ENDOTHELIN RECEPTOR ANTAGONISM AND HYPERTONIC SOLUTIONS IN EXPERIMENTAL ENDOTOXIN SHOCK

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ABSTRACT

Sepsis is a common and serious condition among patients in the intensive care unit. It is characterized by a systemic inflammatory response, resulting in cardiovascular instability and impaired organ tissue oxygenation due to hypoperfusion and inflammatory changes. The supportive treatment includes fluid resuscitation and other measures to promote organ perfusion. Hypertonic saline/dextran (HSD) resuscitation has shown improved haemodynamics in haemorrhage. In addition, immuno-modulatory effects have been reported in experimental studies. These effects would in theory also be of benefit in septic shock. The vasoconstrictive peptide endothelin (ET) is involved in several of the sepsis manifestations. Experimental results of ET antagonism in endotoxemia have demonstrated beneficial effects on cardiac performance and regional perfusion. Hypotension due to vasodilation may however be of concern in septic conditions, requiring measures to secure perfusion pressure.

The overall aim of this thesis was to evaluate two different interventions and their ability to affect vital organ perfusion through effects on systemic and regional haemodynamics in porcine endotoxin shock. Specifically, the cardiovascular effects of HSD resuscitation were further evaluated, with focus on regional perfusion. In addition, effects of the new parenteral ET antagonist, tezosentan, on systemic and regional haemodynamics were investigated. Furthermore, tezosentan was combined with HSD with the purpose of preventing ET antagonism-related hypotension, and possibly augment positive cardiovascular effects. In addition, early pro-inflammatory cytokine response was studied with both treatments to further evaluate their immunomodulating potential.

A model of endotoxin shock in anaesthetized pigs was used in two series of experiments. Haemodynamic responses were assessed using a pulmonary artery catheter, invasive arterial blood pressure and ultrasonic flow probes positioned around the portal vein and the renal and carotid arteries. Plasma TNF-α and IL-6 levels were monitored.

Endotoxemia resulted in a hypodynamic shock including reduced regional blood flow and development of metabolic acidosis. An inflammatory response with increases in cytokine and endothelin-1 levels was elicited. HSD resuscitation transiently improved cardiac index and mean arterial pressure. Regional blood flow increased in the renal and carotid, but not portal circulation. The progression of acidosis was delayed and the macro- and microcirculatory improvements resulted in improved survival rates. Tezosentan treatment resulted in vasodilation, which improved cardiac index, reversed pulmonary hypertension and increased portal and carotid, but not renal blood flow. Addition of HSD to tezosentan did not increase mean arterial pressure, but markedly increased portal and carotid perfusion. None of the interventions affected TNF-α and IL-6 response.

In conclusion, transient beneficial effects of HSD on systemic and renal haemodynamics were confirmed, and improved early survival indicated. From a clinical perspective, HSD resuscitation may gain time for other treatments to have their effects. The immunomodulatory potential of HSD in endotoxemia was not supported
by the results from this study. However, ET antagonism as a means to promote organ perfusion was supported by the results. The improved regional flow demonstrated with combined tezosentan/HSD emphasizes the importance of additional measures to secure perfusion pressure during ET antagonism.

*Keywords: sepsis, endotoxin, fluid resuscitation, hypertonic saline/dextran, endothelin, tezosentan*
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALAT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ASAT</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>BE</td>
<td>Base excess</td>
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<tr>
<td>CI</td>
<td>Cardiac index</td>
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<tr>
<td>CO</td>
<td>Cardiac output</td>
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<tr>
<td>CVP</td>
<td>Central venous pressure</td>
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<tr>
<td>DO₂</td>
<td>Systemic oxygen delivery</td>
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<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>ETₐ</td>
<td>Endothelin receptor type A</td>
</tr>
<tr>
<td>ETₐ</td>
<td>Endothelin receptor type B</td>
</tr>
<tr>
<td>ET-1-LI</td>
<td>Endothelin-1-like immunoreactivity</td>
</tr>
<tr>
<td>EVLW</td>
<td>Extra-vascular lung water</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin concentration</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HS</td>
<td>Hypertonic saline</td>
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<tr>
<td>HSD</td>
<td>Hypertonic saline/dextran</td>
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<tr>
<td>ICU</td>
<td>Intensive care unit</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
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<tr>
<td>MPAP</td>
<td>Mean pulmonary artery pressure</td>
</tr>
<tr>
<td>NaCl</td>
<td>Saline</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NO₃</td>
<td>Nitrate</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Arterial partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PADIAST</td>
<td>Diastolic pulmonary artery pressure</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Arterial partial pressure of oxygen</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end expiratory pressure</td>
</tr>
<tr>
<td>RBF</td>
<td>Renal blood flow</td>
</tr>
<tr>
<td>SvO₂</td>
<td>Mixed venous oxygen saturation</td>
</tr>
<tr>
<td>SVR</td>
<td>Systemic vascular resistance</td>
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<tr>
<td>SVRI</td>
<td>Systemic vascular resistance index</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>VO₂</td>
<td>Systemic oxygen consumption</td>
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1 INTRODUCTION

1.1 SEPSIS

1.1.1 Definitions

Sepsis is considered to be the clinical presentation of patients with a serious infection, who demonstrate a systemic inflammatory response, with or without a positive blood culture. The definition and classification of the sepsis syndromes have been under debate throughout the years, but in 1992 a consensus regarding a universal terminology was reached (Table 1), however, this was modified in 2001.

The source of infection is predominantly pulmonary, followed by abdominal or urinary tract. The causative organisms may either be gram positive or gram negative bacteria, or fungi. The incidences of both gram positive and fungal sepsis have increased over the last few decades.

Table 1. Diagnostic criteria for sepsis (modified from ACCP/SCCM 1992)

<table>
<thead>
<tr>
<th>Systemic inflammatory response syndrom (SIRS):</th>
<th>≥ 2 of the following criteria</th>
</tr>
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<tbody>
<tr>
<td>Temperature &gt;38.3 or &lt;36°C</td>
<td></td>
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<tr>
<td>Heart rate &gt;90/min</td>
<td></td>
</tr>
<tr>
<td>Respiratory frequency &gt;20/min or PaCO₂&lt;4.3 kPa</td>
<td></td>
</tr>
<tr>
<td>Leukocytes &gt;12 or &lt;4 10³ mL⁻¹ or &gt;10% immature cells</td>
<td></td>
</tr>
<tr>
<td><strong>Sepsis</strong></td>
<td>SIRS and infection (confirmed or suspected)</td>
</tr>
<tr>
<td><strong>Severe sepsis</strong></td>
<td>Sepsis and signs of organ dysfunction, hypoperfusion or hypotension</td>
</tr>
<tr>
<td><strong>Septic shock</strong></td>
<td>Severe sepsis and persistent hypotension despite fluid resuscitation</td>
</tr>
</tbody>
</table>

1.1.2 Epidemiology

Sepsis is a common and serious condition among patients in the intensive care unit (ICU). Approximately 12 to 27% of every 100 ICU admissions are due to severe sepsis, and the calculated overall annual incidence of severe sepsis in the adult population ranges from 0.51 to 3.0 per 1000. Over recent decades, the yearly incidence of sepsis has reportedly increased by nearly 9% each year. Mortality from sepsis varies from 20-60%, depending on severity of illness: number of organ dysfunctions and the presence of shock. The predisposing factors for a fatal outcome include increased age, preexisting comorbidity and genetic factors. Even though the mortality rate has decreased over the past decades, the total number of sepsis-related deaths is increasing due to the increased incidence.
1.1.3 Pathophysiology

1.1.3.1 Endotoxin exposure

Endotoxin consists of components of the gram negative bacterial cell wall. Two layers, an inner phospholipid bilayer intersected by transport proteins, and an outer layer of lipopolysaccharide (LPS) and proteins constitutes the cell wall. The lipid A component of the LPS exerts the effects initiating the pathophysiological changes in sepsis. In contrast, gram positive bacteria mainly depend upon exotoxins, acting as superantigens in the initiating process of the sepsis syndrome. In the bloodstream, LPS binds to a circulating LPS-binding protein, which transports the endotoxin to immunocompetent cells (macrophages). The LPS-carrier protein complex interacts with either the membrane bound surface protein CD14 or a soluble pool of the same protein. This ligand complex then binds to a Toll-like-receptor, with subsequent nuclear factor-mediated activation of inflammatory and immune responses. The host response to endotoxin has evolved for protection against infection, but the pathophysiological changes may be overwhelming as the septic syndrome or septic shock develops.

1.1.3.2 Inflammatory response

The activated macrophages and monocytes release cytokines, which, together with a variety of other inflammatory molecules, act to recruit neutrophils. These leukocytes kill microbes by producing highly toxic and unstable oxygen derivatives and other bactericidal substances. The initial pro-inflammatory cytokine response includes tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6) and interferon-γ. TNF-α synthesis and release initiates this response, with subsequent production of the other interleukins. To balance the resulting inflammation, anti-inflammatory cytokines, such as interleukin-4 (IL-4) and interleukine-10 (IL-10) are produced. The different cytokines are ascribed various and specific effects such as inducing fever, capillary leak and vasodilation. A cascade of other inflammatory molecules and systems participate in the inflammatory response, leading to the different pathophysiological changes. Such molecules are nitric oxide (NO), bradykinin, products of arachidonic acid metabolism, toxic oxygen free radicals and several of the factors in the coagulation and fibrinolytic systems. This outlined response is part of the innate immune system, acting immediately and rapidly to gain control of the invading microbes. In addition, the adaptive immune system acts through specialized T- and B lymphocytes generating an antigen-specific response developing over days to weeks.

1.1.3.3 Cardiovascular effects

The haemodynamic manifestations of sepsis and septic shock are characterized by initial vasodilation, resulting in reduced systemic vascular resistance (SVR), in the presence of an increased or normal cardiac output (CO). The vasodilation is mediated by NO, a product of endothelium-derived arginine converted by inducible NO synthase. It is manifested as cutaneous vasodilation with concomitant hypotension. CO is initially increased despite depressed myocardial function, induced by the action of several myocardium depressant factors. TNF-α, IL-1β, NO and cytopathic dysoxia among others have been suggested to exert such effects. As the septic shock
progresses, the relative hypovolemia is aggravated by fluid loss from the intravascular space due to increased capillary permeability, augmenting the hypotension. In this later phase of shock, vasoconstriction ensues, with normalized or increased SVR, and CO falls. Abnormal distribution of systemic and microvascular blood flow further limits effective nutritional blood flow. Microvascular changes include both increased capillary leak and formation of microthrombosis due to alterations in the coagulation cascade 19.

1.1.3.4 Organ damage
The haemodynamic and vascular changes, together with the formation of thrombi and local inflammation (neutrophil sequestration) result in impaired oxygen delivery to peripheral organs with ensuing organ damage. The imbalance between cellular oxygen demand and delivery is augmented by the increase in cellular oxygen demand that occurs during sepsis, with resulting anaerobic metabolism and lactate formation. Cytopathic dysoxia, due to changes in the mitochondrial respiratory chain, further impairs cellular metabolism, ultimately causing cell death.

The resulting organ damage may affect several organs, leading to multiple organ failure. The sepsis related acute lung injury (ALI) is characterized by pulmonary hypertension, deteriorated gas exchange with hypoxemia and reduced lung compliance due to oedema and leukocyte infiltration. Hypoperfusion and hypoxia may also affect the renal and splanchnic circulation, leading to acute renal failure, gut ischemia and hepatic failure, respectively 19.

1.2 THE ENDOTHELIN SYSTEM
The endothelins are a family of isopeptides; endothelin-1 (ET-1), endothelin-2 and endothelin-3, first discovered in 1988 20. Extensive research since then has established that the endothelins are involved in many physiological and pathophysiological processes 21.

1.2.1 Biosynthesis, endothelin receptors and signal transduction
The predominant isoform in human tissue and plasma, involved in cardiovascular physiological and pathophysiological processes, is thought to be ET-1. The gene locus of its precursor is situated on chromosome 6 in the human genome 22. ET-1 is a 21-amino acid peptide synthesized from a prepro-peptide of 212 amino acids, that is cleaved by endopeptidases to form the 38 amino acid molecule big ET-1 23. ET-1 is then finally formed following action by membrane bound metalloproteases – ET converting enzymes 24. ET-1 is mainly produced in the vascular endothelial cells and continuously secreted to the abluminal side of the vessel, acting as a paracrine and autocrine mediator to maintain vascular tone 25. ET-1 is also intracellularly stored, to be released in response to stimuli: ET-1, endotoxin 26, cytokines 27, thrombin, growth factors 28, hypoxia 29, free radicals, catecholamines 30, angiotensin II 30 and shear stress 31. Factors that inhibit the release of ET-1 include NO, prostacyclin, atrial natriuretic peptide and heparin 32. ET-1 is not uniquely synthesized in the endothelium, but is also produced in
vascular smooth cells\textsuperscript{33}, mucosal epithelium\textsuperscript{34}, tracheal epithelium\textsuperscript{35}, renal medulla and hepatic sinusoids and Kupffer cells\textsuperscript{36}, among other cell types.

In humans, ET-1 binds to and mediates its effects through at least two different kinds of G-protein coupled receptor; the ET\textsubscript{A} and ET\textsubscript{B} receptors. Both receptor types are found on vascular smooth muscle cells, where they mediate constriction. The ET\textsubscript{B} receptor is also found on endothelial cells\textsuperscript{37}, mediating vasodilation by the release of prostacycline or NO\textsuperscript{38}. The effect on various vascular beds is however not uniform and depends upon the local receptor population. ET\textsubscript{A} receptor activation leads to activation of a phosphoinositide-specific phospholipase C, which in turn results in activation of two second messengers: membrane-bound diacylglycerol and soluble phosphatidylinositol. Signalling continues by a rapid increase in intracellular calcium (Ca\textsuperscript{2+}) from the sarcoplasmic reticulum\textsuperscript{39}, and protein kinase C activation leading to intracellular alkalinization through stimulation of the sodium/hydrogen (Na\textsuperscript{+}/H\textsuperscript{+}) exchanger\textsuperscript{40, 41}. The increased cytosolic Na\textsuperscript{+} contributes to a further rise in cytosolic Ca\textsuperscript{2+}. The rise in intracellular Ca\textsuperscript{2+} results in contraction of the smooth muscle myofilaments. Activation of the ET\textsubscript{B} receptor initiates a similar intracellular signalling pathway as ET\textsubscript{A} receptor stimulation, but in addition, phospholipase A\textsubscript{2} may also be activated. Arachidonic acid is then processed to prostacycline, mediating vasodilation. Following receptor activation, ET receptors are internalized and ET\textsubscript{A} receptors recycled, whilst ET\textsubscript{B} receptors undergo lysozymal degradation\textsuperscript{42}.

Clearance of plasma ET-1 is rapid with a plasma half-life of about 1-2 min\textsuperscript{43}; the pulmonary circulation being the most important elimination site\textsuperscript{44}. Measured plasma levels of ET-1 only estimate the circulating portion of ET-1, and are not representative of all the interstitial and tissue-bound ET-1. However, in conditions with high levels of circulating ET like sepsis, ET-1 has been shown to function in an endocrine fashion\textsuperscript{45}, and plasma levels have been shown to correlate to severity of illness and mortality\textsuperscript{46}.

1.2.2 Endothelin in sepsis and endotoxemia

Sepsis is the pathological condition known to exhibit the highest levels of plasma ET\textsuperscript{47}. The involvement of ET-1 in septic conditions has been demonstrated in humans and several other species\textsuperscript{46, 48-51}, and plasma levels of ET-1 correlate with both morbidity\textsuperscript{46} and mortality in man\textsuperscript{52}.

1.2.2.1 Cardiovascular effects

Both ET\textsubscript{A} and ET\textsubscript{B} receptors are expressed in the human and porcine\textsuperscript{53} heart, mainly ET\textsubscript{A} on the cardiomyocyte and ET\textsubscript{B} on endothelial and fibroblastic cells\textsuperscript{54, 55}. ET receptors are also found in the coronary arteries, the conducting system and on endocardial cells\textsuperscript{54, 56}. Infusion of ET-1 to humans results in cardiovascular changes partly resembling those seen in sepsis\textsuperscript{57}. CO is decreased, with a negative correlation between plasma ET-1 levels and cardiac index (CI)\textsuperscript{46}. ET has been suggested to act as a mediator of myocardial depression in sepsis, but there is conflicting evidence on the effect of ET on contractility. In vitro studies have indicated both positive and negative inotropy in preparations from different species\textsuperscript{58-61}. In human myocardium, an ET\textsubscript{A} mediated, positive inotropic effect has been reported\textsuperscript{62}, supported by the finding of
impaired systolic function after ET$\alpha$ antagonism in healthy humans exposed to an intra-coronary infusion of ET-1$^{63}$. Other in vivo investigations have demonstrated reduced CO$^{64}$ in response to ET-1. Apart from a possible direct effect on contractility, other mechanisms may contribute to an ET-related reduction in CO; increased afterload due to ET$\alpha$-mediated vasoconstriction$^{64}$ and impaired coronary perfusion leading to ischemia$^{65-68}$. The results are also varied regarding the effects of ET-1 on myocardial diastolic relaxation. In vitro, both improved$^{69}$ and impaired relaxation have been reported$^{70}$, whereas in vivo, ET-infusion reduced diastolic relaxation in healthy humans$^{64}$. The discrepancy between in vitro and in vivo results emphasizes the complexity of mechanisms and factors influencing them. Furthermore, in a heart affected by pathological processes the response to ET-1 may be different from that during normal conditions; several studies report improved CO with dual ET antagonism in sepsis$^{71, 72}$ and beneficial effects are seen also in congestive heart failure$^{73, 74}$.

The predominant vascular effect of ET-1 in sepsis is vasoconstriction$^{57, 75}$ in the pulmonary, renal and splanchnic circulation, contributing to the maldistribution of blood flow seen in septic shock. In contrast, skeletal muscle blood flow is less affected$^{75}$. The increased release of ET-1 during sepsis may serve to maintain blood pressure$^{23}$; therefore the systemic vasodilator properties of ET receptor antagonism may be of concern as they could reduce perfusion pressure to vital organs. So far, the results with non-selective ET receptor antagonism and its effects on mean arterial pressure have been contradictory$^{76-79}$.

1.2.2.2 Pulmonary effects
ET-1 can be related to several of the manifestations of sepsis-induced ALI. The main effect on pulmonary vasculature is vasoconstriction, and the late phase of endotoxin-induced pulmonary hypertension has been shown to be ET dependent$^{80}$. Increased microvascular permeability, leading to oedema formation with increased extra-vascular lung water (EVLW) and deteriorated gas exchange is also related to ET-1$^{71, 81}$. In addition, expression of neutrophil adhesion molecules and promotion of leukocyte migration into the alveoli are increased by ET-1$^{82-84}$. Dual ET receptor antagonism has been shown to ameliorate all these effects$^{71}$.

1.2.2.3 Renal effects
In the kidney, ET-1 has been shown to have negative effects on glomerular filtration rate (GFR) and renal blood flow (RBF) with afferent arteriole constriction, but a diuretic effect has also been reported$^{57, 85-87}$. In human sepsis, plasma levels of the ET-1 precursor big ET-1 have been found to inversely correlate with renal function, expressed as creatinine clearance$^{88}$. The experiences with ET antagonism and renal function during endotoxemia are diverse. Treatment with a non-selective ET receptor antagonist in dogs$^{89}$, as well as use of an ET-1 antibody in rats$^{90}$, has shown improved RBF and renal function. In contrast, the dual ET blocker, bosentan, failed to affect renal haemodynamics in porcine endotoxic shock$^{78}$ and in conscious rats subjected to LPS infusion, a selective ET$\alpha$ receptor antagonist had no effect on renal circulation$^{91}$.
1.2.2.4 Hepatic and splanchnic effects
Regulation of the splanchnic and hepatic circulation appears to involve ET. Both ET\textsubscript{A} and ET\textsubscript{B} receptor activation have been reported to mediate vasoconstriction in the hepatic circulation\textsuperscript{92}, but there are varying results regarding the effects of selective versus non-selective antagonism\textsuperscript{93-95}. Infusion of ET-1 into the portal vein markedly reduces sinusoidal blood flow\textsuperscript{96} and ET-1 released from sinusoidal endothelial cells mediates contraction\textsuperscript{97}. Furthermore, several studies of ET antagonism show improved hepatic blood flow and reduced hepatocellular injury during endotoxemia\textsuperscript{87, 93}. Splanchnic vasoconstriction can be induced by ET-1-infusion\textsuperscript{57} and, in a number of studies, ET antagonism has been proven to counteract endotoxin-induced intestinal vascular effects\textsuperscript{78, 98}.

1.2.2.5 Effects on inflammatory response
ET-1 also exerts direct effects on leukocytes, resulting in activation and promotion of endothelial adhesion\textsuperscript{83, 84, 99}. In vitro studies have shown a mutual interaction between the endothelins and the inflammatory cytokines, where for example, ET-1 induces the production of TNF-\textalpha and IL-6\textsuperscript{47, 100, 101}, whilst TNF-\textalpha and IL-6 in turn increase both gene expression, as well as ET production\textsuperscript{47, 102}. In vitro and in vivo experiments indicate that ET-1-induced TNF-\textalpha and IL-6 production is ET\textsubscript{A} receptor mediated, as selective ET\textsubscript{A} but not ET\textsubscript{B} antagonism will inhibit their release\textsuperscript{101, 103}.

1.3 FLUID TREATMENT IN SEPSIS
1.3.1 General aspects and “early goal directed therapy”
Fluid resuscitation is one of the cornerstones of initial supportive treatment in sepsis, as the septic condition results in intravascular hypovolemia due to vasodilation (venous capacitance) and fluid shifts from the intravascular compartment due to increased vascular permeability\textsuperscript{14, 104}. The importance of early administration of fluids in the course of sepsis, in order to reach certain physiological goals, has also been emphasized\textsuperscript{105}. The debate on which fluid to use for resuscitation is still ongoing, and both crystalloid and colloid solutions are advocated\textsuperscript{106-109}.

1.3.2 Crystalloids versus colloids
A crystalloid is a solution of small, ionic or nonionic particles with low molecular weight (MW< 30 kD). The colloid osmotic pressure is zero, and the solution will freely pass across the vascular membrane and within the extracellular space. Isotonic solutions will distribute throughout the extracellular fluid space, making the effect of fluid resuscitation with isotonic solutions transient, as the solution shifts from the intravascular space. Crystalloid fluid therapy will therefore require larger volumes of replacement fluid. This brings the risk of accumulation of excess fluid in interstitial tissues, resulting in oedema. Examples are isotonic 0.9% saline solution, Ringer’s lactate and Ringer’s acetate\textsuperscript{110}.

A colloid is a high molecular weight substance that largely remains in the intravascular compartment, thereby contributing to the oncotic pressure, leading to plasma volume expansion. The degree of volume expansion is mainly determined by the molecular
weight of the colloid substance, making colloids more effective volume expanders compared to the same volume of a crystalloid. The intravascular persistence of the colloid and its volume expanding effect depends on the elimination of the colloid substance. There are both natural (albumin and plasma) and artificial (dextran, starch and gelatin) forms of colloid. The volume expanding properties vary between <100% for 4% albumin and 3.5% gelatin to 300-500% of the infused volume for 20% albumin. The initial volume expanding effect of 6% dextran 70 is approximately 140%. The hetastarches expand plasma by 100-140% of the infused volume, depending on the molecular weight. The duration of volume expansion ranges from ≤4-6 hours for gelatin and 10% dextran 40, to ≤24 hours for albumin and 6% dextran 70. Reported adverse effects of colloids are alteration of coagulation (dextrans and starches), affected renal function (dextrans, starches and albumin) and allergic or anaphylactoid reactions.

Systematic reviews have not been able to find evidence that fluid resuscitation with either crystalloids or colloids in general is preferable in terms of affecting mortality in the critically ill.

1.3.3 Hypertonic solutions

1.3.3.1 Small volume resuscitation

The concept of small-volume resuscitation refers to infusion of a hypertonic solution, most often 7.2-7.5% NaCl /colloid, in order to rapidly increase intravascular volume. This treatment modality has been advocated and used for almost two decades, mostly in experimental haemorrhage, trauma care and in war scenarios. In sepsis and endotoxemia, the studies and use are more limited. The rationale and advantage include the possibility of restricting the total fluid administration, resulting in reduced oedema formation in the patients, and logistic advantages in prehospital care.

1.3.3.2 Hypertonic saline solutions

Saline solutions of different hyperosmotic concentrations have been evaluated over the years. In the last 20 years, focus has been on the more concentrated hyperosmotic 2400 mOsm L⁻¹ saline solution. The increase in serum osmolality, by approximately 30 to 50 mOsm, generates an osmotic force, which mobilizes fluid from the intracellular compartment through the interstitial space to the vascular compartment. This effect is rapid in onset and maximum volume expansion occurs immediately at the end of infusion of hypertonic saline. Pioneer experimental studies in haemorrhage models reported rapidly restored arterial blood pressure and CO, preventing death with hypertonic saline (HS) resuscitation. The haemodynamic improvements proved to be transient, which incited further experiments with the addition of the colloid dextran to the HS solution, making it hyperoncotic to augment and prolong the intravascular fluid retaining effect. Haemorrhage resuscitation with the 7.5% saline/6% dextran 70 (hypertonic saline/dextran; HSD) solution resulted in higher and more sustained CO and arterial blood pressure than resuscitation with the two separate components alone. Addition of hetastarch (HES) to HS demonstrated similar beneficial haemodynamic effects, although with slightly less volume expansion. The physiological effects of HSD on the heart include increased CO as the result of
increased preload from intravascular volume expansion, reduced afterload through peripheral and pulmonary vasodilation, a positive chronotropic effect and possibly improved contractility. In the peripheral circulation, HSD reduces vascular resistance through arteriolar vasodilation caused by hyperosmolarity-induced smooth muscle relaxation. Reduction in blood viscosity associated with hemodilution may also contribute to the decreased peripheral vascular resistance. Capillary perfusion can be further improved by reduced shock-induced endothelial cell oedema and reduced leukocyte rolling and sticking in the microcirculation; the latter being possibly a dextran mediated effect. Other beneficial effects of HS resuscitation are: reduced pulmonary oedema and decreased lung injury, demonstrated in haemorrhage models. Improvements in renal function have been reported after HS infusion in animal shock models, probably due to increased RBF and improved systemic haemodynamics. Increased diuresis due to natriuresis is another feature of HS treatment.

In addition to the short term effects of HS, with or without colloid, on the cardiovascular system, anti-inflammatory effects and immunomodulation have also been demonstrated. Neutrophil activation, adhesion and signaling are modified and the production and release of pro-inflammatory cytokines reduced. Furthermore, HS has been shown to prevent the immunosuppression following haemorrhage, probably by decreasing the IL-4 and prostaglandin E2 effect on lymphocytes. All these effects may be of potential relevance and importance for the management of sepsis.

The experiences with HS and HSD in sepsis and endotoxemia are more limited. Experimental studies have demonstrated transiently improved systemic circulation with improved cardiac performance, and improved regional blood flow in the renal and splanchnic circulation after resuscitation with HS with or without colloid. In patients, a few studies have shown transiently increased oxygen transport, CO and pulmonary capillary wedge pressure with HS or HSD treatment during stabilized hyperdynamic severe sepsis or septic shock. Previous experiences with HS or HSD and cytokine response after sole endotoxin exposure are limited to a few studies of LPS-exposure in vitro. In these studies, TNF-α expression is suppressed by HSD.

1.4 ANIMAL MODELS OF SEPSIS AND ENDOTOXEMIA

1.4.1 General considerations

Experimental animal models have been widely used in sepsis research. Valuable preclinical information on mechanisms and preliminary effects comes from such studies. The results from different animal models should be combined to provide an overall assessment. However, although the physiology of humans and animals are similar, some considerations should be made in the interpretation and application of results from animals to the human situation. In general, animal studies are conducted in young, healthy and homogenous subjects, with efforts made to keep the study conditions as uniform as possible. In contrast, the ICU patients are heterogeneous with varying age, often elderly and with concomitant systemic disease. The time course also differs between animal studies and the clinical setting. With some exceptions, most animal models study the early phases of sepsis/endotoxemia over a few hours or 1
to 2 days at the most, whilst in the patients the pathophysiology and ultimately mortality, develops over a longer period of time. The welfare of the animals often calls for the use of anaesthesia, which may affect the physiological response to endotoxemia. The species of the laboratory animal in use also has an impact on the interpretation and applicability of results. Porcine models are favorable in studies of haemodynamic effects, as the circulatory system of the pig greatly resembles that of humans. Finally, in the care of the ICU patient, sepsis treatment consists of various organ supportive measures, that are not always included in the experimental animal studies.

1.4.2 Sepsis models

Induction of experimental sepsis or endotoxemia may be accomplished in several different ways. These include parenteral administration of endotoxin or live bacteria and peritonitis by cecal ligation and perforation or implantation of bacteria. Endotoxin administration to humans or animals induces many features of sepsis such as fever, haemodynamic changes, renal dysfunction, cytokine release and coagulopathy. The advantages with endotoxin models are that the experiments are relatively easy to perform and offer a reproducible tool to study changes and mechanisms in the early phase of sepsis. However, while the human cardiovascular response to endotoxin is initially hyperdynamic, the experimental porcine response is typically characterized by a hypodynamic circulation during the first hours. A later hyperdynamic period may be seen after eight to twelve hours. Models using low, sublethal doses of endotoxin in continuous infusion together with aggressive fluid resuscitation have been able to resemble the human hyperdynamic response. In models using high bolus doses of endotoxin or live bacteria the response is often severely hypodynamic, resulting in high mortality rates and short term observations. Peritonitis models, where bacteremia is induced either by cecal perforation or direct intraperitoneal implantation, produce a response that gradually develops into a sepsis syndrome. Both hyperdynamic and hypodynamic circulation have been described. In a comparison between endotoxin and peritonitis models, the former resulted in a more severe condition with higher cytokine levels, whilst the latter demonstrated a higher resemblance to human sepsis. Mortality rates were similar.

1.5 RATIONALE FOR THE CURRENT STUDIES

1.5.1 Hypertonic saline/dextran

The inherent hypertonic and hyperoncotic properties of HSD make it potentially beneficial to use for resuscitation in septic shock. The favourable haemodynamic response seen in haemorrhagic shock, as well as the reported immunomodulatory effects could be applicable to septic conditions. So far, positive systemic haemodynamic effects are indicated, but studies regarding effects on vital organ circulation are limited. Therefore, we wanted to further evaluate circulatory effects of early resuscitation with HSD in porcine endotoxin shock, with focus on regional blood flow. In order to further clarify the potential role of HSD as a modulator of inflammatory and immune responses during endotoxemia, the influence of HSD on early pro-inflammatory cytokine activation was investigated in an in vivo endotoxicosis model.
1.5.2 Tezosentan

To promote nutritional blood flow in vital organs different measures may be used. Apart from optimizing the systemic circulation with fluid resuscitation and vasopressors or inotropic agents to secure perfusion pressure, regional vasodilation may offer another option to improve organ tissue oxygenation during septic shock. Previous investigations with the ET antagonists have indicated beneficial effects on both cardiac function and regional hypoperfusion. Using the novel dual ET receptor antagonist tezosentan, effects and mechanisms of ET antagonism during endotoxemia were further investigated, with focus on systemic and regional haemodynamics. The influence of non-selective ET antagonism on in vivo early pro-inflammatory responses during endotoxin administration is still to be determined. The present thesis therefore studied the TNF-α and IL-6 pattern during tezosentan intervention.

1.5.3 Tezosentan and hypertonic saline/dextran combined

The combination of tezosentan and HSD has not been previously investigated. The rationale for using HSD together with tezosentan was that the combination may potentially enhance the beneficial haemodynamic effects of the individual interventions. An additive effect on both cardiac performance and microvascular blood flow may be hypothesized. When using vasodilation as a means to promote regional blood flow, there is always a risk of an undesired systemic haemodynamic effects. Therefore, we wanted to investigate whether HSD could counteract the reduction in MAP reported with ET antagonism.

In theory, the reported immune/inflammatory modulating abilities of ET antagonism and HSD respectively, may act in concert if they are combined. The possible positive interaction was investigated with regard to the early pro-inflammatory cytokine response.
2 AIMS

The aims of this thesis were to investigate haemodynamic and inflammatory effects of fluid resuscitation with hypertonic saline/dextran and dual endothelin receptor antagonism, in a porcine model of early-phase endotoxin-induced shock.

The specific aims were:

• To further evaluate systemic and regional haemodynamic effects of early fluid resuscitation with hypertonic saline/dextran in experimental endotoxin shock.

• To further evaluate whether additional treatment with hypertonic saline/dextran affects early survival in endotoxin shock.

• To investigate effects of hypertonic saline/dextran on pro-inflammatory cytokine response, endothelin-1 levels, levels of exhaled nitric oxide and nitric oxide metabolites.

• To investigate if hypertonic saline/dextran affects endotoxin-induced lung injury in terms of gas exchange or lung mechanics.

• To study cardio-pulmonary effects of the dual endothelin receptor antagonist tezosentan alone and combined with hypertonic saline/dextran.

• To study effects on regional blood flow and oxygenation of tezosentan alone and in combination with hypertonic saline/dextran.

• To evaluate if dual endothelin receptor antagonism with tezosentan may exert anti-inflammatory effects through modulation of early pro-inflammatory cytokine response.

• To investigate whether combined tezosentan and hypertonic saline/dextran modulate early pro-inflammatory TNF-α and IL-6 response.
3 MATERIAL AND METHODS

3.1 THE PORCINE MODEL

Landrace pigs of 20-25 kg were used in these experiments. A porcine model was preferred because of the great resemblance to humans in terms of anatomy, cardiovascular physiology, ET system and inflammatory response.\(^{48, 53, 168}\)

3.2 ANAESTHESIA AND SURGICAL PREPARATION

All animals arrived at the animal stables of the research facilities in good time prior to the experiment in order to acclimatize. They were fasted overnight with free access to water before the experiment. Anaesthesia was initiated by intramuscular premedication with ketamine 20 mg kg\(^{-1}\) and atropine 20-30 µg kg\(^{-1}\) intravenously. An intravenous cannula was placed in a vein on the ear, and anaesthesia was induced by pentobarbital 6 mg kg\(^{-1}\) h\(^{-1}\) and fentanyl 5 µg kg\(^{-1}\) h\(^{-1}\) throughout the experiment. Additional doses of fentanyl (5-10 µg kg\(^{-1}\)) were administered before starting laparotomy and when needed during the procedure. Muscle paralysis, to prevent shivering, was achieved by a continuous infusion of pancuronium 0.5 mg kg\(^{-1}\) h\(^{-1}\). Prior to the administration of muscle relaxant, anaesthetic depth was evaluated by pain stimuli to the hoof. The animals received an infusion of Ringer’s glucose 2.5% (Na\(^+\) 73.5 mmol L\(^{-1}\), K\(^+\) 2.0 mmol L\(^{-1}\), Ca\(^{2+}\) mmol L\(^{-1}\), and Cl\(^{-}\) 77.8 mmol L\(^{-1}\); Pharmalink, Stockholm, Sweden) at a rate of 20 mL kg\(^{-1}\) h\(^{-1}\) throughout the experiment, including the periods of surgical preparation and rest. The animals were tracheotomized and mechanically normoventilated\(^{169}\) with a gas mixture of oxygen in air (FiO\(_2\) 0.3) and with a positive end expiratory pressure (PEEP) of 4 cm H\(_2\)O. The ventilator settings were adjusted according to the baseline level of arterial partial pressure of carbon dioxide (PaCO\(_2\)), and kept the same throughout the experiment. The body temperature was maintained at 37-39º C using blankets, a heated table and lamps.

The left external jugular vein was cannulated for intravenous access and sampling, and through the right external jugular vein a pulmonary artery catheter (OPTICATH Flow Directed Thermodilution Fiberoptic Pulmonary Artery Catheter Model P575, ABBOTT Critical Care Systems, North Chicago, IL, USA) was introduced, and by pressure guidance positioned in the pulmonary artery. In the right common carotid artery a cannula was introduced for continuous pressure monitoring and blood sampling, whilst a perivascular ultrasonic flow probe (Transonic System Inc., Ithaca, NY, USA) was positioned around the left common carotid artery for regional blood flow measurement. A midline laparotomy was performed and perivascular ultrasonic flow probes were positioned around the left renal artery and portal vein. A catheter was placed in the urinary bladder through a cystotomy for measurement of urinary output. Finally, the abdomen was closed and the animals turned to the left lateral position for a one hour rest before baseline measurements (Fig. 1).
3.3 HAEMODYNAMIC MEASUREMENTS AND CALCULATIONS

The arterial and pulmonary artery catheters were connected to pressure transducers and an electrocardiogram was applied for heart rate (HR) registration. Mean arterial pressure (MAP), central venous pressure (CVP), diastolic pulmonary artery pressure (PADIAST), mean arterial pulmonary pressure (MPAP) and HR were monitored continuously by a Siemens Sirecust 960 (Siemens Elema AB, Solna, Sweden). CO was measured by thermodilution (Abbott Oximetrix 3 SvO₂/CO computer, Abbott Laboratories, North Chicago, IL, USA) and determined as the mean of either two 5-ml (papers I and II) or three 3-mL (papers III and IV) injections of room-temperature isotonic saline. The mixed venous oxygen saturation (SvO₂) was continuously monitored by the Abbott Oximetric 3 SvO₂/CO computer.

CI, systemic vascular resistance index (SVRI), systemic oxygen delivery (DO₂) and systemic oxygen consumption (VO₂) were calculated according to standard formulas, in detail described in the separate papers (I, III and IV). In paper IV, regional oxygen delivery in the carotid, renal and portal vessels respectively was calculated as: regional blood flow x haemoglobin concentration (Hb) x arterial oxygen saturation x 0.0139/1000. In paper I, the expression “normalized CO (NCO)” is used for CO indexed to body weight, whilst in papers III and IV the term CI is used.
3.4 ENDOTOXIN
Throughout the experimental series *Escherichia coli* LPS (serotype 0111:B4, Sigma-Aldrich, Stockholm, Sweden AB) from the same batch was used in order to elicit responses of comparable magnitude. The endotoxin was dissolved in isotonic saline to a concentration of 50 µg mL\(^{-1}\), and heated to prevent precipitate formation. The intravenous infusion was initiated at a rate of 0.625 µg kg\(^{-1}\) h\(^{-1}\) and increased stepwise to a final rate of 5 µg kg\(^{-1}\) h\(^{-1}\) within 30 min, and continued for three hours in total.

3.5 EXPERIMENTAL PROTOCOL
After surgery, one hour of stabilization preceded baseline measurements. Thereafter, the administration of endotoxin was initiated (0 min). One hour after start of endotoxin infusion (60 min), immediately before commencing intervention, the animals were randomised to control or treatment groups, each with eight animals. Intervention was executed as described below (Intervention paragraph) and in the separate papers. The animals were observed for two more hours after the termination of LPS-infusion until 300 min (Fig. 2).

Systemic and regional haemodynamic parameters were recorded continuously and data were collected for analysis every 30 min. Airway pressures and gas volumes were measured by transducers in the ventilator and recorded every half-hour. Urinary output was recorded every 30 min. Blood samples were taken every 30 min for analysis of blood gases, Hb and lactate. Plasma for ET-1, cytokine IL-6 and TNF-α, plasma transaminase activity, creatinine and urea analyses were obtained at baseline (0 min) and hourly thereafter. The maximum sample volume in each experiment was 115 mL. This volume was compensated for with the same volume of isotonic saline solution.

After 300 min the experiments were terminated and the animals still alive received a lethal dose of pentobarbital with potassium.

Fig. 2

![Figure 2 Experimental protocol.](image-url)
3.6 INTERVENTIONS

3.6.1 Hypertonic saline/dextran

HSD solution containing 7.5% saline (NaCl) with 6% dextran 70 (Resqueflow®; Biophausia AB/Pharmalink, Stockholm, Sweden) was administered as a bolus infusion over 5 min at a dose of 4 mL kg\(^{-1}\) (papers I-IV). The same regimen was used both when HSD was given alone and when given in combination with tezosentan (papers III-IV).

3.6.2 Tezosentan

The dual ET receptor antagonist tezosentan (Ro 61-0612) \[5\text{-isopropyl-pyridine-2-sulfonic acid 6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2-(2-1H-tetrazol-5-yl-pyridin-4-yl)-pyrimidin-4-ylamide}\] (Fig. 3) was used in papers III and IV. Tezosentan is a highly specific competitive antagonist to the ET\(_A\) and ET\(_B\) receptors, with 30–fold higher affinity for the ET\(_A\) receptor. It is water soluble, specifically designed for intravenous use, relatively short-acting and has no known active metabolites\(^{170}\). The distribution half-life of tezosentan is 0.1 hours and elimination half-life of three hours in humans\(^{171}\). In paper III and IV, tezosentan administration was initiated one hour after onset of endotoxemia, with an initial bolus dose of 10 mg kg\(^{-1}\), followed by a continuous infusion of 5 mg kg\(^{-1}\) h\(^{-1}\) for the remaining time of the experiment.

Fig. 3

![Chemical structure of tezosentan.](image)

**Figure 3** Chemical structure of tezosentan.

3.6.3 Control group

Isotonic saline was administered as a bolus infusion over 5 min at a dose of 4 ml kg\(^{-1}\) to subjects in the control groups, in order to provide the same volume of fluid as the HSD-treated animals (papers I-IV).
3.7 ULTRASONIC BLOOD FLOW MEASUREMENTS
The perivascular flow probes (Transonic System Inc., Ithaca, NY, USA) measure volume flow by an ultrasonic technique. A wide ultrasonic beam is transmitted between two transducers via a reflector and the velocity of the blood flow is calculated as the difference between upstream and downstream transit times. The velocities from the vessels full cross-sectional area are integrated and this yields the volume flow. Since the transit time is sampled at all points across the vessel diameter, volume flow measurement is independent of the flow velocity profile. Ultrasonic beams that do not intersect the vessel are excluded from the volume flow integral. The ultrasonic flow probes were connected to two Transonic T206/T208 monitors (Transonic System, New York, N.Y., USA) for continuous recording of regional blood flow presented as mL min⁻¹.

3.8 EXHALED NO
Exhaled air was analyzed for NO continuously by a chemiluminescence technique (Lear Siegler Measurement Controls Corp. Model 8841). Gas was sampled from the expiratory gas outlet of the ventilator at a rate of 500 mL min⁻¹ passing through a gas mixing box first to ensure even distribution of contained gases. A 100% oxygen and a gas mixture containing 100 ppb NO (AGA AB, Stockholm, Sweden) was used to calibrate the analyser. The detection limit of the analyser was 0.5 ppb according to the manufacturers’ manual. The response time of the NO device was less than 30 s.

3.9 BIOCHEMICAL ANALYSES
3.9.1 Blood gas and lactate analyses
Blood gases were analysed using a GEM Premier Plus blood gas analyser (Instrumentation Laboratory, Lexington, MA, USA) (papers I-II) or an ABL 70 (Triolab AB, Stockholm, Sweden) (papers III-IV). Lactate levels in whole blood were measured using a Dr Lange Miniphotometer 8 (Dr Bruno Lange GmbH, Berlin, Germany). The precision of the lactate analysis method is reported to be ± 6-8%, with most of the variation in a higher range of values than those represented in the current studies (personal communication (papers I and IV).

3.9.2 NO-derived metabolites
The NO metabolites, nitrite (NO₂) and nitrate (NO₃) were analyzed by the Griess method (paper II).

3.9.3 Endothelin-1
Plasma ET-1-like immunoreactivity (ET-1-LI) was analysed with radioimmunoassay as previously described (papers II and IV).
3.9.4 Cytokine analyses
In papers II and IV, TNF-α and IL-6 were assessed using commercially available porcine ELISA-kits: PTA00 for TNF-α and P6000 for IL-6 (R&D Systems, Minneapolis, MN, USA).

3.9.5 Organ specific markers

3.9.5.1 Aspartate aminotransferase and alanine aminotransferase
Plasma aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were analysed in paper IV as markers of hepatic cellular injury. A Synchron LX multianalyser (Beckman Coulter AB) used an enzymatic rate method to analyse ASAT and a kinetic rate method for the ALAT analysis.

3.9.5.2 Creatinine and urea
Plasma creatinine and urea were analysed in paper IV to assess renal function. A Synchron LX multianalyser (Beckman Coulter AB) analysed creatinine by the Jaffé rate method and urea by an enzymatic rate method (paper IV).

3.10 STATISTICS
Data are presented as mean ± standard error of the mean (SEM) in paper I-II, as mean ± standard deviation (SD) in paper III and as mean (SD) in paper IV.

In papers I-II analysis of variance (ANOVA) for repeated measures was used for comparison between groups and for analysis of changes over time. The time intervals used in the analyses were from 0 to 60 min (before intervention) and from 60 to 150 (paper I) or 180 min (paper II). The 150/180 min time-point was chosen as the endpoint for statistical evaluation due to increasing mortality rate in the control group after that time. Post-hoc Bonferroni correction was performed when appropriate. In papers I-II the computer software program Statistica® (Statsoft Scandinavia, Uppsala, Sweden) was used for statistical calculations.

In papers III and IV linear mixed-effects model, including time as a covariate, was used to analyse the longitudinal change of mean differences between treatments with regard to the different outcomes. The interaction between trend and treatment was also determined. Within the model, planned comparisons between treatments were made at 60 min (before intervention) and 150 min (at the last time point where all groups were sufficiently represented in terms of survivors), in order to determine any differences. All pair-wise tests were adjusted with Bonferroni correction. Since values after 150 min were not included in the mixed model due to considerable mortality in the control group after that time-point, a comparison between the three treatment groups was made at 300 min with a one-way ANOVA and Bonferroni correction (paper III) or with a separate linear mixed-effect model including a planned comparison at 300 min (paper IV).

In papers III-IV all analyses were performed with SAS 9.1.3 (SAS institute, Inc., Cary, North Carolina).
In paper I, survival was illustrated with a Kaplan-Meier graph. In paper III, the time of death of the animals was analysed by Cox’s regression model in order to evaluate if the likelihood of death differed among the treatment groups. The proportional hazard assumption was tested using time by treatment as an effect. No violation of the assumption could be found.

The pooled data from the two series was analysed with repeated measures ANOVA for comparison between groups and changes over time (0-150 min). Planned comparisons were used as post-hoc analysis when appropriate. In the graphs (Fig. 4, 6-7), values are means with 95% confidence intervals. The difference in survival rates was evaluated with the log-rank test and illustrated with a Kaplan-Meier graph.

In all papers a p-value < 0.05 was considered to indicate a significant result.
4 RESULTS

The results are presented as an overview, as detailed results are presented in the separate papers.

4.1 SHAM ANIMALS

The two sham animals survived the whole observation period. After the resting period, MAP was maintained at a stable level throughout the experiment, while CO decreased during the first hour after baseline measurements, but then stabilized. A persistent tachycardia was seen over the observation period. SvO₂ was reduced due to the decrease in CO, and a metabolic acidosis developed during the last hour of observation. Gas exchange remained unaffected. Regional blood flow was preserved in the renal and carotid arteries, whilst portal flow markedly decreased during the first three hours of the experiment. Urinary output was maintained over the entire experiment, ranging from 0.6 to 1.5 mL kg⁻¹ h⁻¹ (paper I).

4.2 EFFECTS OF ENDOTOXIN EXPOSURE

4.2.1 Survival

In the control groups endotoxin exposure resulted in reduced survival; with 3/8 (paper I) and 2/8 (paper III) animals surviving the whole experimental period.

4.2.2 Haemodynamics and urinary output

Endotoxin administration resulted in a progressive circulatory shock response with increased HR and reduced CI and MAP. Concomitantly, systemic and regional DO₂, SvO₂ and regional blood flow decreased. In paper I, a temporary reduction in VO₂ was observed after 120 min of endotoxemia, with recovery within the following two hours in the remaining animals, while in paper IV, VO₂ was unaffected. There was a transient increase in SVRI over the first 60 min of endotoxin infusion, followed by a decrease to (paper I) or below (paper III) baseline level. CVP was unaffected by endotoxemia. In the pulmonary circulation, endotoxin exposure rendered a transient rise in MPAP, which peaked at 60 min and returned to baseline level at 150 min.

Urinary output was approximately 1 mL kg⁻¹ h⁻¹ during the first 120 min of endotoxemia, and then markedly decreased in the remaining animals (papers I and IV).

4.2.3 Lung mechanics and gas exchange

In the lung, compliance decreased in response to endotoxin exposure, with the most pronounced effect during the first 120 min (paper II). Simultaneously, the peak airway pressure increased over time (paper II). Gas exchange was impaired after endotoxin administration with a reduction in PaO₂ and increased PaCO₂ compared to baseline (papers I-III).
**4.2.4 Biochemical analyses**

Endotoxin administration induced an initial haemoconcentration with a rise in Hb and haematocrit. The circulatory changes were accompanied by the development of a metabolic acidosis with reduced pH and base excess (BE) (papers I and III), but there were only discrete changes in lactate levels (papers I and IV).

In response to endotoxin, ET-1-LI increased progressively (0-120 min p<0.01) (papers II-IV) (Fig. 4a). TNF-α levels transiently increased several-fold, with peak values at 120 min (p<0.01) (Fig. 4b), while IL-6 levels increased gradually over the observation period (0-120 min p<0.01) (papers II and IV) (Fig. 4c).

**Figure 4 a)** Endothelin-1-like immunoreactivity (ET-1-LI), **b)** TNF-α, and **c)** IL-6 during endotoxin exposure in control and HSD treated animals from the two series of experiments. The pooled number of animals (n) was 16 in each group at the start of the experiment. In the control group, n was progressively reduced after 150 min, with 5 animals left at 300 min.
There were no changes in exhaled NO after endotoxin administration, but a small decrease in both NO\textsubscript{2} and NO\textsubscript{3} was observed over time.

Discrete changes in liver transaminases were induced by endotoxemia, with slightly increased plasma ASAT and a small decrease in ALAT (paper IV). The plasma creatinine increased by approximately 20%, suggesting reduced renal function, however the urea was essentially unaffected (paper IV).

### 4.3 EFFECTS OF HYPERTONIC SALINE/DEXTRAN

#### 4.3.1 Survival

In the first series (papers I and II), HSD reduced mortality to zero, and in the second study (paper III) the survival rate was 7/8. Fig. 5 illustrates survival rate when data from the two studies are combined; 15/16 with HSD treatment as compared to 5/16 in the control group (p<0.01).

**Figure 5** A Kaplan-Meier graph illustrating pooled survival rates during endotoxemia in control and HSD treated animals from the two series of experiments.

#### 4.3.2 Haemodynamics

HSD treatment rendered a transient circulatory recovery with increased CI (Fig. 6a), MAP (Fig. 6b), DO\textsubscript{2} and SvO\textsubscript{2} and concomitantly decreased HR. Renal and carotid blood flow increased, whereas portal flow (Fig. 6c) was unaffected (papers I and III). The effect on RBF could not be reproduced in paper IV, but there was a significant increase in RBF when data from both series were pooled (Fig. 6d). The effect was most pronounced in the first 30 min, and better than controls after 90 min, but then subsided. HSD intervention had no effect on CVP, MPAP or SVRI. HSD temporarily increased urinary output, the effect lasting for about 1h. These results were seen in both series (papers I and III-IV) and were confirmed when all the collected data were analysed together.
Figure 6 a) Cardiac index (CI), b) mean arterial pressure (MAP), c) portal blood flow and d) renal blood flow during endotoxin exposure in control and HSD treated animals from the two series of experiments. The pooled number of animals (n) was 16 in each group at the start of the experiment. In the control group, n was progressively reduced after 150 min, with 5 animals left at 300 min. *p<0.05, **p<0.01 in post-hoc analysis of differences between groups.
4.3.3 Lung mechanics and gas exchange

Intervention with HSD did not affect pulmonary compliance or peak airway pressures (paper II). The effect of HSD on PaO_2 differed in the two series; no effect was observed in the first series (paper I), whereas improved PaO_2 was seen in the second (paper III). Combined data support improved arterial oxygenation with HSD compared to the control group (p<0.01) (Fig. 7). PaCO_2 remained unaffected (papers I and III).

![Figure 7](image)

**Figure 7** Arterial partial pressure of oxygen (PaO_2) during endotoxin exposure in control and HSD treated animals from the two series of experiments. The pooled number of animals (n) was 16 in each group at the start of the experiment. In the control group, n was progressively reduced after 150 min, with 5 animals left at 300 min. *=p<0.05, **=p<0.01 significant difference in post-hoc analysis of differences between groups.

4.3.4 Biochemical analyses

A hemodiluting effect of HSD treatment was indicated in the two separate studies (papers I and III), and confirmed with an analysis of the combined results. The endotoxin-induced decrease in pH and BE was reduced after HSD, and the metabolic acidosis delayed (papers I and III). No effect on lactate levels was observed (papers I and IV). Intervention with HSD did not affect the levels of ET-1-LI, TNF-α, IL-6 (0-120 min p<0.01) (papers II and IV) (Fig. 4), exhaled NO or NO-metabolites (paper II) compared to control group animals. ASAT, ALAT, creatinine or urea were also not affected.
4.4 EFFECTS OF TEZOSENTAN

4.4.1 Survival

In the animals treated with tezosentan, the survival rate was 6/8 at the end of the experiment, resembling the outcome of the HSD-treatment animals in the same series (paper II).

4.4.2 Haemodynamics and urinary output

Intervention with tezosentan prevented the endotoxin-induced reduction in CI (Fig. 8a), without affecting CVP or the gradual increase in HR. Systemic DO₂ and SvO₂ were also restored to baseline level, but VO₂ remained unchanged (papers III and IV). Tezosentan increased portal vein blood flow and portal DO₂, over time restoring them to baseline levels, whereas no effect was seen on the renal or carotid circulation (paper IV). SVRI was markedly reduced by tezosentan and the endotoxin-induced rise in MPAP was reversed (paper III). MAP decreased to the same extent as with LPS exposure alone (controls) (Fig. 8b). Treatment with tezosentan did not improve diuresis.

![Figure 8a](image)

**Figure 8** Fitted polynomial trend over time, predicted by mixed model analysis, of: a) cardiac index (CI) and b) mean arterial pressure (MAP) during endotoxin exposure in control, tezosentan, HSD and tezosentan/HSD treated animals. *=p<0.05, **=p<0.01 significant difference between trends compared to control group.
4.4.3 Gas exchange
Tezosentan improved PaO$_2$ compared to controls, while PaCO$_2$ remained unaffected.

4.4.4 Biochemical analyses
The endotoxin-induced rise in Hb and haematocrit was reversed by tezosentan and the development of metabolic acidosis was prevented (paper III). ET-1 antagonism with tezosentan resulted in a pronounced increase in ET-1-LI. No effect was seen on TNF-α levels, but the IL-6 response was augmented by about 50% compared to controls after 180 min (p<0.05) (paper IV). Tezosentan did not affect the small endotoxin-induced changes in liver transaminases nor creatinine/urea (paper IV).

4.5 EFFECTS OF TEZOSENTAN COMBINED WITH HYPERTONIC SALINE/DEXTRAN

4.5.1 Survival
In the animals treated with tezosentan/HSD, the survival rate was 6/8 at the end of the experiment.

4.5.2 Haemodynamics and urinary output
Tezosentan/HSD markedly improved CI compared with the control group and with HSD intervention, the effect lasting throughout the observation period (Fig. 8a). As with HSD or tezosentan intervention alone, there was no effect on HR or CVP with the combination (paper III). Systemic DO$_2$ increased to baseline level, with unchanged VO$_2$ (paper IV). The endotoxin-induced reduction in SvO$_2$ was completely prevented by tezosentan/HSD (paper IV). Both portal and carotid blood flow increased markedly to a supra-baseline level, with a concomitant increase in regional DO$_2$ (Fig. 9a). In the renal circulation, there was a similar trend, but it was not statistically significant (Fig. 9b).

SVRI was markedly reduced by tezosentan/HSD as well, whereas the reduction in MPAP failed to be statistically significant compared to the control group (paper III). The tezosentan/HSD group had a higher MAP, although the difference was not statistically significant when compared to the control and tezosentan groups (Fig. 8b). There was a tendency for higher urinary output compared to the control and tezosentan groups (paper IV).
4.5.3 Gas exchange

The combination tezosentan/HSD also improved PaO\textsubscript{2} compared to controls, but was without effect on PaCO\textsubscript{2} (paper III).

4.5.4 Biochemical analyses

The effects on Hb, haematocrit, metabolic acidosis, ET-1-LI and TNF-\alpha were the same for the combination of tezosentan/HSD as for tezosentan alone. The IL-6 response had the same pattern as with tezosentan alone, but failed to reach statistical significance compared to control animals (paper IV).
5 DISCUSSION

5.1 THE ENDOTOXIN MODEL

The experimental endotoxemia model should not be considered as a direct correlate to clinical sepsis. It should rather be viewed as a tool to study, in a reproducible fashion, mechanisms and effects of interventions in the pathophysiology of sepsis. As endotoxin is an important inducer of the septic response, its use in sepsis models can be advocated as a reasonable paradigm for septic syndromes.

An established endotoxin model used in similar studies was adapted to our laboratory facilities. We used a low-dose, continuous infusion of LPS in order to achieve a persistent physiologic response rather than an acute profound reaction. Still, the endotoxin administration resulted in a severe hypodynamic shock response with considerable mortality in the control groups. A hypodynamic response is expected in this kind of model, limiting the direct resemblance to the human hyperdynamic situation. Apart from the direct cardiovascular effects of the LPS, anaesthesia and possibly hypovolemia may have contributed to the hypodynamic state. The use of anaesthetized animals was necessary due to ethical considerations regarding animal welfare. Pentobarbital was used in the same dose as in previous investigations in similar models. However, barbiturates are well-known to exert myocardial depression and may induce hypotension. In the two sham animals, subjected to the same anaesthesia regimen and surgical preparation as animals in the other groups, a reduced CO was noted, but MAP was adequate. The cause of the reduced CO in these animals may be barbiturate-induced myocardial depression. Some degree of hypovolemia cannot be excluded, which could be a possible explanation to the observed tachycardia and relatively low CVP (paper I).

In order to convert a hypodynamic cardiovascular response to a hyperdynamic, some endotoxin models have used aggressive fluid resuscitation, either with a fixed volume kg$^{-1}$ h$^{-1}$ or according to certain haemodynamic goals. Both of these strategies present difficulties; a high continuous fluid administration over the few hours of experimentation would correspond to extreme amounts of fluid in the human clinical situation. For example, 70 mL kg$^{-1}$ h$^{-1}$ during 5 hours in a 70 kg man results in 24.5 L of fluid. When, on the other hand, fluid is administered to achieve predefined haemodynamic endpoints, this may interfere, or at least complicate interpretation of results if the same parameters are influenced by the intervention to be investigated.

In the current studies, a continuous fluid resuscitation of 20 mL kg$^{-1}$ h$^{-1}$ was used, corresponding to 1400 mL per hour in a 70 kg human. As discussed above, some degree of hypovolemia cannot be excluded, but the fact that vasodilatation with tezosentan was associated with increased CO suggests adequate vascular filling (paper III). In addition, apart from the initial (0-90 min) increase in response to endotoxin, Hb and haematocrit did not increase progressively in control animals, precluding ongoing severe absolute hypovolemia (papers I and III). Moreover, in a few pilot experiments a fluid resuscitation of 40 mL kg$^{-1}$ h$^{-1}$ was tested, which resulted in acute pulmonary oedema within one hour.
The endotoxin exposure induced a shock response with reduced CO, MAP and regional blood flow within 1.5 hours. The development of a metabolic acidosis and decreased SvO\textsubscript{2} indicate impaired tissue oxygenation, even though lactate levels were only slightly elevated (papers I and IV). The fact that VO\textsubscript{2}, apart from small changes seen in paper I, remained unaffected suggests that the shock was not "delivery dependent". Despite the severe circulatory impairment, relatively small changes were observed in the gross organ damage markers (ASAT, ALAT, creatinine and urea) (paper IV). This may be explained by the short-term observation, as organ failure develops over a longer period of time. The severe haemodynamic effects resulted in considerable mortality in the untreated control groups. The short-term observation and the high control group mortality suggest that the model is suitable for investigating changes early in the course of endotoxemia.

5.2 HYPTERTONIC SOLUTIONS IN ENDOTOXEMIA

The importance of fluid resuscitation in early sepsis has been emphasized in recent years\textsuperscript{105}. The choice of fluid is still under debate, and either crystalloids or colloids may be preferred\textsuperscript{180, 181}. We used HSD at a dose of 4 mL kg\textsuperscript{-1}, as this dosage has been proven safe and effective in experimental haemorrhage\textsuperscript{182}. In patients the recommended dose in haemorrhage and trauma care is 250 mL\textsuperscript{183}.

5.2.1 Systemic haemodynamics

In the current studies, HSD transiently improved the endotoxin-induced reduction in CI, DO\textsubscript{2} and MAP. HSD improves CO by increased preload and decreased afterload due to reductions in peripheral and pulmonary vascular resistance. In addition, improved contractility in response to the hypertonic solution may contribute, although this effect is disputed\textsuperscript{155, 184, 185}. We observed an immediate reduction in Hb (papers I and III) and haematocrit (paper III) indicating volume expansion and increased preload, even though CVP was unaffected. The increase seen in regional blood flow in the renal and carotid circulation suggests vasodilation and afterload reduction. However, this was not supported by reductions in SVRI or MPAP compared to controls (papers I and III). Our findings confirm those of other studies of HS\textsuperscript{151}, HSD\textsuperscript{152-154, 186} or HS combined with HES in experimental endotoxemia, where transient haemodynamic improvement has been demonstrated in different species. Comparable results have been demonstrated in patients, both in clinical studies of HS in hyperdynamic\textsuperscript{156} and HSD in stabilized\textsuperscript{157} sepsis.

5.2.2 Regional blood flow

The effect on regional blood flow differed between the renal and carotid versus the portal circulation. The reason for this is unclear, and other investigators, using both the same and different measuring techniques, have reported increases in renal\textsuperscript{153} and splanchnic/portal\textsuperscript{153, 154} blood flow. The influence of HSD on carotid blood flow has not been previously described. The fact that increased renal flow in response to HSD was observed in the first study (paper I), but not in the second one (paper IV), might be explained by large variability in data as a significant increase was seen when all animals were pooled.
In general, the regional and microcirculatory improvements are thought to be the result of the macrocirculatory changes combined with endothelial oedema reduction and arteriolar vasodilation. Further contributing mechanisms may be reduced blood viscosity, modulated coagulation and less neutrophil adhesion. The microcirculatory effects may be of longer duration than the observed transient systemic changes, indicated by the delay in the development of metabolic acidosis.

5.2.3 Pulmonary function

HSD did not affect the gross signs of endotoxin-induced lung injury, indicated by the fact that compliance and peak airway pressure were similar in the treatment and control groups. The effect on gas exchange was not unambiguous. The improvement in PaO\textsubscript{2} demonstrated in the second study (paper III) could not be seen in the first (papers I and II). The explanation may be the different statistical methods used or variability in data among the small number of animals. In support of the latter, the beneficial effect reappeared when results from the two studies were combined. Previously, only one study of LPS-induced lung injury in cows has reported favourable effect of HS on hypoxia, but this study differed from the present studies in terms of animal species, endotoxin administration and earlier onset of treatment. Other investigations have demonstrated unaffected PaO\textsubscript{2} after HS or HSD treatment, both in porcine endotoxemia and human sepsis. In a canine endotoxin model, the effect of HSD compared to lactated Ringer’s solution on lung water did not differ, nor did HSD affect gas exchange. The combination of HS and hetastarch also failed to improve gas exchange in both canine and equine models of endotoxemia. Possible mechanisms for improved PaO\textsubscript{2} could be increased CO and pulmonary vasodilation resulting in improved pulmonary perfusion and thereby less dead space ventilation. The increase in colloid osmotic pressure from sodium, as well as the oncotic force of dextran, could explain potential reduction in oedema formation by HSD. In experimental haemorrhage, reduced perivascular oedema as well as attenuated histological lung injury has been demonstrated after HS treatment. The attenuated lung injury is proposed to depend upon suppressed neutrophil activation, infiltration and sequestration. These findings are disputed by both former and more recent studies.

5.2.4 Immunomodulation

The cardio-pulmonary effects of HSD were not associated with an affected early pro-inflammatory cytokine response (papers II and IV). Previous experiences, with HS or HSD and evaluation of cytokine response after sole endotoxin exposure, are limited to a few studies of LPS-exposure in vitro or in vivo as a second hit after systemic inflammation due to burn injury or chemical peritonitis in the rat. In these studies, TNF-\(\alpha\) expression is suppressed by HSD. In haemorrhage/reperfusion or combined haemorrhage/LPS, the ability of HS to reduce neutrophil activation, rolling and adhesion, as well as modulate cytokine response is well described. The pro-inflammatory mediators, TNF-\(\alpha\) and IL-6, have been shown to decrease, whereas anti-inflammatory IL-10 increases in these rat models. Thus, the present finding of essentially unaffected cytokine activation by HSD in endotoxemic pigs.
emphasizes the differences between models, type of trauma and species. Interestingly, in clinical studies of women undergoing elective surgery, preoperative HS infusion did not influence the cellular immune response. In conclusion, the current knowledge suggests that the immune modulating properties of HSD are questionable both in an aggressive in vivo endotoxin model and with a moderate surgical trauma in the clinical setting.

5.2.5 Survival

Survival in short-term endotoxin shock was markedly improved by the additional treatment with HSD. Even though the number of animals in the study was small, the combined results are a strong indication that resuscitation with HSD may reduce mortality in the early phase of endotoxemia. These findings confirm results from previous studies, where others have reported similar survival rates with HSD resuscitation in porcine endotoxin shock. The transient macrocirculatory and the possibly longer lasting microcirculatory effects, slowing haemodynamic deterioration and thereby delaying tissue hypoxia, are the probable causes of the improved survival.

5.3 TEZOSENTAN IN ENDOTOXEMIA

The ET system is activated in several pathological conditions associated with septic shock, such as myocardial dysfunction, acute respiratory distress syndrome, splanchnic hypoperfusion and disseminated intravascular coagulation. Previous investigations with ET antagonism have partly elucidated underlying mechanisms and indicated beneficial effects on cardiac function, lung injury and gut ischemia. In the present studies effects of a new dual ET receptor antagonist of the “sentan-class” (tezosentan) was evaluated in endotoxemia (papers III-IV). Tezosentan was administered in an intermediate dose within the range that currently has been used in endotoxin shock.

5.3.1 Systemic haemodynamics

In paper III, tezosentan improved CI as the endotoxin-induced reduction in CI was prevented. The increase could not be explained by an increase in HR. It was therefore concluded that it results from a higher stroke volume. This is in agreement with other recent investigations, where improved stroke work, as well as signs of improvement in diastolic function of the left ventricle were reported. Recently, Konrad et al. showed, by use of cardiac conductance volumetry in porcine endotoxemia, that administration of tezosentan led to a decrease in contractile function but improved diastolic function. The latter may have contributed to the improved CI observed in our study. It has been suggested that the major mechanism for improved CI seen with tezosentan is due to reduced right and left ventricular afterload. Therefore, our observation of pronounced loss of vascular tone, and as a result reduced left ventricular afterload probably explains much of the increase in CI. The reduced SVRI is consistent with the findings of earlier studies of both bosentan and tezosentan. Moreover, the reduction in MPAP, with return to baseline level, would lead to reduced right ventricular afterload and increased left heart preload, further contributing to the improved CI. Our findings in endotoxemia are consistent
with the hypothesis that the actions of ET-1 may differ between the normal heart and
during pathological conditions. Opposed to the results from ET antagonism in
endotoxemia or congestive heart failure\textsuperscript{74, 209}, there are now several reports where
improved myocardial contraction is demonstrated as a consequence of ET\textsubscript{A} receptor
agonism in healthy hearts or isolated myocytes\textsuperscript{63, 210-212}.

### 5.3.2 Portal blood flow

Tezosentan improved portal blood flow and portal DO\textsubscript{2}. The main mechanism is likely
to be reversal of vasoconstriction in the vein and thereby reduced portal vascular
resistance. Systemic vasodilation together with an unchanged MAP indicates reduced
regional resistance, even though direct monitoring of resistance and pressure in the
peripheral vessel was not included in our model. The response resembles that of the
non-selective ET-blocker bosentan\textsuperscript{78, 213}. Thus, it again confirms the involvement of ET
in the regulation of portal blood flow during endotoxemia. The increase in portal DO\textsubscript{2}
may, in part, be a reflection of the improved systemic DO\textsubscript{2}. However, an independent
increase in portal venous flow with tezosentan intervention is supported by the findings
of Burgener et al.\textsuperscript{214}, who reported restored portal flow during persistently low CO with
tezosentan, in a porcine model of a low CO state due to cardiac tamponade. Less ET-1
induced capillary leakage of plasma and protein could make an additional contribution
to the increased portal blood flow\textsuperscript{82}. Another beneficial effect of the decrease in portal
vein resistance with tezosentan treatment may also be improved splanchnic perfusion,
leading to reduced microcirculatory disturbances in the gut and less intestinal oedema\textsuperscript{78}.
In the present study, only small changes in ASAT and ALAT were observed with no
differences between groups. This is probably due to the short-term observation period
in this model. However, positive effects of tezosentan on endotoxin-induced hepatic
injury with less elevated transaminase activity and decreased intrahepatic infiltration of
neutrophils have previously been reported in a four-hour rat model\textsuperscript{215}. In contrast,
selective ET\textsubscript{A} antagonism resulted in worsened LPS-related hepatic changes and
necrosis\textsuperscript{95}. This suggests that ET\textsubscript{B} receptor activation plays a major role in the
occurrence of liver injury following LPS administration.

### 5.3.3 Renal blood flow

The reports on effects of ET antagonism in the renal circulation during endotoxemia are
contradictory. In the current study (paper IV), tezosentan did not improve blood flow,
diuresis, creatinine or urea. A similar result has been demonstrated with bosentan\textsuperscript{78}.
Investigations using porcine models of other pathological conditions, such as
experimental intra-abdominal hypertension\textsuperscript{216} or experimental low CO state due to
tamponade\textsuperscript{214}, support the finding of decreased RBF during tezosentan treatment. In
contrast, Chin et al. found improved RBF and GFR after tezosentan treatment in
neonatal piglets subjected to endotoxin shock\textsuperscript{205}. The discrepancy between these results
and those in the present study may in part be explained by differences in endotoxin
regimens, fluid administration, lower tezosentan dosage and above all, by a different
monitoring technique. The inability of tezosentan to increase renal perfusion, in
contrast to the effect in the portal circulation, may also suggest that renal vessels are
influenced by other vasoconstrictor mechanisms, such as the renin-angiotensin system
and/or vasopressin. Furthermore, the difference in effect between the portal vein and the renal artery in the present study could also in part be explained by the fact that ET-1 is reported to be a more potent constrictor of large veins than arteries, as well as by the studies in healthy volunteers, where exogenous ET-1 induced a more pronounced vasoconstriction in the splanchnic than in the renal circulation.

5.3.4 Carotid blood flow

The increased carotid flow and DO₂ following tezosentan intervention was interpreted as a reversal of the vasoconstrictor effect from ET also in a vessel supplying skeletal muscle, the head of the animal and other “non vital” peripheral tissues.

5.3.5 Pulmonary function

The endotoxin-induced deterioration in PaO₂ was counteracted by tezosentan (paper III). This improvement is consistent with the findings of other investigators, reporting improved gas exchange and reduced pulmonary oedema. The improved gas exchange induced by tezosentan could, in part, be explained by increased CO and pulmonary vasodilation resulting in improved pulmonary perfusion and thereby less dead space ventilation. The reduction in hydrostatic pressure over the pulmonary capillaries, following tezosentan treatment, also contributes by decreasing extravasation of fluid, resulting in reduced oedema formation. Moreover, ET-1 affects the endothelial barrier and produces a dose-dependent increase in permeability to protein and extravasation of albumin with concomitant haemoconcentration. In our study, the finding of a lower Hb in the animals treated with tezosentan suggests that dual ET antagonism is able to counteract these changes. The proposed anti-inflammatory properties of ET antagonism could also have reduced the inflammatory component of the pulmonary dysfunction.

5.3.6 Pro-inflammatory cytokine response

ET is known to cause monocytes and macrophages to produce pro-inflammatory cytokines such as TNF-α, and ET antagonism may therefore reduce this production. The observation of increased IL-6, without simultaneous changes in the TNF-α response pattern, in tezosentan treated animals was unexpected and contradictory to previous investigations. Earlier studies indicate that pro-inflammatory effects of ET are ETₐ-receptor mediated, and that ETₐ-receptor stimulation is without effect. Therefore, in the present study, the simultaneous inhibition of both receptor subtypes may have influenced our divergent result. No support for the hypothesis that dual receptor antagonism inhibits early pro-inflammatory TNF-α and IL-6 response could be demonstrated in the present study.
5.4 TEZOSENTAN AND HYPERTONIC SALINE/DEXTRAN IN ENDOTOXEMIA

As there was no mortality during the observation period with HSD resuscitation in the first study, an improved survival rate with tezosentan/HSD was not possible. Furthermore, the study was not designed or empowered to investigate differences in mortality. However, there was no increase in mortality observed with the combination.

Endotoxemia causes substantial hypotension, and additional loss of perfusion pressure due to systemic vasodilation would not be favourable in shock. The animals treated with tezosentan did not become more hypotensive than the animals subjected to endotoxin alone (paper III). However, the fact that HSD increased MAP when given by itself but not in combination with tezosentan indicates a tendency to lower blood pressure with tezosentan intervention. The addition of HSD to tezosentan did not counteract this drop in MAP, despite the desired volume effect, as indicated by a significant drop in haematocrit. There could be several explanations for this: the high dosage of tezosentan with subsequent pronounced vasodilation, inadequate intravascular fluid volume or the inherent vasodilator effect of HSD. Konrad et al. proved the cardiovascular effects of tezosentan to be dose-dependent, and our results using an intermediate dose compared to that study, strengthens that assumption. HSD might therefore have opposed hypotension more successfully with a lower dose of tezosentan, but with preserved beneficial cardiac effects. A possible adverse effect of HSD is the reported initial drop in arterial blood pressure. It is however very short-lasting (< 1 min), and most pronounced with rapid infusion. Subsequent to this phase, the peripheral vasodilatation is not associated with reduced MAP.

The combination of tezosentan and HSD resulted in the most pronounced increase in CI compared to controls. This is most likely a volume resuscitation effect associated with increased preload in combination with improved stroke work from tezosentan. A possible inherent positive inotropic effect of hypertonic saline solutions could further contribute to improved contractility.

In the portal and carotid circulation, addition of HSD to tezosentan resulted in further increased blood flow compared to tezosentan alone. These changes were demonstrated concomitantly with the same degree of systemic vasodilation and with similar CI as with tezosentan alone, indicative of regional vasodilation as the major mechanism. These findings suggest additive effects of tezosentan and HSD on peripheral blood flow when combined.

In the renal circulation, there was only a tendency for increased RBF with tezosentan/HSD, most likely explained by the absent/limited effect of ET antagonism. On the other hand, one may speculate that a positive effect of HSD was counteracted by tezosentan-related hypotension. The increased regional DO$_2$ seen with tezosentan/HSD is, in part, a reflection of the improved systemic DO$_2$, but is also influenced by regional flow. The combination of tezosentan/HSD had the same beneficial effects as tezosentan alone on cardiac performance, oxygenation and metabolic acidosis, but did not further augment the responses.
5.5 SUMMARY AND FUTURE PERSPECTIVE

The current recommendations in fluid resuscitation are to administer “fluid challenges” rapidly and repeatedly based on continuous evaluation of the response, preferably to predetermined goals of the haemodynamic parameters. Either a crystalloid or a natural or artificial colloid may be used\textsuperscript{180, 181, 224}. There is insufficient data regarding the use of hypertonic solutions in the critically ill in general\textsuperscript{225}, and even less in septic patients. The haemodynamic improvements and reduced early mortality demonstrated with HSD in the current study suggest a potential role in fluid resuscitation in early septic shock. As early aggressive goal-directed fluid therapy has proven important\textsuperscript{105}, HSD should possibly be included in the initial resuscitation. However transient the effect, such resuscitation may buy time for other treatment to have effect and supportive measures to be established. Before routine clinical implementation, further animal studies need to be performed in clinically relevant models, including longer periods of observation. Patient studies are so far only descriptive, and larger prospective randomized trials of early fluid administration with HSD to predefined clinical end-points are required. Future studies should include determining the effects on the immune and inflammatory systems, as such evidence is still limited in humans.

The major effect of ET antagonism with tezosentan in the present endotoxin shock model was vasodilation in some vital vascular beds. As a result, CO increased, pulmonary hypertension was reversed and hepatic hypoperfusion was prevented. The possible role for tezosentan in sepsis would be organ-protective by promotion of tissue oxygen delivery. It remains to be evaluated if such an increase in nutritional blood flow prevents or limits organ failure both in a short-term and long-term perspective. In light of the recently reported negative effect on systolic contractile function\textsuperscript{208}, the impact of negative side-effects needs to be addressed. In the presence of reduced vascular resistance, blood flow needs to be increased in order to preserve perfusion pressure. The addition of HSD to tezosentan treatment accomplished such an increase, but failed to improve MAP. The critical level of MAP in these conditions has to be determined, and may vary between different vascular beds. In this aspect, the current studies suggest that the renal circulation may be more dependent on adequate perfusion pressure, whilst portal flow responds to vasodilation. Continued efforts in finding other strategies to secure perfusion pressure without inhibiting the positive effect of ET antagonism are warranted. Such efforts should, for example, include tezosentan in combination with different vasopressor agents. The optimum dose and timing of treatment are still to be determined in order to prevent adverse effects. Individualized titration of the tezosentan dose according to effect might be safe and beneficial. In addition, such titration could be conducted in parallel with goal-directed fluid therapy. These are all issues that need to be addressed in future studies.
6 CONCLUSIONS

- Early additional fluid resuscitation with hypertonic saline/dextran transiently improves cardiac output and oxygen delivery in experimental porcine endotoxin shock.

- Treatment with hypertonic saline/dextran transiently improves renal, but not portal blood flow.

- Early additional resuscitation with hypertonic saline/dextran improves early survival in porcine endotoxin shock.

- The improved early survival and circulatory effects seen with hypertonic saline/dextran treatment are not associated with apparent reduction in the early pro-inflammatory cytokines TNF-α and IL-6.

- Treatment with hypertonic saline/dextran does not improve endotoxin-induced lung impairment with regard to lung mechanics. However, a positive effect on gas exchange was indicated.

- The dual endothelin receptor antagonist tezosentan improves cardiac output and oxygen delivery in experimental endotoxin shock.

- Addition of hypertonic saline/dextran to tezosentan treatment does not affect the influence of tezosentan on mean arterial pressure, nor does it further improve cardiac output.

- Tezosentan improves regional blood flow and oxygen delivery in the portal-, but not in the renal circulation in porcine endotoxin shock.

- The combination of endothelin receptor antagonism and fluid resuscitation with hypertonic saline/dextran further improves regional blood flow and oxygen delivery in the portal- and carotid circulation, but does not improve renal perfusion.

- Dual endothelin receptor antagonism with tezosentan could not be demonstrated to exert anti-inflammatory effects through reduction of the TNF-α or IL-6 response.

- The combination of endothelin receptor antagonism with tezosentan and hypertonic saline/dextran does not bring additive anti-inflammatory effects with respect to the TNF-α or IL-6 response.
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8 REFERENCES


