1.6 mL/kg, suggesting that only about 15% of the PV loss can be explained by edema in the traumatized rectus muscle. During the experiment, great care was taken to minimize external fluid losses due to evaporation and bleeding from wound areas during and after surgery. The fact that no bleeding could be observed in the wounds and that Hct increased after trauma suggests that blood loss did not contribute to the observed decrease in PV. Furthermore, urine production in the sham and traumatized animals did not differ. Considering that evaporative losses are small and that only a minor part of the PV loss was localized to the traumatized muscle, the major part of the PV must have been lost to the extravascular space in nontraumatized parts of the body.

The method for measurement of TER for albumin in our study is well established, both in experimental and in human research (2, 10–12). It has been shown that cardiac surgery could increase TER by 100% to 300% from a baseline value of 5% per hour, but TER changes after accidental trauma have not been reported. Normal TER for the anesthetized rat is reported to be in the range of 11% to 14% per hour (10, 13, 14), and the TER value of 13.9% per hour in the sham group thus agrees with the normal values for TER in the rat and supports the reliability of our technique. The TER for albumin is influenced by both the microvascular permeability for albumin and the transcapillary hydrostatic pressure because transcapillary transport of macromolecules occurs by both convective and diffusive mechanisms (7).

Because there was no difference in arterial and central venous pressures between the trauma and the sham groups, it is unlikely that an increase in hydrostatic capillary pressure could explain the trauma-induced increase in TER. The increase in TER after trauma in the present study from 13.9% to 18.5% may therefore be explained mainly by an increase in microvascular permeability, which is likely to be an important mechanism for the observed loss of PV. Considering that

plasma is lost to the whole body, it is likely that the increase in TER reflects an increase in permeability in organs other than the injured muscle. The hypothesis that an isolated trauma may increase permeability also in distant organs is supported by several studies, showing that, for example, brain trauma may increase permeability of both the lung and the intestines (15, 16).

By comparing the results showing an increase in TER for albumin from 13.9% to 18.5% per hour after trauma, with the TER value of 14.2% per hour when the rats were exposed only to a skin incision and a laparotomy, we concluded that the major part of the TER increase is induced by the rectus muscle trauma. As mentioned in the introduction, effects of a nonhemorrhagic intra-abdominal trauma on PV have been previously investigated in the rat (5). In that study, it was shown that the trauma decreased PV by about 3 mL/kg, whereas TER for albumin, in contrast to our study, was unchanged. Considering the small decrease in PV in that study, it is likely that the lack of effect on TER can be explained by a less severe trauma.

Several studies in both rodents and humans have shown that the serum levels of IL-6 increase after surgery, and in humans it has been shown that increases in IL-6 and IL-10 concentrations are correlated to severity of tissue injury, development of multiple organ failure, and mortality (17–22). Our results of an increase in both IL-6 and IL-10 suggest that our model mimics a clinical scenario in which an inflammatory response is triggered by the trauma. IL-6 has been suggested to increase endothelial permeability for albumin *in vitro* and may have contributed to the observed increase in TER after trauma (23). However, the mechanisms influencing microvascular permeability after a trauma are complex, and most likely the inflammatory response is modulated by many factors. Such factors include IL-10, which may counteract cytokine-induced edema formation and permeability-increasing complement factors, which are known to be activated by soft tissue trauma (24–26). Our finding of the absence of change in TNF-α is in line with several previous studies, showing that soft tissue trauma without major hemorrhage does not trigger the TNF-α production (17, 19, 27).

In conclusion, the present standardized nonhemorrhagic skeletal muscle trauma model in the rat confirms the hypothesis that there is a trauma-induced increase in microvascular permeability both in traumatized and in nontraumatized tissues, resulting in a decrease in PV. The model mimics several aspects of a clinical trauma and may be used for evaluation of the effects of different pharmacological and other therapeutic interventions aimed at the correction of hypovolemia.

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ORIGINAL ARTICLE

Prostacyclin reduces plasma volume loss after skeletal muscle trauma in the rat

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BACKGROUND: Trauma induces transcapillary leakage of fluid and proteins because of increased microvascular permeability. Based on studies showing that prostacyclin (PGI_2) has permeability-reducing properties, in the present study, we investigated whether PGI_2 reduces plasma volume (PV) loss after a nonhemorrhagic trauma.

METHODS: The study was performed on anesthetized Sprague-Dawley rats exposed to a controlled standardized blunt trauma to the abdominal rectus muscle. Thereafter, the animals were randomized to treatment with either PGI_2 (2 ng/kg per minute) or 0.9% NaCl. PV was estimated before and 3 hours after the trauma using ^{125}I-albumin as tracer. In separate experiments, the transcapillary escape rate of ^{125}I-albumin was calculated and plasma concentrations of cytokines were measured after both treatments.

RESULTS: Average PV at baseline was 41.6 mL/kg ± 2.5 mL/kg and 42.3 mL/kg ± 1.7 mL/kg in the PGI_2 and NaCl animals, respectively. PV was decreased by 22% ± 8% in the NaCl animals and by 11% ± 9% in the PGI_2 animals 3 hours after the trauma ($p < 0.05$). Trauma induced a decrease in mean arterial blood pressure and an increase in hematocrit in both groups. There were no differences in urine production and mean arterial blood pressure between the PGI_2 and NaCl animals. The transcapillary escape rate for albumin was calculated for one hour starting 30 minutes after the trauma and was 15.1% ± 2.4% per hour in the PGI_2 animals and 17.4% ± 3.3% per hour in the NaCl animals ($p = 0.09$). Interleukin 6 concentration 3 hours after the trauma was lower in the PGI_2 animals than in the NaCl animals ($p < 0.05$).

CONCLUSION: We conclude that PGI_2 attenuates PV loss after blunt muscle trauma. The vascular effects of PGI_2 are associated with a modulation of the trauma-induced inflammatory response. (*J Trauma Acute Care Surg*. 2012;73: 1531–1536. Copyright © 2012 by Lippincott Williams & Wilkins)

KEY WORDS: Prostacyclin; trauma; microvascular permeability; plasma volume; rat.

S evere trauma is associated with an inflammatory response, an increase in microvascular permeability, and an increased transvascular leakage of plasma.[1–4] This may adversely affect organ oxygenation through a decrease in cardiac output and through a hypovolemia-induced activation of the baroreceptor reflex. Furthermore, tissue edema may increase oxygen diffusion distances and increase tissue pressure. While fluid substitution with the objective of preserving a normal plasma volume (PV) is essential to restore cardiac output and organ perfusion, it may have the disadvantage of aggravating the interstitial edema. Pharmacologic interventions, with the objective of reducing fluid and protein leakage by decreasing microvascular permeability, may therefore be beneficial after trauma.

According to the Starling equation, transvascular fluid exchange is influenced not only by permeability for fluid and macromolecules but also by transcapillary hydrostatic and osmotic pressures and the area available for fluid exchange.[5] In addition, the capacity of the lymphatic system for return of fluid and macromolecules to the circulation will be of importance. Accordingly, an experimental analysis of the net effect on PV of a permeability-reducing drug must be evaluated in a whole-animal model.

Prostacyclin (PGI_2) is a labile arachidonic acid metabolite that is mainly produced by the endothelium. It is a vasodilator, inhibits blood cell aggregation and endothelial adhesion of platelets and leukocytes, reduces microvascular permeability, and has scavenging and anti-inflammatory effects.[6–11] Interestingly, the permeability-decreasing effect of PGI_2 has been demonstrated during intravenous administration in the range of 0.5 to 2 ng/kg per minute.[11,12] In these doses, PGI_2 does not affect blood pressure, as was shown in animal experiments[12,13] and in patients,[14] indicating that PGI_2 is a potential substance to decrease permeability and to reduce PV loss without causing hypotension.

The objective of the present study was to determine whether PGI_2, given in a relatively low but clinically relevant dose, has PV-sparing effects during trauma-induced inflammation and whether it can counteract a trauma-induced increase in microvascular permeability and an increase in the cytokine release. For this purpose, we used a recently described trauma model in which a systemic inflammatory response was induced in rats after exposure to a standardized blunt skeletal

muscle trauma.[15] To our knowledge, this is the first study to evaluate whether a substance that has been shown to reduce permeability may also counteract trauma-induced hypovolemia.

MATERIALS AND METHODS

Materials and Anesthesia

The study was approved by the Lund University Ethics Committee for Animal Research (M87-90), and the animals were treated in accordance with the guidelines of the National Institutes of Health for Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (N = 71) weighing 358 g ± 21 g were used. The animals had free access to water and food until anesthesia was induced by placing the rats in a covered glass container with a continuous supply of isoflurane (Isoba Vet; Intervet AB, Stockholm, Sweden). After tracheostomy, the animals were connected to a ventilator (Ugo Basile; Biological Research Apparatus, Comerio, Italy) using a positive end-expiratory pressure of 3 to 4 cm H_2O. Anesthesia was maintained by inhalation of 1.6% to 1.8% isoflurane through the tracheal cannula. Body temperature, measured rectally, was kept at 37.1°C to 37.3°C via a feedback-controlled heating pad. End-tidal P_{CO_2} was monitored continuously and kept between 4.8 kPa and 5.5 kPa (Capstar-1000; CWE, Artmore, PA). The left femoral artery was cannulated for measurement of mean arterial blood pressure (MAP) and to obtain blood samples for measurement of arterial partial pressure of oxygen and carbon dioxide (Pa_{O_2}, Pa_{CO_2}) electrolytes, and hematocrit (Hct) (I-stat; Abbott Point of Care Inc., Abbott Park, IL). The right jugular vein was cannulated and used for injections and was kept open with a continuous saline infusion of 0.2 μL/min. The left femoral vein was cannulated and used for infusion of PGI_2 or NaCl. Urine was collected in a glass vial placed at the external meatus of the urethra from the end of the preparation until the end of the experiment, when the bladder was emptied by external compression. After the experiment, the animals were killed with an intravenous injection of potassium chloride.

Experimental Trauma

The experimental trauma has been described previously.[15] Briefly, after an intravenous bolus dose of fentanyl 25 μg/kg (Braun Melsungen AG, Melsungen, Germany), a laparotomy was performed along the linea alba followed by a blunt trauma of the rectus muscle induced by clamping the muscle in a standardized manner at 12 different locations, six on each side of the midline, using a pair of anatomical forceps. To reduce evaporation, the time of exposure of the wound area to the atmosphere was kept to a minimum. The abdomen was closed with surgical clips.

Measurement of Plasma Volume

PV was determined by measurement of the increase in radioactivity per milliliter of plasma after intravenous injection of a known amount of activity of human ^{125}I-albumin (GE Health Care; Bio-Science, Kjeller, Norway). The increase in radioactivity was calculated by subtracting the activity in a 250-μL blood sample taken just before the injection from one taken 5 minutes after the injection. With this technique, the PV measurement was independent of remaining radioactivity

from previous injections. To determine the exact dose injected, the radioactivity in the emptied vial, in the syringe, and in the needle was subtracted from the total radioactivity in the prepared dose. The amount of unbound radioactivity in the injected ^{125}I-albumin was measured regularly by precipitation with 10% trichloroacetic acid and was found to be less than 1%.

Measurement of Transcapillary Escape Rate for Albumin

Transcapillary escape rate (TER) (percentage of albumin leakage per hour) was estimated by measurement of the reduction in the radioactivity per time unit after injection of a bolus dose of human ^{125}I-albumin.[15] Blood samples of 250 μL were taken 5, 15, 30, 45, and 60 minutes after the ^{125}I-albumin injection. After centrifugation, radioactivity in a PV of 100 μL was measured with a gamma counter (Wizard 1480; LKB-Wallace, Turku, Finland). TER was determined as the slope of the line, that is, the relative reduction in radioactivity in the plasma samples over time.

Cytokines

The plasma concentrations of interleukin 6 (IL-6) and IL-10 were measured from 250-μL arterial blood samples, and cytokine concentrations were determined with a flow cytometer using cytometric bead array kits specific for the respective cytokines (BD Biosciences, Franklin Lakes, NJ).

Experimental Protocol

The study included three groups, the PV group, the TER group, and the cytokine group as defined below. The preparation was the same for all groups and included anesthesia and cannulation, which lasted for about 40 minutes. The animals were then left undisturbed for a period of 15 minutes to measure baseline values and to ensure that there was hemodynamic stability. This period was followed by the experimental trauma, which lasted for about 25 minutes.

Thereafter, the animals were randomized to receive an infusion of either PGI_2 (Flolan; GlaxoSmithKline, Brentford, United Kingdom) at a rate of 2 ng/kg per minute, which was dissolved in glycine buffer and diluted with 0.9% NaCl in the ratio 1:7 or 0.9% NaCl given at the same infusion rate (0.5 μL/min). The infusions were started directly after the trauma. The glycine vehicle does not influence microvascular permeability at these infusion rates.[16] Animals did not receive any additional resuscitation fluids.

In the first group, denoted the PV group, the PV was calculated before and 3 hours after the trauma as described above (Fig. 1). We have previously shown that PV after a sham procedure in terms of anesthesia, cannulation, and tracheostomy (but no surgical trauma) remains unchanged after 3 hours in this model.[15] In the second group, denoted the TER group, the TER calculation started from 30 minutes after the trauma because it takes some time for the increase in capillary permeability to develop, and the blood sampling was performed during the following 60 minutes, as described above (Fig. 1). In the third group, denoted the cytokine group, blood samples were taken before trauma and at 1 hour and 3 hours after trauma for measurement of plasma concentrations of the cytokines IL-6 and IL-10, as described above (Fig. 1). We have previously
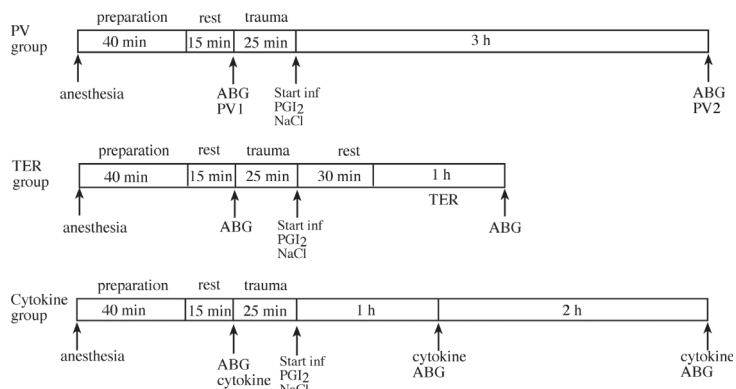
Figure 1. Time course for the experiments in the PV group, the TER group, and the cytokine group. ABG (arterial blood gases, electrolytes, and hematocrit).

shown that plasma concentrations of IL-6 and IL-10 are increased after the trauma in this model.[15] Samples for arterial blood gases and electrolytes were taken just before trauma (baseline) and at the end of the experiment for all three groups (Fig. 1). The authors were blinded to all analysis results until all experiments have been completed.

Statistical Analysis

Physiological parameters, laboratory values, PVs, and TER values passed tests for normality and equal variance and were analyzed with unpaired *t*-tests or paired *t*-tests as appropriate. Cytokine values did not appear to be normally distributed and were analyzed with the Mann-Whitney U-test. Values of $p < 0.05$ were considered significant. GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA) was used for the analysis. Data are expressed as mean ± SD if normally distributed and otherwise as median with the first and third quartiles.

RESULTS

Physiological Data

Sodium, potassium, pH, base excess, Pao_2, and $Paco_2$ did not differ between the PGI_2 and the NaCl animals at baseline and 3 hours after trauma in the PV group. Potassium was higher and sodium was lower 3 hours after trauma than at baseline in

both PGI_2 and NaCl animals ($p < 0.01$). MAP did not differ between the PGI_2 and NaCl animals at baseline, and there was a reduction in MAP in both groups after the trauma compared with baseline ($p < 0.01$), with no difference between the PGI_2 and NaCl animals. There was no difference in Hct between the two treatment groups at baseline, and Hct increased after trauma in the NaCl animals but not in the PGI_2 animals ($p < 0.01$) (Table 1). There was no difference in urine production between animals that received PGI_2 (2.4 ± 0.4 mL/kg) and those that received NaCl (2.8 ± 0.7 mL/kg) (values given for the whole study period). Physiological data for the TER and cytokine groups showed the same pattern as those in the PV group and are not presented.

Plasma Volume

PVs at baseline and after trauma in the PV group are presented in Figure 2. PV at baseline was 41.6 mL/kg ± 2.5 mL/kg in the PGI_2 animals (n = 13) and 42.3 mL/kg ± 1.7 mL/kg in the NaCl animals (n = 14). PV decreased in both groups after trauma and was 37.0 mL/kg ± 4.6 mL/kg and 33.0 mL/kg ± 3.1 mL/kg in the PGI_2 animals and the NaCl animals, respectively; it was significantly lower in the NaCl animals ($p < 0.01$). PV decreased by 11.2% ± 8.5% in the PGI_2-treated animals and by 21.8% ± 8.0% in the NaCl-treated animals. The PV loss was significantly smaller in the PGI_2 animals than in the NaCl animals ($p < 0.05$).

TABLE 1. Data for Na^+, K^+, Hct, pH, $Paco_2$, Pao_2, and MAP for the PGI_2 Animals (n = 13) and the NaCl Animals (n = 14) at Baseline and at the End of the Experiments for the PV Group

	Na^+, mmol/L	K^+, mmol/L	Hct, %	pH	$Paco_2$, kPa	Pao_2, kPa	MAP, mm Hg
PGI_2 baseline	136 ± 1	4.6 ± 0.3	38 ± 2	7.47 ± 0.04	5.2 ± 0.5	11.6 ± 1.2	92 ± 9
NaCl baseline	135 ± 1	4.5 ± 0.4	42 ± 2	7.50 ± 0.03	4.9 ± 0.4	11.6 ± 0.7	102 ± 12
PGI_2 end	132 ± 2*	6.4 ± 0.5*	40 ± 2	7.46 ± 0.02	4.8 ± 0.3	11.7 ± 1.6	74 ± 9*
NaCl end	132 ± 3*	6.1 ± 0.5*	45 ± 2*	7.46 ± 0.05	4.9 ± 0.5	11.3 ± 1.1	75 ± 8*

*$p < 0.01$ compared with baseline.
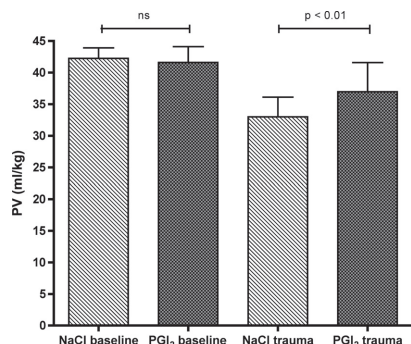Na^+, sodium; K^+, potassium.

Figure 2. PV at baseline and 3 hours after trauma. PV at baseline was not significantly different between the PGI$_2$ and the NaCl animals. Three hours after trauma, PV was significantly lower in the NaCl animals than in the PGI$_2$ animals ($p < 0.01$).

Transcapillary Escape Rate

The TER values after trauma and treatment with either PGI$_2$ or NaCl are presented in Figure 3. TER in the PGI$_2$ animals (n = 10) showed a tendency for lower values than in the NaCl animals (n = 10): at 15.1% per hour ± 2.4% per hour and 17.4% per hour ± 3.3% per hour, respectively ($p = 0.09$).

Cytokines

The plasma concentrations of IL-6 and IL-10 at baseline, at 1 hour, and at 3 hours after the trauma in the cytokine group are presented in Figure 4*A* and *B*. At baseline, the median concentration of IL-6 was 4.5 (0–7.1) pg/mL in the PGI$_2$ animals (n = 11) and 0 (0–21.4) pg/mL in the NaCl animals (n = 11). The corresponding concentrations for IL-10 were 22.3 (13.7–41.0) pg/mL and 22.3 (9.7–46.4) pg/mL. The IL-6 and the IL-10 concentrations at baseline and 1 hour after the trauma did not differ between the PGI$_2$ animals and the NaCl animals. Three hours after the trauma, the concentration of IL-6 was lower in the PGI$_2$ animals (43.7 pg/mL; range, 37.5–54.3 pg/mL) than in the NaCl animals (62.3 pg/mL; range, 46.7–91.4 pg/mL) ($p < 0.05$). The concentration of IL-10 did not differ between the PGI$_2$ and the NaCl animals at 3 hours. One PGI$_2$ animal and one NaCl animal were excluded because of technical failure regarding the baseline analysis.

DISCUSSION

The present results showed that a blunt muscle trauma induced a reduction in PV and a decrease in MAP. PV loss was attenuated by treatment with PGI$_2$. The smaller PV loss in the PGI$_2$-treated animals than in the NaCl-treated animals was associated with a tendency toward a decrease in TER for albumin and a simultaneous reduction in the plasma concentration of IL-6.

The use of ^{125}I-albumin as tracer is an established method for measurement of PV.[17] The technique gives reproducible results during both normal and inflammatory states, and the PV at baseline in the present study was in the same range as previously reported for the rat.[15,18,19] The trauma model used in this

study has been presented in detail previously,[15] and the results from the present study, with a trauma-induced reduction in PV and a tendency toward an increased TER, are in agreement with those from our previous study, thus supporting the reliability and reproducibility of the model.[15] The present observation of an increase in Hct and a simultaneous decrease in PV is also similar to that reported previously.[15] This, and the fact that no hemorrhage was observed during and after the trauma, supports the hypothesis that plasma is mainly lost through transcapillary leakage and not because of trauma-induced hemorrhage.

There was no difference in urine production and in arterial blood pressure between the PV groups in the present study. Furthermore, there are no indications from the current literature that PGI$_2$ affects lymphatic return of fluid from the interstitial space to the circulatory system. Therefore, the demonstrated reduction in trauma-induced PV loss by PGI$_2$ is most likely caused by a decrease in extravasation of plasma fluid, an interpretation supported by the trend toward a reduction in TER by PGI$_2$. The large variations in TER values in each group and the fact that the study was not powered to detect a difference of 2% per hour to 3% per hour between the groups may explain why the difference in TER values between the two groups did not reach statistical significance. Assuming that the true difference in TER is in the range of 2% per hour to 3% per hour, this would lead to a 6% to 9% difference in extravasation of albumin 3 hours after the trauma, which is compatible with the observed reduction in PV loss after 3 hours in the present study.

Transvascular transport of fluid and macromolecules, the latter reflected by changes in TER, is influenced by both capillary hydrostatic pressure and microvascular permeability.[20,21] Capillary hydrostatic pressure in turn is determined by arterial pressure and venous pressures and the ratio of precapillary to postcapillary resistance (Rv/Ra).[22] Our finding that MAP was not significantly different between the PGI$_2$ group and the NaCl group confirms previous results showing that the presently used dose of PGI$_2$ does not affect blood pressure.[12] Furthermore, there are no data indicating that PGI$_2$ has
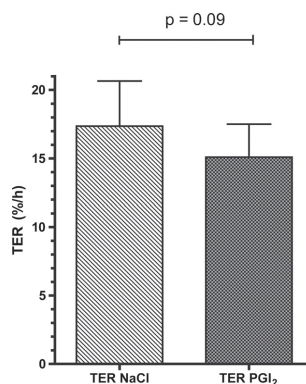

Figure 3. TER for albumin after trauma. A trend toward a decreased TER in the PGI$_2$-treated animals could be observed animals ($p = 0.09$).
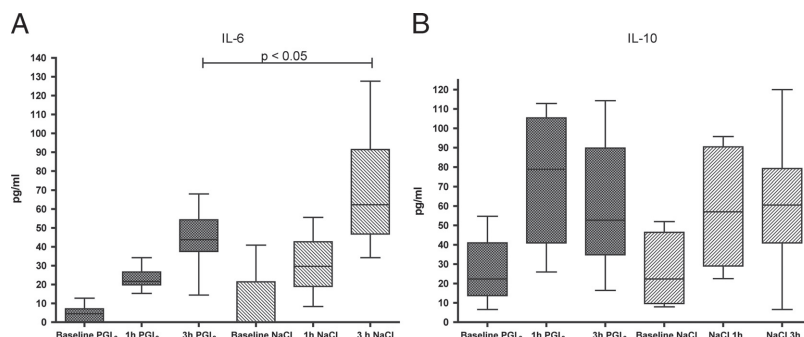
Figure 4. Plasma concentrations of IL-6 (*A*) and IL-10 (*B*) at baseline and 1 hour and 3 hours after the trauma for the NaCl and the PGI$_2$ animals. After 3 hours, IL-6 was significantly lower in the PGI$_2$ animals than in the NaCl animals ($p < 0.05$). No significant difference in IL-10 levels could be detected between the PGI$_2$ and NaCl animals at any time point.

an effect on central venous pressure or the Rv/Ra ratio, which suggests that differences in hydrostatic capillary pressure cannot explain the difference in PV loss. Instead, the tendency toward a reduction in TER by PGI$_2$ may be explained by a decrease in microvascular permeability. This conclusion is supported by previous studies showing that PGI$_2$ reduces permeability both to water and to macromolecules without any effects on capillary hydrostatic pressure at a dose of 2 ng/kg per minute.[10–12]

It has already been shown that the present trauma model induces not only a localized increase in microvascular permeability in the traumatized skeletal muscle but also a systemic increase in permeability.[15] It is therefore reasonable to assume that the PGI$_2$-induced reduction in PV is not only a local effect in traumatized tissues but also a more generalized systemic effect.

PGI$_2$ has been used previously at an infusion rate of 2 ng/kg per minute, both in human and experimental studies, without any adverse effects on blood pressure.[13,14] In experimental studies, permeability-reducing effects of PGI$_2$ have been demonstrated at an infusion rate of 2 ng/kg per minute.[12,13] The infusion rate of 2 ng/kg per minute was chosen with the objective of having a maximal effect on microvascular permeability without affecting blood pressure. The rationale for starting the treatment 30 minutes after trauma was to mimic a clinical scenario with short transport times. Previous studies have shown that treatment with PGI$_2$ in a similar dose attenuates trauma-induced increases in macromolecular permeability in skeletal muscle when initiated as long as 5 hours after the trauma.[11] These data indicate that PGI$_2$ treatment may be effective also if initiated at a later time point than in the present study.

Trauma rapidly initiates a systemic inflammatory reaction, and we have previously shown that the cytokine response in the present soft tissue trauma model is associated with an increase in IL-6 and IL-10.[15] The cytokine response is similar to that in a previous study analyzing the cytokine response after a combination of soft tissue and skeletal trauma.[23] This indicates that our results may even be valid after more complex injuries. IL-6 is thought to increase and IL-10 to decrease microvascular permeability,[24,25] and increases in these cyto-

kines have been correlated with the severity of the injury and with poor outcome in clinical studies.[26,27] PGI$_2$ is thought to reduce microvascular permeability by acting on the G-protein–coupled PGI$_2$ (IP) receptor with a release of cAMP, in turn resulting in reduced tension in the cytoskeleton of the endothelial cell.[28] The reduction in IL-6 by PGI$_2$ indicates that the relatively higher PV in the PGI$_2$-treated group may be mediated in part by a reduction of the proinflammatory action of IL-6 and indicate that PGI$_2$ may also act through mechanisms other than direct action on the endothelial cytoskeleton via the IP receptor. In this respect, it is of interest to note that PGI$_2$ has recently been shown to modulate the inflammatory response to ischemia and reperfusion injury through activation of intracellular receptors belonging to the peroxisome proliferators–activated receptor family.[29]

CONCLUSIONS

We conclude that PGI$_2$ attenuated the loss of PV after a blunt muscle trauma, and that this is most likely caused by a decrease in microvascular permeability. The effect is associated with a modulation of the inflammatory response induced by the trauma. Treatment with PGI$_2$ may be a potential therapy for reduction of transcapillary leakage of plasma in traumatized patients.

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Paper 3

Effect of charge on microvascular permeability in early experimental sepsis in the rat

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ABSTRACT

A key feature of sepsis is hypovolemia due to increased microvascular permeability. It has been suggested that the negative charge of albumin and of the endothelial glycocalyx is important for maintenance of the normally low permeability for albumin. Here we tested the hypothesis that charge effects contribute to the increased permeability in sepsis. Transcapillary escape rate (TER) and initial distribution volume for ^{125}I-labeled bovine serum albumin (BSA, isoelectric point pH 4.6) and for ^{131}I-labeled charge modified BSA (cBSA, average isoelectric point, pH 7.1) was measured 3 h after sepsis was induced by cecal ligation and incision (CLI) ($n = 11$) and in control animals ($n = 12$). The importance of charge for permeability in sepsis was estimated by comparing the ratio between TER for cBSA and TER for BSA during control conditions to that after CLI. Plasma concentration of the glycocalyx component glycosaminoglycans (GAGs) was measured in separate control and CLI animals. The initial distribution volume for BSA and cBSA in control animals was 38 ± 3 ml/kg and 47 ± 4 mL/kg and decreased by 17% and 19%, respectively, following CLI. TER for BSA increased from $16.7 \pm 4.1\%$ in the controls to $20.1 \pm 1.9\%$ following CLI. Corresponding values for cBSA were $26.7 \pm 5.6\%$ and $29.8 \pm 3.5\%$, respectively. The ratio between TER for cBSA and TER for BSA was 1.62 ± 0.1 in the control group and 1.49 ± 0.1 following CLI ($p < 0.05$). Plasma GAG concentrations were higher in CLI animals than in the control group. We conclude that CLI induce hypovolemia secondary to increased microvascular permeability. Negative charge contributes to the normally low permeability of albumin and the importance of charge is decreased in early experimental sepsis. The observed charge effects are associated with CLI-induced breakdown of the glycocalyx.

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Introduction

Sepsis is a serious condition with a reported mortality of about 30% and an increasing incidence (Angus et al., 2001). A key pathophysiological feature of sepsis is hypovolemia due to increased microvascular permeability, which may induce hemodynamic instability and necessitate resuscitation with large volumes of fluids, both of which adversely affect outcome (Bagshaw et al., 2008).

It has long been recognized that the luminal side of endothelial cells is covered with the glycocalyx, which is composed mainly of negatively charged polysaccharides called glycosaminoglycans (GAGs). Based on studies showing that neutralized macromolecules have a higher microvascular permeability than corresponding anionic native proteins, it has been suggested that the glycocalyx impedes the passage of negatively

charged plasma macromolecules, and by that contributes to the normally low permeability to these molecules (Brenner et al., 1978; Gandhi and Bell., 1992; Haraldsson et al., 1983; Swanson and Kern., 1994; Vehaskari et al., 1982). It has recently been suggested that the glycocalyx can be shed in pathophysiological states such as ischemia and in sepsis, indicating that changes in endothelial charge may contribute to the increased microvascular permeability to macromolecules observed in these conditions (Mulivor and Lipowsky., 2004; Rehm et al., 2007; Nelson et al., 2008). Such a theory is supported by studies suggesting that experimental sepsis is accompanied by a decrease in luminal endothelial negative charge (Gotloib et al., 1988).

The present study was designed to test the hypothesis that the increased permeability in early sepsis is associated with charge effects and that these effects could involve degradation of the glycocalyx. The study was performed in rats using a cecal ligation and incision method to induce abdominal sepsis (Scheiermann et al., 2009; Hubbard et al., 2005). Microvascular permeability was estimated by measuring the transcapillary escape rate of radiolabeled albumin and the impact of charge on permeability in sepsis was evaluated by comparing permeability to charge-modified bovine serum albumin to that of normal bovine serum albumin during normal conditions and during sepsis. Degradation of the glycocalyx was estimated by measuring GAG levels in plasma during normal conditions and during sepsis.

Materials and methods

Anesthesia and surgical preparation

The study was approved by the Lund University Ethics Committee for Animal Research (Dnr M87-09), and the animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals.* 39 Sprague–Dawley rats (Scanbur BK, Sollentuna, Sweden) weighing 354 ± 12 g were used. Anesthesia was induced with 4% isoflurane (Schering-Plough Animal Health, Ballerup, Denmark) in a closed container, and after induction the animals were removed from the container and anesthesia was maintained with 1.6–1.8% isoflurane in air delivered via a mask. After tracheostomy, the animals were mechanically ventilated (Ugo Basile Animal Ventilators, Comerio, Italy) to an end-tidal CO_2 concentration of between 4.8 and 5.5 kPa using a volume-controlled mode and a positive end expiratory pressure of 3 cm H_2O. Body temperature was maintained at 37.2–37.5 °C with a feedback-controlled heating pad throughout the experiment. The left femoral artery was cannulated for continuous measurement of arterial pressure and blood sampling. The left femoral and the right internal jugular vein were cannulated, and following administration of an intravenous bolus of fentanyl (25 µg/kg, Braun Melsungen AG, Melsungen, Germany) and the start of a continuous fentanyl infusion (0.5 µg/kg/min), isoflurane was reduced to 1.0–1.2%. After a 10-min equilibration period, mean arterial blood pressure was recorded and blood samples for analysis of arterial blood gases, electrolytes, hematocrit, and lactate were collected (I-stat; Hewlett Packard, Böblingen, Germany) (Fig. 1).

Animals were then randomized by lottery either to a sham procedure or were subjected to a cecal ligation and incision (CLI) procedure as described elsewhere (Fries et al., 2008; Scheiermann et al., 2009). Briefly, following a 3–4 cm midline abdominal incision the cecum was mobilized while carefully avoiding damage to blood vessels. The cecum was ligated with a 3.5 silk ligature and a 1-cm incision was made in the ligated cecum with a scalpel blade. The abdomen was then closed with metal clips. The sham-operated animals were not laparotomized. We have previously shown that laparotomy per se does not increase microvascular permeability in rats during these experimental conditions (Bansch et al., 2010). Three hours after CLI, arterial blood gases, hematocrit, and lactate were measured. At the end of the experiment, the animals were killed with an intravenous injection of 3 M KCl. Urine was collected in a glass beaker for the duration of the experiment, starting after the tracheostomy. Immediately after injection of 3 M KCl, the bladder was emptied by external compression.

Preparation of charge-modified albumin

Bovine serum albumin was charge-modified using the method first described by Hoare and Koshland (1967) and later modified by Wiig et al. (2003). The principle of this method is activation of carboxyl groups in albumin with a carbodiimide, followed by amidation with glycine methyl ester. Briefly, 150 mg of bovine serum albumin was dissolved in 15 mL of 0.133 M glycine methyl ester and the pH was adjusted to 4.75 by adding HCl. The reaction was started by adding 5 mL of 0.04 M 1-ethyl-3, (3-dimethylaminopropylcabodiimide) hydochloride. Temperature was maintained at 20 °C on a feedback-controlled heating plate and pH was maintained by adding either HCl or NaOH while continuously stirring. The reaction was stopped after 30 min by adding 20 mL of 4 M sodium acetate and the albumin solution was dialyzed against distilled water for 24–36 h. Charge-modified albumin was then labeled with ^{131}I by using 1,3,4,6-tetrachloro-3α, 6 α-diphenylglycouril (Iodo-Gen, T0656, Sigma) as described in detail previously (Wiig et al., 2003). Stock solutions were stored in darkness at 4 °C and before each experiment radioactive low-

molecular-weight compounds were removed using centrifugal filtration (Micron 30 filters, Millipore, Bedford, MA, USA).

The isoelectric pH (pI) of ^{125}I-labeled bovine serum albumin and ^{131}I-labeled charge-modified BSA was determined by isoelectric focusing (IEF) on precast IEF gels (pH 5–8, Bio-Rad, Sundbyberg, Sweden). Gels were run at a 100 V for 1 h, at 200 V for 1 h and were then fixed and stained with Coomassie blue staining solution (Bio-Rad, Sundbyberg, Sweden). The pI of the albumin tracers was then determined by comparison with a standard containing proteins with pI ranging from 4.45 to 9.6. In preliminary experiments it was shown that the pI of the probes did not change within 2 weeks after labeling. All experiments were performed within 2.5 weeks after labeling of the probes and pI was determined in the middle of the experimental period.

The molecular size of ^{125}I-labeled bovine serum albumin (BSA) and of ^{131}I-labeled charge-modified BSA (cBSA) was determined by HPLC using a silica-based gel filtration column with an exclusion range of 5000–700 000 Da (BioSep-SEC-S3000 7.8 × 300 mm, Phenomenex, Torrance, CA, USA) and standards containing proteins of a known size. Elution was performed with 50 mM phosphate buffer, pH 7.4, at 1.0 mL/min. Detection was performed by measuring UV absorbance (280 nm) and radioactivity (3" × 3" NaI (Tl)-detector) on-line.

Measurement of initial distribution volume and transcapillary escape rate

Three hours after CLI or the sham procedure the animals received a bolus injection of about 25 kBq BSA (0.05 mg/kg) and 12.5 kBq cBSA (0.05 mg/kg) dissolved in 0.1 mL of 0.9% NaCl in the internal jugular vein and the venous line was then flushed with 0.4 mL of 0.9% NaCl. To determine the exact dose injected, the remaining radioactivity in the emptied vial, the syringe, and the needle was subtracted from the total radioactivity in the prepared dose. At 5, 15, 30, 45, and 60 min after the injection, 0.25 ml arterial blood samples were collected in brown glass vials (Scantec Lab, Partille, Sweden) and centrifuged at 8000 rpm for 7 min. Plasma was then collected and counted in a gamma counter (Wizard 1480, LKB-Wallac, Turku, Finland) and corrections for both spillover and background were made automatically. The amount of unbound radioactivity in the bolus dose in the plasma samples and in the urine was determined by measuring the activity in the supernatant following precipitation with 10% trichloroacetic acid and centrifugation at 12 000 rpm for 10 min. Unbound activity was<1% in plasma samples and all values were corrected for unbound activity.

The initial distribution volumes for BSA and cBSA were calculated by dividing the injected dose with the resulting plasma concentration 5 min after injection (Dubniks et al., 2007). A previous study has shown that plasma disappearance is linear between 10 and 60 min after injection of tracer (Bent-Hansen, 1991) Transcapillary escape rate (TER) for BSA and cBSA was calculated by fitting the plasma concentrations at 15, 30, 45 and 60 min post injection to a linear function using least square regression. The slope of the curve represents TER and is expressed as %/h.

Measurement of plasma concentrations of glycosamininoglycans (GAGs)

Plasma GAG concentrations were measured in separate animals prepared as described above. Arterial blood samples (0.25 ml) were obtained after the 10 min equilibration period and 3 h after CLI or the sham procedure. Isolation and detection of sulphated GAGs from plasma was performed using a kit from Euro-diagnostica (Malmö, Sweden) and as previously described (Björnsson, 1998). Briefly, 10 µl of plasma sample or standard (CS-A at 0, 1.25, 1.875, 2.5, 3.75, 5, 7.5, 10, 15 or 20 µg/ml) was added in duplicate to 20 µl of an acidulous buffer and gently agitated for 15 min at room temperature. Two hundred microliters Alcian blue solution was added and the mixture was left to precipitate for 1 h. A polyvinylidene fluoride membrane

(PVDF, Amersham) was prewetted in ethanol, blocked for 1 h in 1% (v/v) Triton X-100 and assembled in a 96-well slotblot apparatus (manufactured in house). Two hundred-microliter prewash-buffer was added to each well and 100 μl was evacuated. The samples were transferred to the slot blot apparatus and passed through the membrane by vacuum followed by two washes with 300 μl 50% (v/v) ethanol in 0.05 M MgCl$_2$. The membrane was then removed and dried. The membrane was mounted between two plastic sheets and scanned in reflectance-color mode in a Canon CanoScan LiDE210 scanner. The red channel of the output RGB-matrix was used for the calculations performed in Matlab 7.11.0.

Statistics

Physiological parameters, laboratory values, plasma volumes and TER values passed tests for normality and equal variance and were compared with paired *t*-tests within groups. Plasma volumes, TER values between groups were compared with unpaired *t*-test. GAG values did not appear to be normally distributed and were analyzed with Wilcoxon matched pairs test within groups and Mann–Whitney test between groups. Analyses were performed using GraphPad Prism version 5.0a for Macintosh (GraphPad Software, San Diego, CA). *p*-Values <0.05 were considered statistically significant. Data are expressed as mean ± S.D. if normally distributed and otherwise as median with first and third quartiles.

Results

Tracer characteristics

The mean pI for the ^{131}I-labeled charge-modified bovine serum albumin (cBSA) was 7.1 (range 6–7.4) and for the ^{125}I-labeled bovine serum albumin (BSA) it was 4.5 (Fig. 2). The molecular radii of cBSA and BSA were 33.9 Å and 35.7 Å, respectively, as determined by HPLC (Fig. 3a). Approximately 95% and 92% of the total radioactivity of cBSA and BSA solutions, respectively, could be attributed to molecules of these sizes (Fig. 3b). The remaining radioactivity was bound to larger molecules with a weight corresponding to albumin dimers.

Physiological parameters

Physiological parameters for the animals in which TER and plasma volume were measured are presented in Table 1. Mean arterial pressures, arterial blood gases, potassium, and lactate were similar in the CLI and control groups at baseline. At 3 h after the CLI procedure hematocrit, lactate, and potassium had increased and pH had decreased compared to baseline. Urine production during the experimental period was lower in the CLI group than in the control group, and was 3.5 ± 1.1 mL/kg and 6.0 ± 1.7 mL/kg, respectively (*p*<0.05). Physiological parameters and urine production in control and CLI groups at the different time points in the GAG animals were similar to those described above (data not presented).

In the CLI group, 0.008 ± 0.01% of the injected dose of BSA and 0.002 ± 0.007% of the injected dose of cBSA was detected in the urine. Corresponding figures for the control group were 0.001 ±

0.02% and 0.0009 ± 0.02% for BSA and cBSA, respectively. The fraction of the injected dose recovered in the urine did not differ between the respective tracers or between the CLI group and the control group.

TER and distribution volume

Plasma concentration curves for cBSA and BSA during control conditions and in sepsis are presented in Fig. 4. TER for BSA was lower than that for cBSA in the control group and was 16.7 ± 4.1% and 26.7 ± 5.6%, respectively (Fig. 5, *p*<0.05). Also in the CLI group, TER for BSA was lower than that for cBSA and was 20.1 ± 1.9% and 29.8 ± 3.5%, respectively (*p*<0.05). TER for BSA following CLI was 20% higher than in the control group (*p*<0.05) while TER for cBSA was 11% higher than in the control group and did not differ significantly from control. The ratio of TER for cBSA to TER for BSA was in 1.62 ± 0.12 the control group and 1.49 ± 0.13 in the CLI group (Fig. 6, *p*<0.05).

Distribution volumes for BSA and cBSA in the control group were 38 ± 3 mL/kg and 47 ± 4 mL/kg, respectively (Fig. 7). Distribution volumes for BSA and cBSA in the CLI group were 31 ± 4 mL/kg and 38 ± 5 mL/kg, respectively.

GAG concentrations

Plasma concentrations of GAGs at in the control group and in the CLI group at baseline did not differ and were 2.1 (1.6–3.0) μg/mL and 1.6 (1.4–3.6) μg/mL, respectively (Fig. 8). At 3 h plasma concentrations of GAGs did not differ from baseline in the control group and had increased compared to baseline in the CLI group and were 1.8 (1.6–3.6) μg/mL and 3.5 (1.6–6.8) μg/mL, respectively (*p*<0.05). At 3 h plasma concentrations of GAGs were higher in the CLI group than in the control group.

Discussion

The present results show that the cecal ligation and incision model of sepsis is associated with hypovolemia, an increase in plasma concentrations of GAGs and an increase in TER for both normal albumin and charge-modified albumin. The increase in TER following CLI was larger for the normal albumin than for the charge-modified albumin.

TER and plasma volume during normal conditions

Initial distribution volume for radiolabeled BSA is a standard method for plasma volume measurement; it is used both clinically and in laboratory experiments with highly reproducible results (Margarson and Soni, 2005). The potential errors in the technique, such as effects of poor mixing of the tracer in plasma and effects of transcapillary escape during the 5-min mixing period, have been discussed previously and found to be small (Dubniks et al., 2007). The distribution volume for BSA of about 38 mL/kg in the present study is similar to values presented by others and us previously, and supports the reliability of the technique (Bansch et al., 2010; Lee and Blaufox., 1985; Lundin et al., 1981).

TER for albumin is influenced by both surface area of the capillary network and by the transcapillary transport of albumin per unit area. According to the two-pore theory, transcapillary transport of albumin
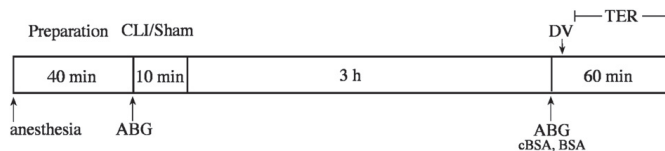

Fig. 1. Schematic drawing of the experimental protocol. ABG, arterial blood gas; CLP, cecal ligation and puncture; DV, measurement of initial distribution volume for respective tracer; TER, transcapillary escape rate, cBSA; charge modified albumin; BSA, bovine serum albumin.
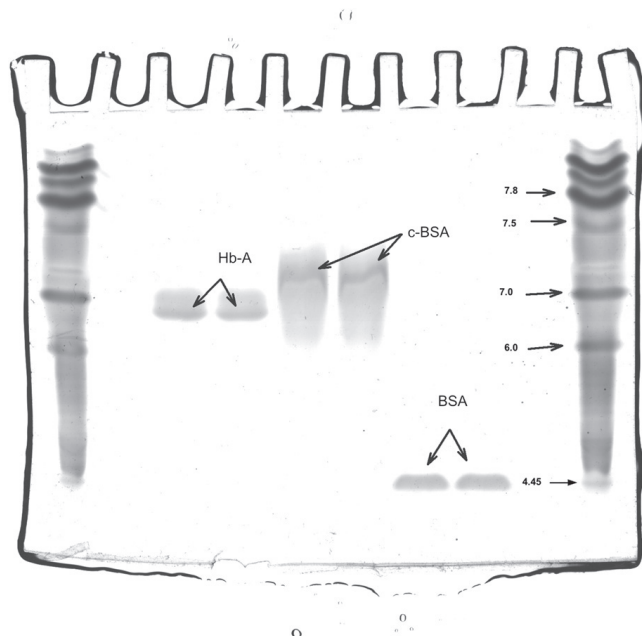
Fig. 2. Isoelectric focusing gel containing standards of known isoelectric pH (pI), BSA, ^{125}I-labeled bovine serum albumin; c-BSA, ^{131}I-labeled charge-modified bovine serum albumin; Hb-A, hemoglobin A (pI 6.8).

is both diffusive and convective, and the latter is dependent on capillary hydrostatic pressure, which may increase with increasing mean arterial pressure (Parving et al., 1974). The observation that the TER value for normal albumin is in the upper range of previously reported TER values of 11–17%/h in healthy rats may therefore be explained by a higher mean arterial pressure in the present experiments than in most previous studies (Bansch et al., 2010; Zakaria and Rippe, 1995; van den Born et al., 1997; Oturai, 1999; Åkerström et al., 1989).

Our finding of a 60% higher TER for charge-modified albumin than for normal albumin is similar to results presented in a previous *in vivo* study using charge modified albumin with a pI of around 7 in the rabbit. In that study, a 20% higher permeability in skin and 50% higher permeability in skeletal muscle was shown (Gandhi and Bell., 1992). In contrast, in the isolated perfused rat lung, albumin with a pI of about 7.5 was found to have an approximately 340% higher permeability than normal albumin (Swanson and Kern, 1994). A similarly large difference in permeability between the negatively charged lactate dehydrogenase (LDH) 1 isoform and the slightly positive LDH 5 isoform has also been reported in the artificially perfused rat hindquarter (Haraldsson et al., 1983). It could be speculated that the reason for this apparent discrepancy between the latter studies and the present one and the Gandhi and Bell study (1992) may be related to the differences in perfusion mode and possibly the extensive surgical trauma during preparation in the studies by Haraldsson et al. (1983) and Swanson and Kern (1994).

According to the two-pore model, the slightly smaller molecular size of the charge-modified albumin may have influenced our results. Assuming a small pore radius of 47 Å and a large pore radius of 250 Å, it can be calculated that the difference in size could account for about 30% of the difference in permeability for the different tracers at

baseline (Rippe and Haraldsson., 1994) meaning that we may have overestimated the importance of charge slightly. As mentioned in the introduction, charge has been suggested to be important for the normally low permeability for albumin in the glomerulus (Brenner et al., 1978; Vehaskari et al., 1982) and it could be argued that the difference in TER for the different tracers could be attributed mainly to a difference in glomerular filtration. However, glomerular filtration of albumin only accounts for 1.5% of TER during normal conditions and corresponding figure for cBSA is about 13% (Rippe et al., 2007). Based on these figures it can be calculated that at most 30% of the difference in TER between the differently charged tracers at baseline can be explained by a difference in renal clearance.

Furthermore, even if previous investigators have suggested that charged-dependent differences in permeability during control conditions can be referred to effects on diffusive and convective transport through large and small pores (Gandhi and Bell., 1992; Haraldsson et al., 1983; Swanson and Kern, 1994) we cannot exclude that vesicular transport may have contributed to the observed differences in TER between the differently charged tracers.

Our finding that the initial distribution volume of the charge-modified albumin was about 20% higher than that of BSA could be explained by the observation that the negatively charged luminal glycocalyx limits intravascular distribution of normal albumin (Vink and Duling., 2000). It may also be that a modified protein such as cBSA is cleared more rapidly by the reticuloendothelial system, and by that influence the volume of distribution. However, previous studies showing increased tissue uptake of charge modified albumin in isolated lung as well as in rabbit skeletal muscle and skin in vivo suggest that uptake by the RES cannot explain the whole difference in TER between BSA and cBSA (Gandhi and Bell., 1992; Swanson and Kern., 1994).
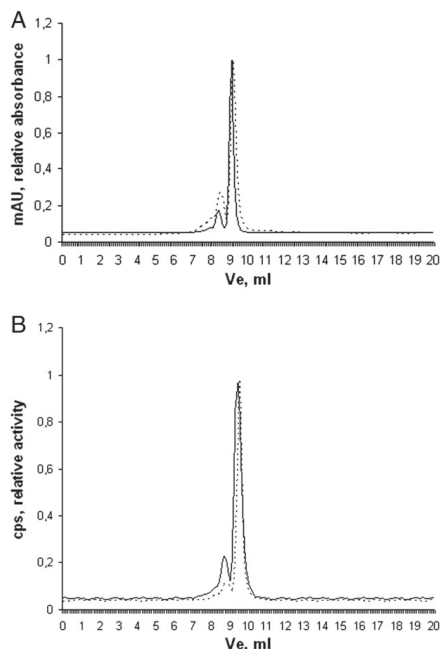
Fig. 3. (a) Representative tracings of ultraviolet absorbance for ^{125}I-labeled bovine serum albumin (BSA) and ^{131}I-labeled charge-modified bovine serum albumin (c-BSA, dotted line) following HPLC in a size-exclusion column. (b) Radioactivity tracings for ^{125}I-labeled bovine serum albumin (BSA) and ^{131}I-labeled charge-modified bovine serum albumin (c-BSA, dotted line) following HPLC in a size exclusion column.

TER and sepsis

The cecal ligation and incision model of abdominal sepsis in the rat has previously been shown to result in gram-positive bacteremia within hours after incision, and has a mortality within the first 24 h of about 90% (Otero-Antón et al., 2001). Our result of a decrease in urine production, mean arterial pressure, and pH and an increase in lactate 3 h after CLI illustrates the progressive hemodynamic instability induced by sepsis in this model. Our finding that the initial distribution volume for normal albumin decreased by about 20% suggests that the hemodynamic instability, at least in part, is due to a sepsis-induced decrease in plasma volume. This is also supported by the observation that hematocrit consistently increased following CLI. Based on the finding that TER for albumin increased after CLI, it is reasonable to conclude

Table 1
Hematocrit (Hct), arterial pH (pH), arterial pCO$_2$ (pCO$_2$) arterial pO$_2$ (pO$_2$) arterial lactate and mean arterial pressure (MAP) for the control ($n = 12$) and sepsis groups ($n = 11$) at baseline (T$_0$) and during the TER measurement (T$_0 + 3$ h). Significant differences within groups at the different time points are indicated. $^*p < 0.05$.

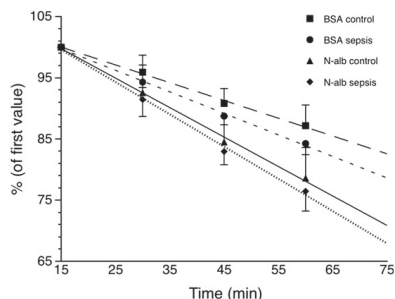|	Hct (%)	pH	pCO$_2$ (kPa)	pO$_2$ (kPa)	Lactate (mmol/l)	MAP (mm Hg)
Control T$_0$	40 ± 2	7.49 ± 0.03	5.4 ± 0.4	12.5 ± 0.9	1.9 ± 0.4	109 ± 16
Control T$_0$ + 3 h	39 ± 2*	7.44 ± 0.02	5.4 ± 0.3	9.9 ± 1.5	2.1 ± 0.8	111 ± 12
Sepsis T$_0$	40 ± 2	7.51 ± 0.04	5.1 ± 0.4	12.2 ± 1	1.8 ± 0.6	109 ± 19
Sepsis T$_0$ + 3 h	43 ± 3*	7.39 ± 0.03*	5.3 ± 0.4	11.3 ± 1	3.1 ± 0.8*	99 ± 19*

Fig. 4. Plasma disappearance curves for ^{125}I-labeled bovine serum albumin bovine (BSA) and ^{131}I-labeled charge-modified bovine serum albumin (c-BSA) during control conditions ($n = 12$) and following induction of sepsis ($n = 11$).

that the hypovolemia is causally related to an increased extravasation of plasma macromolecules. Our finding that mean arterial pressure decreased after CLI and previous results showing a decreased mean arterial pressure and increased vascular resistance suggest that transcapillary hydrostatic pressure is decreased following CLI (Fries et al., 2008). Taken together with a previous study showing a reduced number of perfused capillaries following CLI (Fries et al., 2008), i.e. decreased surface area available for transvascular albumin transport, it is likely that the increase in TER is caused by an increase in permeability.

The presently observed increase in TER for normal albumin of about 20% is comparable to the 30% increase in TER reported for pigs 4 h after induction of endotoxemia (Marx et al., 2002) and in the same range as the 50% increase in TER observed in rats 24 h after induction of sepsis (Ruot et al., 2003). While this increase seems small, it is clearly biologically important and has the potential to rapidly alter the Starling equilibrium as shown by the 20% lower distribution volume for both tracers in septic animals. In this respect it should be noted that in a whole animal in vivo model, several compensatory mechanisms strive to counteract changes in TER for albumin and by that underestimate the true change in permeability induced by sepsis. Hypovolemia will activate the baroreceptor reflex, which increases precapillary resistance and decrease transcapillary hydrostatic pressure causing a decreased convective transport of albumin. The decrease in capillary pressure is augmented by the observed decrease in blood pressure due to hypovolemia.

It could be argued that the effects of CLI on TER and distribution volume for albumin are due to the surgical trauma induced by the laparotomy and not secondary to sepsis. However, in a previous study we were unable to demonstrate an effect of the laparotomy per se on TER and distribution volume for albumin (Bansch et al.,
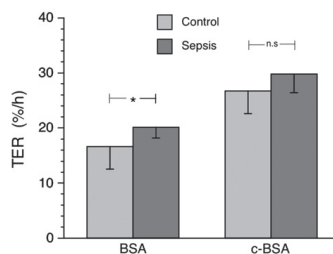

Fig. 5. Transcapillary escape rate (TER) for ^{125}I-labeled bovine serum albumin (BSA) and ^{131}I-labeled charge-modified bovine serum albumin (c-BSA) during control conditions ($n = 12$) and following induction of sepsis ($n = 11$). $^*p < 0.05$.
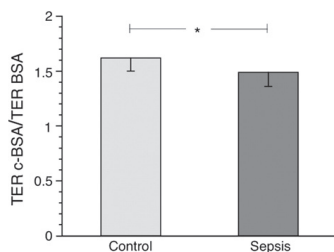
Fig. 6. Ratio of ^{125}I-labeled bovine serum albumin (BSA) to ^{131}I-labeled charge-modified bovine serum albumin (c-BSA) during control conditions or following induction of sepsis. *$p < 0.05$.

Fig. 8. Box plot of plasma concentrations of glycosaminoglycans (GAGs) at baseline (T_0) and at 3 h (T_3) in control animals ($n = 14$) and in sepsis animals ($n = 14$). *$p < 0.05$.

2010). This result indicates that the circulatory effects of CLI are primarily mediated by the induction of sepsis and that any contribution of the laparotomy to circulatory effects of CLI, if present, is likely to be small.

Charge and sepsis

The finding of a decreased ratio between TER for normal albumin and that of charge-modified albumin supports our hypothesis that charge effects are involved in the sepsis-induced increase in permeability and tentative mechanistic explanations are presented below.

The observed CLI-induced increase in plasma concentration of GAGs indicates a sepsis-induced change in GAG turnover as previously reported also in septic patients (Nelson et al., 2008). The mechanisms underlying the increase in plasma concentration of GAGs were not studied in the present study, however, a previous study has shown that increased levels of GAGs in ischemia are associated with a reduction in endothelial glycocalyx thickness (Rehm et al., 2007). Taken together, our results are compatible with the hypothesis that shedding of the negatively charged endothelial glycocalyx contributes to the observed charge effects following CLI.

In addition, previous studies have suggested that the increased permeability observed in sheep lung following administration of endotoxin or *E. coli* is associated with increased number of both small and large pores (Bradley et al., 1988; Smith et al., 1987). If the ratio of large to small pores is maintained this will not affect the ratio of cBSA to BSA. However, it has been hypothesized that the importance of charge effects is dependent on both size of the charged molecule as well as pore size and that the negative charge of albumin mainly restricts transport through small pores, whereas transport through
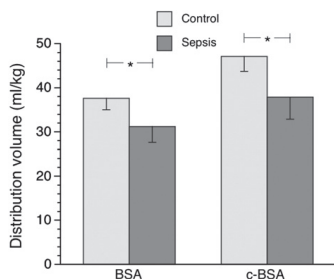
large pores is much less influenced by charge. Thus, if number of large pores increases more than number of small pores the ratio of cBSA to BSA may change.

Conclusions

We conclude that CLI induces hypovolemia secondary to increased microvascular permeability. Negative charge contributes to the normally low permeability of albumin and the importance of charge is decreased in early experimental sepsis. The observed charge effects are associated with a CLI-induced increase in plasma GAG levels, most likely reflecting breakdown of the endothelial glycocalyx.

Author contributions

Study concept and design: Peter Bentzer. *Collection, analysis, and interpretation of the data, and drafting of the manuscript:* Peter Bansch, Peter Bentzer, Axel Nelson and Tomas Ohlsson.

Fig. 7. Distribution volume for ^{125}I-labeled bovine serum albumin (BSA) and ^{131}I-labeled charge-modified bovine serum albumin (c-BSA) during control conditions ($n = 12$) or following induction of sepsis ($n = 11$).
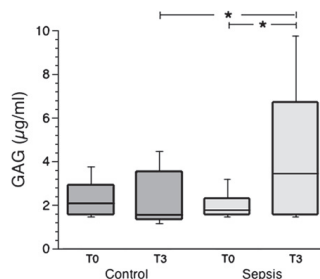
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Paper 4

Perioperative changes in the sublingual microcirculation during major surgery and postoperative morbidity: An observational study

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Abstract
Background: Little is known about perioperative microcirculatory changes during major abdominal surgery, and the main objectives of this study were to evaluate perioperative microcirculatory alterations in this setting, and if changes in microcirculatory parameters are associated with post-operative morbidity and/or with changes in parameters reflecting oxygen delivery.
Methods: Patients scheduled for major abdominal surgery with an estimated P-POSSUM-score of >30 and operation time >3h were eligible for inclusion. Perioperative microcirculatory alterations were evaluated in the sublingual mucosa using Sidestream Dark Field imaging (SDF). Perfused vessel density (PVD), vessel perfusion (microvascular flow index, MFI) and flow heterogeneity (heterogeneity index, HI) were analyzed. $ScvO_2$ and lactate were measured simultaneously. During a 30-day follow up period, postoperative complications were registered according to predefined criteria.
Results: 42 patients with a median P-POSSUM of 33 were included in the study. MFI was higher during anaesthesia than pre- and post anaesthesia. PVD and HI did not change during the observation period. Lactate and $ScvO_2$ increased during surgery. Perioperative lactate and $ScvO_2$ values were not correlated with microcirculatory parameters. Complications occurred in 16 patients. No difference in microcirculatory parameters was detected between patients with and without complications.
Conclusions: Perioperative changes in microcirculatory parameters appear to be minor and no association with outcome in major abdominal surgery could be demonstrated. Changes in $ScvO_2$ and lactate do not reflect sublingual microcirculatory alterations in this setting.

Introduction
It is commonly believed that inadequate tissue oxygenation is a risk factor for the development of post-operative complications following major surgery, and several studies demonstrating an association between reduced global oxygen delivery or related parameters and poor outcome after major surgery support this hypothesis.[1-3] In addition, intervention studies with the objective to either increase global oxygen delivery above a threshold value or to maintain markers of hypoxia such as lactate below a threshold value have been shown to reduce the incidence of complications following major surgery.[4-9] These results have led to the recommendation to use global hemodynamic parameters as targets for the perioperative resuscitation during major surgery with the objective to normalize tissue perfusion.

Emerging evidence, however, indicates that normal macrocirculatory parameters may not always reflect a normal microcirculation. In a subset of septic ICU patients it has been shown that persistent microcirculatory pathology is correlated with poor outcome despite correction of macrocirculatory parameters.[10] It was also recently suggested that impairment of microvascular perfusion postoperatively after major abdominal surgery is associated with complications in patients with normal values of lactate and central venous oxygen saturation.[11] It is currently unknown if

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microcirculatory alterations occur intra-operatively during major abdominal surgery and if a similar disassociation between macro- and microcirculatory parameters can be found intra-operatively. If so, microcirculatory variables may offer an opportunity to detect circulatory disturbances of importance for outcome and may represent goals for perioperative resuscitation.

The present study was designed to investigate if microcirculatory changes can be detected perioperatively during major abdominal surgery and to test the hypothesis that microcirculatory disturbances may be related to increased morbidity and/or mortality after major abdominal surgery, and that microcirculatory parameters may change independently of parameters reflecting global oxygen delivery. Patients undergoing major abdominal surgery were investigated perioperatively using Sidestream Dark Field imaging (SDF) of the sublingual mucosa. Microcirculatory parameters were related to central venous saturation, lactate and to complications during the first postoperative month.

Material and Methods
Study design
The study was approved by the Human Research Ethics committee of the University of Lund (Dnr 309/2008) and was performed at Lund University Hospital, Lund, Sweden. Written informed consent was obtained prior to the surgical procedure. The identification number of the study at ClinicalTrials.com is NCT01037803.

Patient selection
Between October 2008 and September 2010, patients scheduled for major upper gastrointestinal surgery were screened for eligibility and included in a non-consecutive fashion whenever a member of the research team was available. An interim analysis was planned and based on the result, the decision weather or not to proceed with patient inclusion was to be made (se below). Inclusion criteria were: age above 18 years, operation expected to last more than 3 hours, invasive arterial blood pressure measurement and central venous catheter placement planned as part of routine management and an Portsmouth Physiological and Operative Severity Score for

the enUmeration of Mortality and Morbidity (P-POSSUM) expected to be above 30.[12] Exclusion criteria were lack of consent and intra-operative decision by the surgeon not to proceed with planned surgery.

Clinical management
Anaesthesia was performed according to local protocol. Propofol was used as induction agent and isoflurane or desflurane were used for maintenance. Intravenous fentanyl and in some cases epidural mepivacaine was used for intra-operative analgesia. Rocuronium or succinylcholine was used for intubation and rocuronium was used thereafter. Intra-operative fluid management consisted of a basal infusion of Ringers acetate at a rate of 1-2 ml/kg/h to cover evaporative losses and basal fluid requirements. Blood loss was replaced by colloids in a 1:1 ratio until a transfusion trigger level (9-10g/dl) of haemoglobin was reached. Postoperative fluid therapy consisted of a basal infusion of 5 % dextrose solution at a rate of 1 ml/kg/h. Both intra- and postoperatively, additional crystalloids and colloids were administered with the objective to maintain urine output of > 0.5 ml/kg, a mean arterial pressure > 60 – 65 mmHg, a $ScvO_2$ > 70 % and lactate < 2 mmol/l. Noradrenaline or Dopamine (< 10µg/kg/min) were administered if the patient was considered as a non-responder to fluid (pulse pressure variation < 12% or no effect of bolus dose of colloid infusion on hemodynamic parameters). Intra-operative fluid management in pancreatic and liver cases was targeted to maintain a central venous pressure of < 5 mmHg until the resection was completed. This was accomplished by restricting fluid administration and a by reducing positive end expiratory pressure (PEEP) to zero. In cases where this was insufficient, a nitroglycerin infusion was started. Thoracic epidural or patient controlled intravenous administration of morphine was used for postoperative analgesia. The result of the microcirculatory analysis was not available to the anaesthesiologist caring for the patients.

Study protocol
Sublingual SDF imaging of the sublingual mucosa was performed by a person not involved in patient care with an SDF-camera with a 5x lens (Microvision Medical, Amsterdam, Netherlands) on five different

occasions: before anaesthesia (T0), after induction of anaesthesia (T1), during the last hour of surgery (T2), within 2 hours after arrival at the recovery room (T3) and on the first postoperative morning (T4). At each time point, microcirculation was filmed at 5 locations for 20 seconds per sequence. Great care was taken to avoid pressure artifacts and to remove saliva for optimal image quality. The T2 examination was performed > 30 minutes after completion of resection in all liver cases.

Arterial and central venous blood samples were obtained simultaneously as SDF measurements except at T0, when arterial and central venous cannulation had not yet been performed. Arterial lactate, central venous saturation were measured within 10 min of sampling (Radiometer® 720, Copenhagen, Denmark). Correct positioning of the central venous catheter in the superior vena cava was verified by chest x-ray the day after surgery.

Data analysis
Flow parameters for vessels < 20 μm in diameter were analyzed using Microscan Analysis Software (Microvision Medical, Amsterdam, Netherlands) according to guidelines proposed by a consensus conference.[13] Average flow velocity was estimated by calculating the Microvascular Flow Index (MFI). This is a semi-quantitative flow index obtained by dividing the images into 4 quadrants and each quadrant is assigned a value of 0-3, where 0 stands for no flow, 1 for intermittent flow, 2 for sluggish flow and 3 for continuous flow, depending on the dominant form of flow in that quadrant. MFI is the averaged flow of the quadrants at each time point.[14, 15] Microvascular flow heterogeneity was estimated by using the heterogeneity index (HI), which is calculated by subtracting the lowest MFI of any quadrant from the highest MFI, divided by the average MFI of all quadrants.[15] Perfused vessel density (PVD) was calculated by calculating number of perfused vessels crossing a grid pattern containing 3 equidistant horizontal and vertical lines across the image, divided by the total grid length.[16] Analyzes were performed by one researcher. Intra-observer variability for the microvascular parameters was evaluated by calculating the coefficient of variation for PVD values and a weighted kappa score for MFI values in ten randomly selected films. Coefficient of variation for PVD was found to be 4.2 % and the weighted kappa score was 0.7. A weighted kappa score above 0.6 is considered to indicate good agreement.[17]

Measures of outcome
Complications during the first 30 postoperative days were used as an outcome measure. A research nurse actively sought complications during the 30-day follow up. Recorded complications included infectious complications, respiratory complications, cardiovascular complications, gastrointestinal complications and renal complications. Definitions of complications are given in the appendix.

Interim analysis
No power analysis was performed before initiation of the study because of uncertainties with regard to the prevalence of complications and to the precision of the microcirculatory data in the present material. Instead, an interim analysis after data collection from 40 patients was planned prior to initiation of the study. Previous studies in sepsis and in postoperative patients have shown that differences in MFI of 0.3-0.8 and differences in HI of 0.5 are associated with differences in outcome.[11, 15] It was assumed that differences of this magnitude would be of clinical interest and the interim analysis was performed to estimate the probability of a difference in MFI or HI of ≥0.3 and ≥0.5, respectively, between the group with and without complications.

Statistics
The Student's t-test, the Mann-Whitney test and Fisher's exact test were used as appropriate to compare the groups with regard to demographic data. One-way repeated measures ANOVA was used to analyze changes in microcirculatory parameters over time. Two-way repeated measures ANOVA was used to analyze microcirculatory parameters in the group with and the group without complications. Correlation between microcirculatory parameters and $ScvO_2$ and lactate intra- and postoperatively was evaluated using a Spearman correlation analysis. Prism 5.0c was used for the analysis. Data are presented as median and range unless stated otherwise. P-values of < 0.05 were considered significant.

Table 1.
Demographic data for patients with and without complications. Data are presented as median and range if applicable.

	Complications (n = 16)	No complications (n=26)	p-value
Age	66 (43-86)	64 (43-86)	0.38
Gender (female/male)	9 / 7	11 / 17	0.39
P-Possum score	33 (27-42)	32 (25-42)	0.70
P-Possum surgical score	15 (9-26)	14 (8-26)	0.76
Duration of surgery (h)	7.3 (3.5-13)	6.6 (3.5-10.5)	0.35
Liver surgery (number and percentage of patients)	6 (37%)	14 (54 %)	0.57
Pancreatic surgery (number and percentage of patients)	8 (50%)	11 (42%)	0.35
Gastric/esophageal surgery (number and percentage of patients)	2 (12%)	1 (4%)	0.30

Table 2.
Perioperative fluid loss, fluid- and drug administration and hospital stay for patients with and without complications. * Statistically significant difference. Data are presented as median with range if applicable

	Complications (n=16)	No complications (n=26)	p-value
Intraoperative iv fluids (mL)	4000(1500-5500)	3900 (1500-7500)	0.68
Total iv fluids (mL)	5900 (3000-7250)	5600 (3000-9000)	0.75
Estimated blood loss (mL)	915 (250-4000)	740 (50-4500)	0.29
Transfused volume of erythrocytes (mL)	250 (0-3250)	0 (0-2000)	0.09
Number and percentage of transfused patients	8 (50%)	7 (27%)	0.19
Hospital stay (days)	15(9.5-16)	10(8-10.5)	*<0.01
Vasoactive drugs			
- Noradrenaline (number and % of patients receiving drug)	5 (31%)	11 (42%)	0.31
- Dopamine (number and % of patients receiving drug)	1 (6%)	5 (19%)	0.25
- Nitroglycerin (number and % of patients receiving drug)	1 (6%)	7 (27%)	0.10

Table 4.
Microvascular flow index (MFI), heterogeneity index and perfused vessel density, central venous saturation (ScvO$_2$) and lactate in the groups with and without complications. Measurements were performed prior to surgery (T0), following induction of anaesthesia (T1), during the last hour of surgery (T2), within two hours after arrival at the recovery room (T3) and in the morning of the first postoperative day (T4). Estimated difference is presented as mean ± 95% confidence interval and all other values are presented as median and range.

	T0	T1	T2	T3	T4
MFI					
Complications	2.7 (2.1-3.0)	2.8 (2.2-3.0)	2.8 (2.4-3.0)	2.7 (1.9-3.0)	2.7 (2.0-3.0)
No complications	2.7 (2.0-3.0)	2.8 (2.4-3.0)	2.8 (2.3-3.0)	2.7 (2.1-3.0)	2.7 (2.0-3.0)
Estimated difference	0 (-0.24 - 0.26)	0 (-0.26-0.25)	0 (-0.29 - 0.22)	0 (-0.22 - 0.29)	0 (-0.27 - 0.25)
Heterogeneity Index					
Complications	0.14 (0-0.31)	0.12 (0-0.48)	0.14 (0-0.35)	0.16 (0-0.54)	0.14 (0-0.43)
No complications	0.13 (0-0.32)	0.10 (0-0.25)	0.09 (0-0.45)	0.18 (0-0.49)	0.17 (0-0.42)
Estimated difference	-0.01 (-0.1 - 0.1)	-0.02(-0.1 - 0.1)	-0.05(-0.1 - 0.0)	0.02 (-0.1 - 0.1)	0.03 (0.1 - 0.1)
PVD (n/mm)					
Complications	12.6 (11.4-15.1)	12.6 (10.4-17.0)	12.8 (10.7-14.7)	12.4 (10.2-15.2)	12.7 (8.8-16.6)
No complications	12.7 (9.7-14.9)	12.8 (10.5-14.5)	13.2 (11.3-15.7)	12.4 (9.7-14.6)	12.5 (10.0-14.8)
Estimated difference	0.0 (-0.7 - 0.8)	0.2 (-0.7 - 1.2)	0.3 (-0.5 - 1.1)	0.1 (-0.9 - 1.0)	-0.2 (-1.3 - 0.9)
ScvO$_2$ (%)					
Complications		76 (69-89)	77 (63-86)	78 (67-84)	71 (59-81)
No complications		77 (67-88)	81 (66-89)	74 (64-84)	71 (55-82)
Estimated difference		1 (-3 - 6)	4 (0 - 8)	-4 (-9 - 1)	0 (-4 - 5)
Lactate (mmol/L)					
Complications		1.2 (0.5-3.1)	2.6(0.7-5.8)	2.5 (0.9-4.0)	1.7 (0.8-3.2)
No complications		1.2 (0.4-3.6)	2.3(0.8-4.2)	2.1 (0.6-4.6)	1.5 (0.5-2.9)
Estimated difference		0.0 (-0.4 - 0.5)	-0.3(-1.0 - 0.5)	-0.4(-1.2 - 0.4)	- 0.3(-0.7 - 0.1)

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Type of complication	n
Infectious	12
- wound infection	7
- abscess	4
- fever + CRP rise with unclear focus	1
Respiratory	2
- prolonged postoperative ventilation	1
- pleural effusions	1
Cardiovascular	3
- atrial fibrillation	1
- postoperative hypotension	2
Gastrointestinal	3
- gastrointestinal bleeding	1
- prolonged postoperative ileus	1
- anastomotic leakage	1
Renal	3
- injury	2
- oliguria	1

Results
Demographic data and complications
A total of 49 patients were included. In 7 patients, the surgeon did not proceed with the planned procedure leaving 42 patients for analysis. The 95 % confidence interval of the estimated differences in MFI and HI at the different time points calculated at the interim analysis suggested that differences in MFI and HI of $\geq$ 0.3 and $\geq$ 0.5, respectively were unlikely to exist and the study was stopped (table 4). Median age was 66 yrs (43-86). The indication for surgery was malignancies. The type of surgery was liver surgery, pancreatic surgery and gastric/esophageal surgery in 48%, 45% and 7% of the cases, respectively. Demographic data and data on fluid and vasoactive drug administration for the group with and without complications are presented in tables 1 and 2. Hospital stay was longer in the group with complications. Of the 42 analyzed patients, 16 (38%) developed a total of 23 complications. No patient died during the observation period. The nature and frequency of complications are summarized in table 3.

Circulatory data
In the whole group, MFI increased from pre- to intraoperative and decreased postoperatively (Fig. 1). No change in the heterogeneity index and the perfused vessel density could be detected during the observation period. No differences between the group with and the group without complications could be detected in any of the microcirculatory parameters at

any of the time points (table 4). Both, $ScvO_2$ and lactate increased during the operation and decreased postoperatively (Fig. 1). No differences in $ScvO_2$ or lactate could be detected between the group with and the group without complications. Arterial oxygen saturation, mean arterial blood pressure, heart rate, central venous pressure, temperature, haemoglobin and base excess did not differ between the groups with and without complications (data not shown). There was no correlation between microcirculatory parameters and $ScvO_2$ or lactate (data not shown).

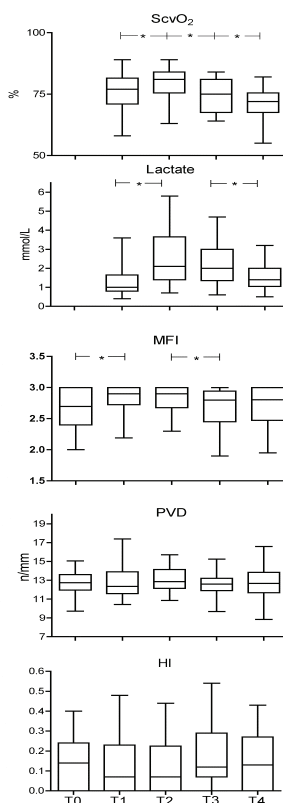Figure 1: Box plot of central venous oxygen saturation (ScvO2), lactate, microvascular flow index (MFI), perfused vessel density (PVD) and the heterogeneity index (HI) before anaesthesia (T0), after induction of anaesthesia (T1), during the last hour of surgery (T2), within 2 hours after arrival at the recovery room (T3) and on the first postoperative morning (T4). * Indicates $p < 0.05$ using repeated measures ANOVA.

Discussion

Our results showed that sublingual microvascular perfusion increased after induction of anaesthesia and decreased postoperatively whereas no change in microvascular perfusion could be detected intra-operatively in patients subjected to major abdominal surgery. The observed changes in microcirculatory parameters did not differ between patients with and without postoperative complications. Perioperative changes in lactate and $ScvO_2$ did not correlate with the microcirculatory parameters.

Previous studies have shown that the methodology used to quantify the microcirculation has a high inter- and intra observer agreement.[14] Our result of an intra-observer weighted kappa of 0.7 for MFI values and a coefficient of variation of about 4 % for PVD values further supports the robustness of the methodology. Due to limited tissue penetration, side stream dark field imaging can only be performed on mucosal surfaces, and most commonly, the sublingual circulation is evaluated. Previous studies have demonstrated that alterations in sublingual microcirculation correlate with changes observed in intestinal microvessels in sepsis.[18, 19] In addition, several studies have reported a correlation between a disturbed sublingual microcirculation and mortality in sepsis, indicating that this site is of pathophysiological relevance.[15, 16] Taken together, these data indicate that generalized microvascular dysfunction is likely to be detected by monitoring the sublingual microcirculation.

Our observations that MFI increased following induction of anaesthesia an decreased after discontinuing anaesthesia are in agreement with a previous study studying sublingual microcirculatory alterations following induction of anaesthesia in patients subjected to cardiac surgery.[20] The mechanisms mediating the increase in microcirculatory parameters are likely to be related to both, vascular dilatation caused by anaesthetic agents as previously described in experimental models and in human vessels, and reduced sympathetic influence on vascular tone.[21,22] Interestingly, the results are in contrast to the decrease in the proportion of perfused small vessels following induction of anaesthesia reported by another group.[23,24] It could be speculated that differences in the anaesthetic technique may have contributed to the observed difference in microcirculatory response following induction of anaesthesia. In the two studies reporting a decreased microvascular perfusion a propofol infusion was used for maintenance in contrast to the use of volatile anaesthetics for maintenance in the present and the study by den Uil et al (2008), respectively. [20]

It should also be noted that perioperative fluid therapy has been shown to influence microcirculatory parameters and may contribute to differences in microcirculatory response to anaesthetics or other vasoactive drugs .[25, 26] Thus, protocol driven fluid administration with the objective to maximize stroke volume has been shown to improve microvascular parameters in patients undergoing major abdominal surgery, and that adding dopexamin does not improve microcirculation further.[25] A similar finding was reported for resuscitated sepsis patients in which nitroglycerin administration did not improve microvascular perfusion, [27] whilst in less aggressively fluid resuscitated septic patients, nitroglycerin increased microvascular perfusion.[28] Taken together, these results indicate that the microcirculatory effect of vasoactive agents may be dependent on the volume status of the patient. This in turn illustrates that the external validity of our results may be dependent on the perioperative resuscitation protocol.

Based on this it could be argued that the fact that intra-operative fluid management in patients undergoing pancreatic and liver surgery differed from that in the patients undergoing gastric/esophageal surgery may constitute a limitation of the study. The latter patients, however, only contribute with 7% to the study population and it can be calculated that the omission of data from these patients in table 4 would not influence the conclusions above. Furthermore, the postoperative protocol for fluid and vasoactive drug administration was the same for all patients.

The result of no difference in microvascular parameters at any time point between the groups with and without complications differs from the correlation between postoperative decreases in microvascular parameters and

complications previously reported in an observational study following major abdominal surgery.[11] Baseline characteristics of patients, type of surgery and rate of complications are similar in present study and the study by Jhanji et al. and the reasons for the difference between the results are not readily apparent. In the previous study, microcirculatory parameters were evaluated every second hour for the first eight postoperative hours, which may have increased the probability to detect microcirculatory disturbances of importance for development of complications compared to the present study. However, the first postoperative measurement in the present study was generally performed at about 2 hours post surgery, the time point at which the most pronounced microcirculatory alterations were observed in the study by Jhanji et al.[11]

Our hypothesis that perioperative microcirculatory variables may be altered despite normal lactate and $ScvO_2$ was not supported by our result that microcirculatory variables remained close to baseline values despite significant alterations in both lactate and $ScvO_2$. This finding contrasts with previous studies showing that microcirculatory variables may be altered despite normal macrocirculatory variables after major abdominal surgery and in resuscitated sepsis patients.[10, 11] In addition, microvacular perfusion indices have been shown to correlate with macrocirculatory parameters in early sepsis, and a correlation between intraoperative lactate levels and microvascular perfusion has been reported in cardiac surgery patients while on by-pass.[15, 23] The difference in results between the different studies which have investigated the relationship between microcirculatory variables and parameters reflecting global oxygen delivery and hypoxia is most likely explained by differences with regard to the nature and severity of patophysiological disturbances and illustrates a complex relationship between the macro- and microcirculation. It is likely that our result of no or a small change in microcirculatory parameters can be explained by relatively stable haemodynamic conditions and by the fact that abdominal surgery is likely to induce a lesser inflammatory response than cardiac surgery or severe sepsis. Had our patients been severely haemodynamically compromised it is

unlikely that microcirculatory would have remained unaltered.

The relatively small number of patients included in this study could be considered as a limitation and it could be argued that a larger study could have detected an association between microcirculatory parameters and outcome. As can be seen in table 4, the 95% confidence intervals for the difference in microvascular parameters between patients with and without complications clearly show that differences in microvacular parameters of a similar magnitude as those observed previously in sepsis and postoperatively are unlikely to be present in our cohort.[11, 15] This conclusion may also be supported by a retrospective power analysis showing that with the present incidence of complications and precision in the measurement of microcirculatory parameters, the study had a power of 80 % or more to detect a difference in MFI of more that 0.3 between the groups at any time point using a t-test. On the other hand, we cannot exclude that smaller differences may exist and could have been detected if more patients had been included. In this respect, it could be noted that the smaller the difference between the groups, the less useful will microvascular parameters be to discriminate between patients that are likely to suffer complications and those with an uneventful postoperative period.

We conclude that perioperative changes in microcirculatory parameters appear to be minor and that no association with outcome in major abdominal surgery can be demonstrated. Changes in $ScvO_2$ and lactate do not reflect sublingual microcirculatory alterations in this setting. Our results do not support the hypothesis that sublingual microcirculatory variables may represent a clinically useful resuscitation endpoint in the setting of major elective abdominal surgery.

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Appendix: Definition of complications

Infectious:	
Pneumonia	X-ray signs + clinical signs or increase in CRP/temperature + treatment with antibiotics
Abdominal infection	Clinical signs + increase in CRP/temperature (>38.5° C) + treatment with treatment with antibiotics
Wound infection	Clinical signs (rubor, calor, dolor, functio laesa) + increase in CRP/temperature or positive culture + treatment with antibiotics
Urinary tract infection	Leucocytes + nitrate on urine sticks or positive culture + treatment with antibiotics
Catheter infection	Local irritation + Clinical signs or positive culture + treatment with antibiotics
Sepsis	2 out of 4 SIRS criteria + likely infection + treatment with antibiotics
Septic shock	Sepsis necessitating inotropic support
Infection with unclear focus	CRP rise + fever + treatment with antibiotics
Cardiovascular:	
Myocardial infarction	1. Increase in Trop T + one of the following: typical symptoms for at least 15min or new infarction signs on ECG (Q-wave in at least 2 leads, new LBBB, new ST-T changes) or loss of viable myocardium as judged by new movement anomaly on cardiac ultrasound.
Postoperative hypotension	Mean arterial pressure <65 despite adequate volume transfusion, necessitating inotropic/vasopressor support
New arrhythmia	New persistent arrhythmia on ECG necessitating treatment
Pulmonary oedema	Clinical signs + x-ray
Stroke	New neurological deficit
Respiratory:	
Pleural effusion	X-ray or ultrasound
Pulmonary embolism	CT scan or lung scintigraphy
Prolonged need for respiratory support	Reintubation/NIV or delayed extubation > 2 h postoperatively
Secretions necessitating interventions	Clinical signs + intervention (deep suctioning, extra physiotherapy, NIV, intubation)
ALI/ARDS	Sudden onset + bilateral infiltrates on x-ray (in absence of left heart failure) + PaO2/FiO2 < 300/200
Abdominal:	
Prolonged paralytic ileus	No bowel movement > 6 days postoperatively
Intraabdominal hypertension	>20 cmH$_2$O surgical intervention necessary
Abscess	x-ray + clinical signs
Intestinal ischemia	Visual diagnosis during reoperation
Anastomostic leakage	Visual diagnosis during reoperation
Wound dehiscence	Surgical intervention necessary (in the ward or in theatre)
Renal:	
Renal Injury	Rise in creatinine by 2 x preoperative value
Need for dialysis	Dialysis
Oliguria	<0.5mL/Kg/h averaged over the first 24h postoperatively
Bleeding disorders:	
Gastrointestinal bleeding	Clinical signs + pharmacologic or surgical intervention
Coagulopathy	PK>1.8 + APTT>60 sec or platelets count < 80.000/uL
Unspecified bleeding	Transfusion of >1 unit of erythrocytes postoperatively
Prolonged need of postoperative ICU/HDU	Still in recovery/ICU after 10.00 a.m. (esophagectomy 14.00 a.m.) due to need for prolonged observation

Paper 5

Plasma volume expansion of 5% albumin relative to Ringer's acetate during normal and increased microvascular permeability. A randomized trial in the rat.

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Abstract

Objective: To test the hypothesis that the plasma volume expanding effect of 5% albumin relative to that of a crystalloid solution is reduced during increased microvascular permeability. **Design:** Prospective and randomized animal study. **Setting:** University hospital laboratory. **Subjects:** 58 adult male Sprague-Dawley rats. **Interventions:** In the normal permeability group, animals were subjected to a controlled hemorrhage of 8ml/kg and immediately resuscitated with either 5% albumin (8ml/kg) or Ringer's acetate (36ml/kg). In the high permeability group, abdominal sepsis was induced by cecal ligation and incision (CLI). Three hours after induction of sepsis animals were resuscitated with either 5 % albumin or Ringer's acetate in a ratio of 1:1 or 1:4.5, respectively, to the measured plasma volume loss. **Measurements and Main results:** Plasma volumes were measured with a radiolabelled albumin tracer technique. Average plasma volume at baseline was 39.8±2.0 ml/kg in the hemorrhage and sepsis groups and decreased to 32.4±3.1 ml/kg at 3 hours after CLI. After resuscitation, plasma volumes were lower in the sepsis group than in the hemorrhage group. In the sepsis group, plasma volumes 15 min after resuscitation with albumin were higher than in the Ringer's acetate group but did not differ in the hemorrhage group. At 2 h post resuscitation, plasma volumes in the hemorrhage group were unchanged and did not differ between animals resuscitated with albumin or Ringer's acetate. In the sepsis group, plasma volume had decreased to 29.6±3.2 ml/kg and to 30.6±3.4 ml/kg at 2 h post resuscitation and to 27.4±5.8 ml/kg and 28.3±4.2 ml/kg at 4 h post resuscitation, in the animals resuscitated with albumin and Ringer's acetate, respectively. **Conclusions:** The plasma volume expanding effect of both albumin and crystalloids is dependent on the prevailing pathofysiological conditions. The present study did not provide support for the hypothesis that the plasma volume expanding effect of albumin relative to that of crystalloids is decreased in pathophysiological conditions characterized by an increased permeability.

Introduction

Maintenance of normal intravascular volume is universally considered to be a cornerstone in the treatment of hemodynamically compromised sepsis patients, but the optimal type of fluid used to reach this therapeutic goal has been debated for a long time (Cannon 1923, Rackow et al., 1983). Proponents of colloids have argued that less volume is required for equal plasma volume expansion and that crystalloids may compromise organ function secondary to edema formation.

Based on studies both in postoperative patients and trauma victims as well as in experimental models of hemorrhage it has been suggested that 4 - 4.5 times the volume of crystalloid solutions required to obtain the same plasma volume expansion as a given volume of albumin (Lamke et al., 1976, Shoemaker, 1976, Persson and Grände, 2005). The

difference in the distribution volumes for the different fluids is commonly attributed to the fact that microvascular permeability to small solutes is high whereas permeability to colloids is low. This means that during conditions of increased permeability such as sepsis it is plausible that the distribution volume of a colloid approaches that of a crystalloid solution as also suggested previously (Shoemaker, 1976). If so, this may contribute to explain the observation that in recent randomized controlled studies the ratio between crystalloids and colloids is reported to be 1: 1-1.3 (Finfer et al., 2011, Perner et al., 2012, Myburg et al., 2012).

To our knowledge, only one previous study has presented data on the ratio of colloid to crystalloids with regard to plasma volume expansion in sepsis (Ernest et al., 1999). It was shown that in septic patients the ratio of 5% albumin to normal saline was about 1:5 one hour after resuscitation was completed, indicating that the ratio between saline and albumin in sepsis is similar to that observed during normal conditions. However, plasma volumes and oxygen delivery were in the normal range before administration of fluid and no data suggesting an increased permeability were presented. It could also be argued that the observation time after resuscitation was to short for detecting clinically relevant decrease in plasma volume expansion by albumin secondary to an increased transcapillary escape rate for albumin.

Based on these considerations the present study was designed to test the hypothesis that the difference in volume of a colloid or a crystalloid required for equal plasma volume expansion decreases during conditions that are associated with increased permeability. For this purpose, rats subjected to either a volume-controlled hemorrhage or abdominal septic shock were randomized to receive resuscitation with either 5% albumin or Ringer's acetate in a ratio of 1:4.5. Plasma volume was measured up to four hours after resuscitation by measuring the initial distribution volume of radiolabelled albumin.

Materials and Methods

Materials and anesthesia

The study was approved by the Lund University ethics committee for animal research (M87-09), and the animals were treated in accordance with the guidelines of the National Institutes of Health for Care and Use of Laboratory animals. Adult male Sprague-Dawley rats (n = 58) weighing 354 ± 13 g were used. The animals had free access to water and food until anesthesia was induced by placing the rats in a covered glass container with a continuous supply of isoflurane (Isoba® Vet; Intervet AB, Sweden). After a tracheostomy, the animals were connected to a ventilator (Ugo Basile; Biological Research Apparatus, Comerio, Italy) and ventilated with tidal volumes of 6 ml/kg with a positive end-expiratory pressure of 3–4 cm H_2O. Anesthesia was maintained by inhalation of 1.6–1.8% isoflurane in humidified air through the tracheal cannula. Body temperature, measured rectally, was kept at 37.1– 37.3°C via a feedback-controlled heating pad. End-tidal pCO_2 was monitored continuously and kept between 4.5 and 5.5 kPa (Capstar-1000; CWE, Artmore, PA). The left femoral artery was cannulated for measurement of mean arterial blood pressure (MAP), pulse pressure variation and to obtain blood samples. The right jugular vein and the left femoral vein were cannulated and used for injections and to measure central venous pressure (CVP) intermittently. Following administration of an intravenous bolus of fentanyl (25 µg/kg, Braun Melsungen AG, Melsungen, Germany) and the start of a continuous fentanyl infusion (0.5 µg/kg/min), isoflurane was reduced to 1.1 - 1.3%. After a 10-min equilibration period, MAP, CVP and pulse pressure variation (PPV) were recorded and baseline values for arterial blood gases, electrolytes, hematocrit (Hct), and lactate were measured (I-stat; Hewlett Packard, Böblingen, Germany). PPV was calculated by measuring pulse pressure variation over a single respiratory cycle: PPV [%] = (PPmax - PPmin)/((PPmax + PPmin) / 2) x 100 and is presented as the mean of 3-4 calculations. A PPV above 13% has previously been shown to be highly predictive for preload responsiveness in the rat (Sennoun et al., 2007)

Urine was collected in a glass vial placed at the external meatus of the urethra from the end of

the preparation until the end of the experiment, when the bladder was emptied by external compression. Urine production is presented as ml/kg/h (total production divided by length of collection period) for easier comparison between the different groups. After the experiment, the animals were killed with an intravenous injection of potassium chloride.

Measurement of plasma volume

Plasma volume (PV) was estimated by determination of the initial distribution for human serum albumin (HSA, CSL Behring, King of Prussia, Pennsylvania, USA) labeled with ^{125}I as described in detail previously (Wiig et al., 2003). This was accomplished by measuring the increase in radioactivity following injection of a known amount ^{125}I-HSA by subtracting the activity in a 250-µL blood sample taken just before the injection from the activity 5 min after the injection. The radioactivity in the emptied vial, in the syringe, and in the needle was subtracted from the total radioactivity in the prepared dose. By dividing the administered dose with the resulting concentration, the distribution volume for the tracer could be calculated. The amount of unbound radioactivity in the injected ^{125}I-albumin was measured regularly after precipitation with 10% trichloroacetic acid, and was found to be less than 1%. All samples were counted in a gamma counter (Wizard 1480, LKB-Wallac, Turku, Finland).

Experimental protocol

Hemorrhage group

In the hemorrhage group, animals were bled a total of 8 ml/kg in 5 minutes. Animals were then resuscitated with either 5 % albumin (8 ml/kg) (CSL Behring, King of Prussia, Pennsylvania, USA, Na^+ 155mmol/l, caprylate 4mmol/l, N-acetyltryptophan 4mmol/l, Cl^- approx 150mmol/l) or Ringer's acetate (36 ml/kg) (Fresenius Kabi, Uppsala, Sweden, 131 mmol/l Na^+, 4 mmol/l K^+, 2 mmol/l Ca^{2+}, 1 mmol/l Mg^{2+}, 112 mmol/l Cl^-, 30 mmol/l Acetate, osmolality 270 mosmol/kg) during a 30 min resuscitation period. Plasma volumes were measured at baseline, 15 min after resuscitation was completed and again 2h later. Arterial blood gases, lactate, Hct and electrolytes were measured at baseline, after hemorrhage and at 2h post-resuscitation. Plasma volumes directly after hemorrhage

were calculated as follows: [Baseline -(8ml x (1-Hct))]. MAP, CVP, PPV were measured at baseline, after hemorrhage, 15 min and 2 h post resuscitation (Fig. 1).

Sepsis groups

Following surgical preparation and baseline measurements as described above, animals were subjected to a cecal ligation and incision (CLI) procedure. The CLI procedure has been described in detail previously (Fries et al., 2008, Otero-Antón et al., 2001). Briefly, following a 3 - 4 cm midline abdominal incision the cecum was mobilized while carefully avoiding hemorrhage. The cecum was ligated with a 3.5 silk ligature, and a 1-cm incision was made in the ligated cecum with a scalpel blade. The abdomen was then closed with metal clips. Three hours after the CLI procedure, MAP, CVP and PPV were recorded and blood samples for analysis of plasma volume, arterial blood gases, electrolytes, Hct, and lactate were collected. Plasma volume loss was calculated and the loss was replaced by the same volume of 5% albumin or by 4.5 times the lost volume of Ringer's acetate during the following 30 min. Plasma volume was measured again 15 minutes and 2 h after completion of the infusion (Fig. 1). MAP, CVP, PPV were measured immediately before plasma volume measurements, and arterial blood gases were measured again at the end of the experiments. On a post-hoc basis, a second set of experiments was performed in septic animals, using an identical protocol except for the fact that the last plasma volume measurement was performed 4 hours post resuscitation instead of 2 hours post resuscitation (Fig. 1).
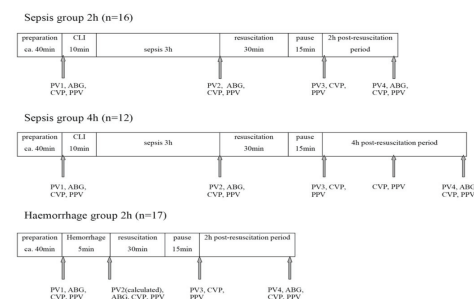

Fig 1. Schematic figure of the experimental protocol in the Sepsis and Hemorrhage group. (CLI = cecal ligation and incision, PV = plasma volume, ABG = arterial blood gas, CVP = central venous pressure, PPV = pulse pressure variation).

Statistics

Following tests for normality with the Kolomogorov-Smirnov test, treatment effects of albumin and Ringer's acetate within the hemorrhage and sepsis groups respectively were analyzed using two-way-repeated measures-ANOVA followed by Bonferroni post-hoc test. To test for differences in plasma volume between the hemorrhage and the sepsis animals immediately after resuscitation the student´s t-test was used. Data are presented as mean ± SD. P-values of < 0.05 were considered significant. Prism 5.0c was used for the analysis.

	Sepsis		Hemorrhage	
	Albumin	**Ringer's**	**Albumin**	**Ringer's**
Baseline				
MAP (mmHg)	110 ± 15	106 ± 14	109 ± 27	111 ± 23
PPV (%)	8 ± 2	8 ± 3	11 ± 3	10 ± 5
Hct (%)	43 ± 3	43 ± 2	45 ± 2	45 ± 2
Lactate (mmol/L)	2.0 ± 0.5	2.2 ± 0.3	2.1 ± 0.4	2.3 ± 0.3
Base excess	6 ± 2	5 ± 2	5 ± 1	5 ± 1
3h after CLI / 5min after hemorrhage				
MAP	104 ± 15	102 ± 14	77 ± 23 *	80 ± 24 *
PPV	15 ± 4 *	17 ± 7 *	17 ± 7 *	17 ± 5 *
Hct	48 ± 3 *	47 ± 2 *	41 ± 2 *	41 ± 2 *
Lactate	3.5 ± 0.9 *	3.3 ± 0.8 *	2.2 ± 0.5	2.3 ± 0.5
Base excess	0 ± 2	1 ± 2	4 ± 1	5 ± 1
15min post-resuscitation				
MAP	109 ± 14	100 ± 11	110 ± 11 *	103 ± 16 *
PPV	12 ± 3	13 ± 5	9 ± 6 *	9 ± 4 *
2h post-resuscitation				
MAP	90 ± 13	88 ± 17	106 ± 17	100 ± 17
PPV	17 ± 6	21 ± 9	9 ± 2	13 ± 5
Hct	48 ± 5	43 ± 7	35 ± 2 *	36 ± 3 *
Lactate	2.3 ± 0.4 *	2.8 ± 1.0	1.9 ± 0.5	1.9 ± 0.7
Base excess	-2 ± 3	-4 ± 2 *	2 ± 2 *	0 ± 2 *
4h post-resuscitation			n.a.	n.a.
MAP	56 ± 7 *	65 ± 15 *		
PPV	30 ± 10	18 ± 8		
Hct	45 ± 7	47 ± 4		
Lactate	5.7 ± 2.7 *	4.0 ± 2.1 *		
Base excess	-10 ± 5 *	-7 ± 3 *		

Table 1. Mean arterial pressure, Pulse pressure variation, Hematocrit, Lactate and Base excess in the animals resuscitated with 5% albumin or Ringer's acetate. Data are presented as mean ± S.D. * p < 0.05 compared to previous measurement in the same treatment group (n.a. = not applicable).

Results

Hemorrhage group
Physiological and laboratory data

A total of 17 animals were included in the hemorrhage group. No animal died during the experiment. MAP decreased after bleeding and increased after fluid resuscitation in both treatment groups. MAP then remained unchanged until the end of the experiment. PPV increased after bleeding in both groups (p<0.05). PPV decreased in both groups after fluid resuscitation (p<0.05) and remained unchanged until the end of the experiment (Table 1). The Ringer's acetate group had a higher urine production than the albumin group with 1.8 ± 0.2 ml/kg/h and 1.3 ± 0.2 ml/kg/h respectively (p<0.05).

Hct decreased after bleeding and decreased further until the end of the experiment in both treatment groups with no difference between the groups (p<0.05). No changes in lactate levels were observed during the experiment. BE decreased in both treatment groups towards the end of the experiment with no changes between the groups (p<0.05) (Table 1). No differences between the groups with regard to arterial blood gases, sodium, potassium concentrations or CVP could be detected at any of the time points (data not shown).

Sepsis groups
Physiological and laboratory data

A total of 28 animals were included in the sepsis groups. In the 2 hour group, 4 animals (20%) died and in the 4 h group, 9 animals (36%) died prior to completion of all measurements and data from these animals were not included in the analysis. No difference in mortality between the animals treated with albumin or Ringer's solution could be detected. At baseline, there were no differences with regard to MAP, PPV, Hct, lactate or Base excess (BE) (Table 1). MAP remained unchanged until the last measurement (2h or 4h), when it had decreased compared to the measurement 15min post resuscitation in both treatment groups. PPV was increased at 3 h after CLI in both groups with no further changes at later time points. Average MAP from start of resuscitation until the end of the 2 h experiment was 107 ± 10mmHg in the albumin group and 93 ± 12 in the Ringer's acetate group. The respective values for the 4 h experiments were 86 ± 12 mmHg and 87 ± 15 mmHg. Urine production was 0.8 ± 0.1 ml/kg/h and 0.9 ± 0.2 ml/kg/h in the albumin and Ringer's acetate group, respectively.

Hct and lactate increased and BE decreased in both treatment groups 3 h after the CLI procedure (p<0.05). At 2 hours post resuscitation, lactate had decreased in the albumin group whereas no change was detected in the Ringer's acetate treated group (p<0.05). At 4 hours, lactate had increased again in both treatment groups (p<0.05). BE decreased both 2 and 4 hours after resuscitation with Ringer's acetate. No significant changes in BE could be detected in

the albumin group after 2 hours, but BE had decreased at 4 hours after resuscitation (p<0.05)(Table 1). No differences between the groups with regard to arterial blood gases, sodium, potassium concentrations or CVP could be detected at any of the time points (data not shown).
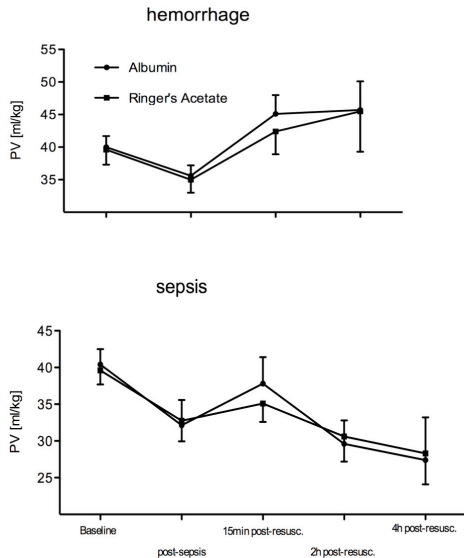

Fig 2. Upper panel: Plasma volumes at baseline, immediately following a controlled hemorrhage of 8 ml/kg, 15 min and 2 hours after resuscitation with either 5% albumin (n=8) or Ringer's acetate (n=9).
Lower panel: Plasma volumes at baseline, 3 hours after cecal ligation and incision, at 15 min, 2 hours and 4 hours after resuscitation with either 5% albumin or Ringer's acetate (n=28 until 15 min post-resuscitation, n=16 for 2h-group and n=12 for 4h-group with equal amount of animals/treatment group).

Plasma volumes
Following hemorrhage, plasma volumes decreased from 40.0 ± 1.7 ml/kg to 35.6 ± 1.6 ml/kg in the albumin group and from 39.6 ± 2.3 ml/kg to 35.0 ± 2.0 ml/kg in the Ringer's acetate group (p<0.01 for both). Plasma volume increased by 9.5 ± 2.3 ml/kg to a volume of 45.1 ± 2.9 ml/kg 15 min after the completion of resuscitation with albumin (p<0.01), and was 45.7 ± 4.4 ml/kg after 2 hours (Fig. 3). In the animals resuscitated with Ringer's acetate, plasma volume had increased by 7.4 ± 2.9 to 42.4 ± 3.5 ml/kg at 15 min after resuscitation (p<0.01) and was 45.5 ± 6.2 ml/kg after 2h (Fig 3). There was no difference between the plasma volumes after resuscitation with either albumin or Ringer's

solution at 15 min or 2 h after resuscitation (Fig. 2).

In the sepsis groups, plasma volumes had decreased from 40.4 ± 2.1 ml/kg to 32.1 ± 3.4 ml/kg in the albumin group and from 39.6 ± 1.9 ml/kg to 32.7 ± 2.8 ml/kg in the Ringer's acetate group at 3 h after the CLI procedure (p<0.01). Plasma volume increased by 5.7 ± 2.9 ml/kg to 37.8 ± 3.6 ml/kg 15min after the completion of the 30 min-period of resuscitation with albumin, and decreased to 29.6 ± 3.2 ml/kg and 27.4 ± 5.8 ml/kg at 2h and 4h, respectively (p<0.01)(Fig. 2). In animals resuscitated with Ringer's acetate, plasma volume increased initially by 2.4 ± 3.0 ml/kg to 35.1 ± 2.5 ml/kg at 15 min after resuscitation and decreased to 30.6 ± 3.4 ml/kg and to 28.3 ± 4.2 ml/kg at 2h and 4 h, respectively (p<0.05 for both)(Fig 2). The increase in plasma volume 15 min after resuscitation was higher in the albumin group (p<0.01), a difference that was not maintained after 2 or 4 hours (Fig 2, 3). Plasma volume expansion by both albumin and Ringer's acetate was higher in the hemorrhage group than in the sepsis groups at 15 min after completion of resuscitation (p<0.05).
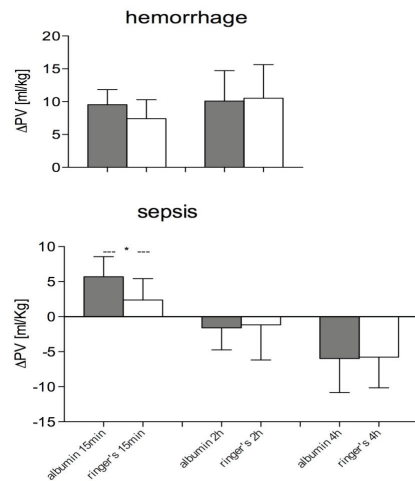

Fig 3. Upper panel: Change in plasma volume in animals subjected to a controlled hemorrhage of 8 ml/kg at 15 min and 2 h after resuscitation with either 5 % albumin or Ringer's acetate.
Lower panel: Change in plasma volume in animals subjected to cecal ligation and incision at 15min, 2 hours and 4 hours after resuscitation with either 5% albumin or Ringer's acetate (n=28 until 15 min post-resuscitation, n=16 for 2h-group and n=12 for 4h-group with equal amount of animals/treatment group)(*=p<0.01).

Discussion

Our results showed that resuscitation with albumin or Ringer's acetate in a ratio of 1 to 4.5 results in equal plasma volume expansion following hemorrhage. Resuscitation with albumin or Ringer's acetate in the same ratio in septic animals results in better plasma volume expansion by albumin immediately after resuscitation whereas plasma volumes at 2 and 4 h after resuscitation did not differ. The increase in plasma volume by albumin and by Ringer's acetate immediately after resuscitation is higher after hemorrhage than in sepsis.

Plasma volume measurement using radiolabelled albumin is a well established method both experimentally and in clinical practice (Margason and Soni, 2005). The potential errors in the technique, such as effects of poor mixing of the tracer and effects of transcapillary escape of tracer during the 5-min mixing period, have been discussed previously and found to be small (Dubniks et al., 2007). Our result of a baseline plasma volume of about 40 ml/kg is similar that published by others and us previously and illustrate the reliability of the methodology (Bansch et al., 2010, Lee and Blaufox, 1985, Lundin et al., 1981). The cecal ligation and incision method has been shown to result in a gram-positive bacteremia within hours with a high mortality rate (Otero-Antón et al., 2001). The presently observed decrease in plasma volume of about 7ml/kg prior to resuscitation in combination with hemoconcentration suggest that the model induces plasma leakage secondary to an increased microvascular permeability. The continuing plasma loss after resuscitation in the present study and the previously reported increase of the transcapillary escape rate of albumin after cecal ligation and incision further support the hypothesis that microvascular permeability is increased (Bansch et al., 2011). Taken together these data support that the plasma volume expanding properties of albumin and Ringer's acetate were evaluated in a model with an increased permeability.

In the hemorrhage group animals were bleed 8 ml/kg and the rationale for this volume of bleeding was to achieve a similar depletion of intravascular volume as was expected in the sepsis animals (Bansch et al., 2011). It could be argued that hemorrhage may have induced a systemic inflammatory response syndrome, which in turn could have increased microvascular permeability. However, a hemorrhage of 8 ml/kg only constitutes 11 % of total blood volume in the rat and corresponds to a class I hemorrhage as defined by the Advanced Trauma and Life Support (ATLS) guidelines and is unlikely to have increased permeability. This notion may be supported by our result that plasma volume was unchanged 2 h after resuscitation in the hemorrhage group. Based on this, it is reasonable to conclude that microvascular permeability was in the normal range in the hemorrhage group.

As expected, the ratio of Ringer's acetate to albumin of 4.5:1 resulted in an equal plasma volume expansion in the hemorrhage group. The adequacy of this crystalloid to colloid ratio is supported by a previous study showing that if Ringer's acetate is administered in a lower ratio to albumin, plasma volume expansion will be significantly lower than in the group resuscitated with 5 % albumin (Jungner et al., 2010). Similar ratios between 0.9% NaCl and albumin and have been reported in hemorrhage models indicating that, with respect to plasma volume expansion, 0.9% NaCl and Ringer's solutions are very similar (Persson and Grände, 2005). The poor plasma expanding properties of crystalloid solutions is also supported by clinical studies showing that only about 20 % of 0.9 % NaCl or a Ringer's solution remains intravascularly immediately after resuscitation (Ernest et al., 1999, Lamke and Liljedahl, 1976, Shoemaker, 1976).

Our hypothesis that 5% albumin would be a relatively less potent plasma volume expander than a crystalloid in sepsis with increased plasma leakage compared to conditions with a normal microvascular permeability was not supported by our result that plasma volume was equal in the Ringer's acetate and albumin groups at 2 hours post resuscitation, and initially even better in the albumin treated animals. Based on the concern that 2 hours post resuscitation was too short a time for an increased permeability to affect the plasma volume expanding properties of albumin, we added a second group of sepsis animals on a post-hoc basis in which plasma volume was evaluated 4 hours post resuscitation. Also the 4-hour data failed to demonstrate significant

differences in plasma volume between the Ringer's acetate and the albumin group. It could be argued that by measuring plasma volume at even later time points after resuscitation, a difference in the plasma volume expanding effect could have been detected. However, given that more than 1/3 of all animals died before the end of the 4-hour period, longer experiments were not considered feasible. In this respect it should be noted that the surviving animals in the 4 h group are likely to represent a subgroup of animals with a less severe sepsis and possibly less severe plasma leakage.

Extravasation of albumin is dependent on both diffusion and convection and it is possible that the decrease in mean arterial blood pressure may have decreased convective transport of albumin, which in turn may have influenced any change in the plasma volume expanding effect of albumin caused by an increase in permeability (Parving et al, 1974). However, average mean arterial blood pressures during experimental period were clearly above the 65mmHg threshold for sepsis patients suggested by the Surviving Sepsis Guidelines (Dellinger et al., 2013).

Our result that the plasma volume expansion by both albumin and Ringer's acetate is lower in the sepsis group than in the hemorrhage group at 2 hours is expected and probably reflects the ongoing loss of plasma in the sepsis animals. However, our result of a difference in plasma volume expanding effect of both albumin and Ringer's acetate during sepsis compared to that observed after a hemorrhage already 15 min after resuscitation is unlikely to be explained only by plasma leakage, and several mechanisms may contribute to this result. Following a hemorrhage, homeostatic mechanisms such as activation of the baroreceptor reflex will immediately strive to normalize intravascular volumes by mobilizing fluid from the extravascular compartment. The mobilized fluid is added to that given during the resuscitation. This notion may be supported by our result that the isooncotic 5 % albumin solution increased plasma volume by more than the infused volume. As mentioned above, there is ongoing plasma leakage secondary to increased microvascular permeability in the sepsis animals. Plasma leakage during the 3

hours prior to resuscitation was about 7ml/kg and is unlikely to explain the difference in plasma volume of 5ml/kg only 15 min after resuscitation if maintained a similarly slow rate. However, given that autoregulation of capillary pressure is likely to be depressed in sepsis, it is possible that an the increased blood pressure seen during resuscitation will be transferred to the exchange vessels and transiently increase plasma leakage and thereby contribute to the reduced volume expanding properties of both colloids and crystalloids in sepsis (Terborg et al., 2001, Radaelli et al., 2013).

Potential clinical implications
The SAFE trial suggested that the large difference in plasma volume expanding effect between albumin and crystalloids shown both following surgery and in trauma patient does not seem to be readily apparent at bedside and that in the context of that study the ratio of albumin to crystalloid was about 1:1.4 (Finfer et al. 2004). Similar results have recently been reported for hydroxyethyl starches both in a mixed ICU population and in sepsis patients (Myburg et al. 2012, Perner et al., 2012). Our results do not support the hypothesis that the small difference between the volume of albumin and crystalloids administered clinically can be explained by increased microvascular permeability for colloids.

Conclusion
The present study do not support the hypothesis that pathophysiological conditions associated with an increased microvascular permeability change the plasma volume expanding properties of 5% albumin relative to that of crystalloids and suggest that also in severe sepsis, the ratio of albumin to crystalloid may be about 1:4.5.

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