

Adjunctive Therapies in a Clinical Relevant Ovine Model of Septic Shock

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To my wife, Yang I am deeply grateful to her heartfelt love And to my parents I am equally grateful for their long term nurture and support

The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity.

> Albert Einstein (1879-1955)

Proclamation

The author states that this thesis contains no content that has been accepted as part of a work for a degree or diploma and, to the best of his understanding, contains no material published by or originating from other persons, except where appropriate references are cited.

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Summary

Sepsis has been defined as a systemic response to an infection. With an incidence of 3 per 1000 population per year or about 750 000 cases a year, this syndrome ranks as the 10th leading cause of death in the United States (1). Increasing severity of sepsis correlates with increasing mortality, which rises from 30-40% for severe sepsis up to 40-60% for septic shock. This thesis examines the effectiveness of adjunctive therapies, including activated protein C, hypercapnia and acidosis, and sodium selenite, in a clinically relevant ovine model of septic shock. The results from these studies can provide valuable information for future clinical trials on sepsis.

This thesis is divided into four sections: 1) sepsis overview; 2) an autologous fecal peritonitis model in sheep and its evaluation; 3) the series of studies on adjunctive therapeutics; and 4) ongoing studies and future perspective.

In the first section, a broad overview gives a rough introduction to delineate many aspects of sepsis syndrome such as terminology, etiology, epidemiology, pathophysiology and current guidelines for management. Hemodynamics in sepsis are especially elaborated since these are major observations throughout the studies presented later.

In the second section, the general characteristics of the sepsis models used in this thesis are elucidated. Data on hemodynamics, lung mechanics, gas exchange, etc. are presented to feature the ovine peritonitis model. The results of laboratory examinations for hematology, coagulation, bacteriology, biochemistry and hormonology are also presented. And then, I review currently used sepsis models with regards to their advantages and disadvantages.

The third section discusses three studies with their objectives, the methods used, the major findings, and the potential clinical implications.

- 1) Beneficial effects of recombinant human activated protein C in experimental septic shock. Activated protein C has a multitude of beneficial effects in severe sepsis and septic shock, including anti-inflammation, anti-coagulation, profibrinolysis, antiapoptosis and endothelial protection. A clinical Phase III trial demonstrated that the administration of recombinant human activated protein C improved survival in patients with severe sepsis. However, doubts on the protective effects of activated protein C have persisted and been refueled by the recently published negative trials in less severely ill patients and in children. In the light of these ambiguities and uncertainties, we reinvestigated the effects of activated protein C in experimental septic shock.
- 2) Acute hypercapnia improves indices of tissue oxygenation more than dobutamine in *septic shock*. Hepercapnia has been found to possess beneficial effects in diverse acute inflammatory states independent of protective lung mechanics. To prove the hypothesis that acute hypercapnia has similar or superior hemodynamic effects to those of a dobutamine infusion, which may be particularly relevant in the presence of hemodynamic instability associated with respiratory failure, we investigated the effects of hypercapnia, which induced by inspiring extrinsic carbon dioxide in experimental septic shock.
- 3) *High bolus dose of sodium selenite prolongs survival in an ovine model of septic shock.* Selenite has both pro- and anti-oxidant effects. The administration of high dose sodium selenite may improve survival in septic shock patients. The benefit may be greater with the administration of a bolus (to achieve higher concentrations) rather than a continuous infusion. To test this hypothesis, we examined the effects of a high dose bolus administration of sodium selenite in experimental septic shock.

The fourth and final section talks about currently ongoing studies and offers some perspective on future direction.

Chapter I: An Overview of Sepsis

1.1. Evolution and Current Definition

Sepsis can be simply defined as the host response to local infection. The concept of "sepsis" dates at least as far back as the time of Hippocrates and was viewed as a process of dangerous, odorous biologic decay, or putrefaction (1). In the 19th century, Louis Pasteur and Robert Koch proposed the "germ theory of disease" and established the microbial disease causal relationship that overturned the "spontaneous generation", a mainstay theory of sepsis for more that 2000 years (2). Modern belief highlights that sepsis is a dynamic course of host-pathogen interactions (3). In 1992, a North American consensus conference created the simple definition of systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock (Table1.1) (4). However, ongoing dispute and discussion persisted surrounding the definition of sepsis, especially regarding the concept of SIRS, which was criticized for being oversensitive, not reflecting the pathophysiology, and non-specific (5). These discussions resulted in the suggestion of an updated system, called PIRO, in 2001, characterized by individual pathogens and recognizable host-response profiles (Table 1) (3). Undoubtedly, with advances in underlying molecular knowledge and clinical practice, a more precise, mechanism-based definition will be developed in the future helping with the development of more effective treatment strategies for sepsis.

1.2 Epidemiology

Very large epidemiological studies of up to 6 million people give an incidence of 30 to 50 per 10,000 population per year or about 750 000 cases a year in the United States (6). The incidence rate is particularly high among the elderly population due to increased susceptibility to infections when aging, higher co-morbidity, and use of invasive surgical techniques. Increasing severity correlates with increasing mortality, which reaches 30-40% for severe sepsis and 40-60% for septic shock. The average length of hospital stay and cost per patient were 19.6 days and \$22,100, respectively. The total national cost in the United States for severe sepsis has been estimated at \$16.7 billion annually.

1.3 Etiology

A large-scale prospective study by Sands et al. (7) in 12,758 patients disclosed that Gram-negative bacteria accounted for approximately 40% of the cases of sepsis syndrome in which an etiologic agent was identified. Of these bacteria, 67% were attributable to the *Enterobacteriaceae* family and 25% to *Pseudomonas* spp. The most frequent sites of infections are the respiratory tract (42%), primary bacteremia (12%), genitourinary tract (11%), and the abdomen (10%). Bacteremia occurred in 45% of the patients with sepsis syndrome attributable to Gram-negative bacteria. In another large epidemiological investigation, Brun-Buisson et al. (8) reported similar results, with *Escherichia coli* identified as the predominant etiological agent in Gram-negative sepsis and severe sepsis.

However, more recent data indicate that Gram-positive pathogens are increasingly frequently identified as causative sources of sepsis, particularly *Staphylococcus aureus* and *Streptococcus pneumoniae* (9, 10).

1.4 Pathophysiology

The pathophysiological course of sepsis is characterized by 1) an imbalanced response of the host's innate and adaptive immune systems under the influence of genetic factors, 2) coagulation activation with concurrent down-regulation of anti-coagulant systems and fibrinolysis, 3) endothelial dysfunction and microcirculatory alterations, 4) lymphocyte apoptosis.

When microbial pathogens from the exogenous or endogenous environment gain systemic access to the host, their cell wall component, lipopolysaccharide (LPS), in synergy with other constituents, like DNA and lipoprotein, binds to the pattern recognition receptors on the surfaces of monocytes, macrophages, neutrophils and endothelium and activates the recipient cells via a series of inflammatory cascades (11, 12). Subsequently, a large number of inflammatory cytokines, chemokines, oxygen free radicals and complement components are generated and released into the infected sites. Tissue factors are also triggered and exposed on the membranes of activated inflammatory cells and endothelium, initiating the extrinsic pathway of coagulation (13). These processes, when properly coordinated, usually confine and eliminate the invading microorganisms and maintain homeostasis. However, an overwhelming systemic host response can cause widespread tissue injury and organ dysfunction, a cluster of clinical manifestations defined as sepsis (14).

Recent advances have demonstrated the complicated interplay of inflammation and coagulation in sepsis, in which endothelial activation and dysfunction play a pivotal role and interface (15). Pro-coagulant components, like tissue factor and microparticles, are predominantly released in response to the inflammatory stimuli, while activated protein C, anti-thrombin, tissue factor pathway inhibitor, and fibrinolysis are down-regulated systemically. Likely, the components of the protein C system, fibrinolytic system and anti-thrombin have a significant impact on inflammation, partly via modulation of protease-activated receptor (PARs) activation by thrombin (16).

Microcirculatory function is severely impaired in sepsis, characterized by heterogeneous abnormalities in blood flow within tissues (17), disturbed endothelial signal transduction pathways and smooth muscle control (18), arteriovenous shunts, altered hemorheology (19) and coagulation. It is believed that microcirculatory dysfunction, when not corrected for prolonged periods of time, can act as a motor of organ failure and death. Mitochondrial dysfunction has also been postulated to contribute to respiratory distress and cellular dysoxia, especially in the late stage of sepsis (20, 21). Therefore, immediate monitoring and early-goal directed therapies aimed at the microcirculation are essential to optimize diagnosis and treatment in sepsis.

Other hallmarks in sepsis are early and widespread lymphocyte apoptosis that is widely observed in septic animals and patients (22, 23) and neutrophil hyperactivity due to the delayed apoptosis and prolonged nuclear factor-kappa B (NF- κ B) activation (24). A dysregulation of this cellular process between diverse inflammatory cells contributes to the pathogenesis of sepsis. In this respect, it is worth mentioning that an evolving body of evidence has questioned the fundamental theory of a hyper-inflammatory systemic response in the pathophysiology of sepsis, after the failure of more than 30 clinical trials to treat sepsis by controlling inflammatory mediator responses (25). Animal studies in sepsis have shown that the initial hyper-inflammatory response quickly transited to a hypo-inflammatory state that is termed "immunoparesis" (26, 27). It is quite possible that many septic patients who survive the initial days develop an immunosuppressed state, manifested by an insufficiency to eliminate the primary infection and develop a new nosocomial infection. Therefore, clinical treatment in these cases must oppose the traditional anti-inflammatory therapy and focus on "immuno-support", like targeting antiapoptotic therapy to specific lymphocyte populations and restoring immune function. New advances in this field certainly prompt a great step forward in the understanding of sepsis.

1.5 Systemic Hemodynamics in the Sepsis Syndrome

Severe sepsis and septic shock are characterized by a marked decrease in peripheral vascular resistance and a hyperdynamic state (28). Although cardiac output is usually high (especially after fluid resuscitation), clinical studies have shown that biventricular systolic and diastolic function are usually impaired, with an increase in end-diastolic and end-systolic volume (29, 30). The circulatory volume is reduced as a result of increased capillary leakage and venodilation, leading to hypovolemia and hypotension (31). Clinical manifestations of sepsis include decreased urine output, altered mental status, altered skin perfusion, increased intramucosal PCO_2 gap and hyperlactatemia, indicative of tissue hypoperfusion.

Septic shock is the most common form of distributive shock. The regional maldistributions of cardiac output has been observed in animal studies, with a decreased blood flow towards the myocardium, skeletal muscle and splanchnic areas, while blood flow to the brain and kidney is preserved (32-35). The decreased gut mucosal blood flow is a consistent finding in sepsis and may act as a motor to organ failures.

1.6 Current diagnosis of sepsis syndrome and severity assessment

Current widely used diagnostic definitions of sepsis and complications were developed in the International Congress in 1992. They used explicit criteria to classify the subsets of septic patients and identify progression of infections along with appropriate responses (4). However, these criteria exist considerable overlaps. They are clearly inadequate in terms of allowing detection of severe infections in routine daily practice. For example, Vincent (36) criticized the legitimization of the concept of SIRS because of problems with oversensitivity, lack of linkage with pathophysiology, loss of definition specificity, and unclear benefit for patient care and advancement in research.

It is highly desirable to find specific and sensitive markers that allow early detection of sepsis and correlate with patient outcome. Unfortunately, no single such marker has been identified so far, though some of them have certain values in differentiating sepsis from other non-sepsis illnesses or determining severity of sepsis.

In critically ill patients, increased concentrations of serum endotoxin have been associated with the development of sepsis, disease severity, and mortality (37). Detectable levels of LPS are identified in up to 75 per cent of patients with sepsis in intensive care setting (38). So it seems logical to use endotoxin as a measurable marker. However, because of the extreme potency, poor immunogenicity, and short serum half-life of LPS, highly sensitive bioassays are needed to measure plasma LPS. The Limulus amebocyte assay is technically difficult and has problems with plasma inhibitors and cross-reacting elevation by fungal elements. Recently, the new rapid endotoxin activity assay' (39). This assay measures the oxidative burst that is produced by activated neutrophils and compares this activity with an LPS-free control sample and a sample maximally activated by endotoxin. It is anticipated that this or similar rapid assays will permit a convenient measure of circulating LPS levels for clinical trials and as a guide to early institution of specific anti-LPS treatments.

Blood lactate level was proposed to assess the severity of sepsis. A single venous lactate measurement above 4 mmol/l as a marker for severe tissue hypoperfusion predicted short-term and in-hospital risk for death in patients with suspected infection (40). Some groups suggested that lactate clearance was a better prognostic factor than a single lactate determination performed on ICU admission, identifying those patients who will respond to treatment and have a favorable outcome (41, 42).

Attempts to understand the role of inflammation in sepsis have prompted studies of the value of cytokines on outcome. Small studies have demonstrated links between risk of death and cytokine levels, such as elevated serum interleukin-6 (43, 44) or tumor necrosis factor (TNF) levels (45). However, because the range of cytokine levels from survivors and nonsurvivors typically overlap, such tests are of poor discriminative value.

Other innovative diagnostic biomarkers like procalcitonin and biphasic waveform analysis of APTT proved to be of prognostic value for mortality, provided that some intrinsic limitations are recognized. (46, 47).

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Table 1.1 The definitions of sepsis syndromes in the 1992 and 2001 consensus conferences

Definitions, 1992

Systemic Inflammatory Response Syndrome (SIRS)

Two or more of the following: Body temperature 38.5°C or <35.0°C Heart rate >90 bpm Respiratory rate >20 breaths per minute or arterial CO2 tension <32 mm Hg or need for mechanical ventilation White blood cell count >12 000/mm3 or <4000/mm3 or immature forms >10%

Sepsis: SIRS + documented infection

Severe sepsis: sepsis with organ dysfunction, such as oliguria, lactic acidosis, alterations in skin perfusion, alterations in mental status, coagulation abnormality, hypoxemia or renal dysfunction

Septic shock: severe sepsis with persistent hypotension despite fluid resuscitation, need for vasopressor therapies

Proposed PIRO scheme: 2003

Predisposition

Underlying illness Genetics Host-pathogen interaction

Insult/infection

Specific pathogens Source control Virulence factors

Response

SIRS, shock, CRP IL-6, TNF-α, protein C levels

Organ dysfunction

Number of failing organs or composite score Apoptosis, cytopathic hypoxia, cell stress

Chapter II Current Guidelines for Management of Severe Sepsis and Septic Shock

2.1 Introduction

My study is aimed at investigating adjunctive therapies in the condition of sepsis. Severe sepsis and septic shock remain a leading cause of mortality in the intensive care unit (1). Similar to polytrauma, acute myocardial infarction, or stroke, early recognition and prompt treatments after severe sepsis develops are likely to greatly improve outcome (2). Generally, the current guidelines in the management of patients with septic shock include rapid administration of appropriate antibiotic therapy (3) as well as source control at the site of infection (4). Early goal-directed fluid resuscitation to restore organ perfusion and improve oxygen delivery to tissues are considered to be important (5). Extensive supportive care of failing organs like careful fluid management and optimized vasopressor support all contribute to improved outcomes in sepsis (6, 7). Consideration is also given to the use of rhAPC for patients with multiorgan failure, at high risk of death, and low risk of bleeding complications (8). Low-dose corticosteroids to reverse the pathophysiology of severe sepsis (9), and tight glycemic control are reasonable supportive measures based upon the most recent evidence available (10).

2.2 Infection Source Control

Source control represents a key component of successful interventions in sepsis. Early detection of infection sites helps to identify the potential microbiologic cause and facilitates eradication by source control measures (11). It includes drainage of infected fluids, debridement of infected soft tissues, removal of infected devices or foreign bodies, and finally, definite measures to correct anatomic derangement resulting in ongoing microbial contamination and to restore optimal function (12). A significant improvement on survival has been observed when surgical removals (eg, debridement, fasciotomy, amputation) are undertaken early for necrotizing skin and soft tissue infections (13, 14). Intravascular catheters should be removed and cultured if there are signs of infection at the insertion site (eg, drainage of pus, erythema) or there is evidence of severe sepsis/septic shock with no other source of infection. Moreover, when source control is required, the effective intervention associated with the least physiologic insult be employed (e.g., percutaneous rather than surgical drainage of an abscess) (2).

2.3 Antimicrobial Therapy

Like source control, antimicrobial therapy should be initiated as early as possible once the microbial origin of sepsis is ascertained. Appropriate cultures should be obtained before initiating antibiotic therapy but should not interfere with immediate prescription of antimicrobial therapies with broad spectrum (2). Antimicrobial regimens for patients with severe sepsis/septic shock must refer to pathogen prevalence, susceptibility patterns, and results of clinical trials (12).

Principally, the initial selection of antimicrobial therapy must have a broad spectrum of action to cover all likely pathogens until the causative organism and its antibiotic susceptibilities are defined. The failure to initiate appropriate therapy (i.e., therapy with activity against the pathogen that is subsequently identified as the causative agent) correlates with increased morbidity and mortality (15, 16). The prescription regimen of empirical antibiotics depends on comprehensive considerations of patient's history, including drug intolerances, underlying disease, the clinical syndrome, and also susceptibility patterns of pathogens in the community that previously have been documented to colonize or infect the patient.

Although antimicrobials should be prescribed in a full loading dose, concerns must be considered in terms of drug toxicity because septic patients usually have compromised hepatic or renal functions, and abnormal volume distribution due to aggressive fluid resuscitation (17, 18).

2.4 Fluid Resuscitation

Fluid resuscitation is of great importance to correct sepsis-induced hypovolemia and tissue hypoperfusion before any other hemodynamic support is given. Early goaldirected resuscitation has been shown to improve survival for patients with septic shock in a randomized, controlled, single-center study (19). In our study, we use clinically recommended criteria for fluid resuscitation with some revisions. Those include Mean arterial pressure (MAP) >65 mm Hg, Urine output >0.5 mL/kg/hr, mixed venous oxygen saturation >70%, and pulmonary artery occlusion pressure at baseline level (2).

In terms of fluid types, no difference in outcome has been demonstrated when using colloids compared to crystalloids with respect to mortality or hospital length of stay (20, 21). We previously performed a study to compare clinical effects of different fluid types (22). We found the choice of fluid resuscitation with Ringer's lactate, albumin, gelatine and hydoxyethyl starch has limited effect on outcome in terms of survival time, hemodynamic response and lung mechanics, etc. But the combined use of colloid and crystalloid is superior to crystalloid may limit total fluid volumes and resultant intraabdominal hypertension and pulmonary edema.

2.5 Inotropic and vasopressor therapy

Dobutamine is the first choice inotrope for patients with myocardial dysfunction in the presence of adequate left ventricular filling pressure and adequate mean arterial pressure (2). Adequate preload is necessary for optimal benefit from dobutamine therapy and should be titrated in a goal-directed fashion (23). Because the vasodilatory effect of dobutamine could worsen hypotension, it should be used in combination with vasopressors for patients with persistent hypotension (24). Although some clinical studies suggested that dobutamine could improve splanchnic perfusion and oxygenation, and increase gut mucosal pH in sepsis (25, 26), these effects are inconsistent (27, 28). In a previous study, we did not observe a significant increase in mesenteric blood flow despite of high cardiac output in experimental sepsis, either.

In the presence of hypotension and tissue hypoperfusion that is refractory to fluid resuscitation, vasopressor therapies should be administered to maintain mean arterial pressure above 65mmHg (2). Up to now, there is no clear evidence to support one vasopressor superior to another. Norepinephrine, at a dosage of 2 to 20 μ g/minute, or dopamine, at a dosage of 5 to 20 μ g/kg/ minute, have been advocated as first-line agents in septic shock patients (29). Epinephrine, at a dosage of 1 to 10 μ g/minute, is considered as the last-resort therapy used in septic patients unresponsive to norepinephrine or dopamine. Its use can be deleterious, because of the concern that it may impair splanchnic circulation and increase lactate acidosis (30).

Vasopressin levels in septic shock have been reported to be lower in septic shock (31). Low doses of vasopressin, 0.01 to 0.04 U/minute, may be effective in raising blood pressure in patients refractory to other vasopressors and may have synergistic effects with other vasoactives (32).

2.6 Protective Ventilatory Strategy

Septic patients are frequently complicated by acute lung injury and acute respiratory distress syndrome (ARDS) (33). The large clinical ARDSnet trial showed a 9% decrease of all-cause mortality in patients with ALI or ARDS ventilated with tidal volumes of 6 mL/kg of predicted body weight (PBW), as opposed to 12 mL/kg, and aiming for a plateau pressure \leq 30 cm H₂O (34). The use of lung-protective strategies for patients with ALI is supported by clinical trials and has been widely accepted, but the precise choice of tidal volume for an individual patient with ALI may require adjustment for such factors as the plateau pressure achieved, the level of positive end-expiratory pressure chosen, the compliance of the thoracoabdominal compartment, and the vigor of the patient's breathing effort. Moreover, airway plateau pressure should be limited less than 30 cm H₂O in mechanical ventilated patients (2). If plateau pressure remains >30 after reduction of tidal volume to 6 mL/kg PBW, tidal volume should be reduced further to as low as 4 mL/kg PBW.

Protective lung strategies result in a relatively high level of $PaCO_2$, so called permissive hypercapnia. Traditionally, the beneficial effect of ventilatory strategies incorporating permissive hypercapnia is considered to be only due to a decrease in alveolar ventilation, with hypercapnia allowed in order to achieve this goal (35). However, large quantities of experimental studies demonstrated the potential protective effects of hypercapnic acidosis by direct improvements in gas exchange and modulation of inflammatory events (36-38). These were contradicted by clinical trials, in which impaired ventilation/perfusion matching and oxygenation were observed (39, 40). A possible explanation for these disparities lies in the manner by which hypercapnia was obtained. In animal studies, CO_2 was added to the inspired gas mixture, while in human studies hypercapnia was achieved by reducing tidal volume and minute ventilation. Indeed, Dr. Sinclaire et al. demonstrated that it was the reduced tidal volume rather than hypercapnia per se that was responsible for the increased shunting and atelactasis, since no changes in shunting was detected in either hypercapnic condition or normocapnia in their study if tidal volume was kept same (41). In terms of this controversy, we carried out a prospective study to investigate the effects of inspired hypercapnia in the condition of sepsis. We showed that moderate hypercapnia and acidosis is a safe and promising maneuver to the management of septic patients.

Prone positioning is also suggested as a supportive maneuver to improve oxygenation in ARDS patients (42-44). Some accidental events associated with this treatment like unexpected dislodgment of the endotracheal tube and central venous catheters must be avoided with carefulness.

2.7 Recombinant Human Activated Protein C (rhAPC)

Recombinant Human Activated Protein C (rhAPC) (Drotecogin alfa, Xigris, Eli Lilly and company, IN, USA) is the first therapeutic agent that significantly improve survival rate in the patients of severe sepsis and septic shock (45). An evolving body of preclinical studies and clinical trials illustrated that APC has possessed multiple beneficial effects like anticoagulant, profibrinolytic, antiinflammatory, and antiapoptotic (46-48). Although the PROWESS trial showed exciting result, doubts still raised in the subgroup analyses. The subsequent ADDRESS trial disclosed that rhAPC did not improve survival in septic patients at low risk for death (49). Similar results were demonstrated in children (RESOLVE trial, 50). Moreover, increased bleeding events, especially severe intracranial hemorrhage, were associated with the use of APC. Therefore, the current recommendation is that APC should only be administered in adult patients with Acute Physiology and Chronic Health Evaluation (APACHE) II >25 or multiple organ failure, without contraindication (2).

In view of the inconsistent efficacy of APC, we thought it is necessary to reexplore the effect of APC in relevant experimental condition, especially the early use, which was believed to carry a larger survival advantage (51).

2.8 Corticosteroid

Many septic patients have inadequate adrenal function manifested by an inadequate response when challenged with adrenocorticotropic hormone or corticotrophin-releasing hormone (52, 53). Relative adrenal insufficiency, defined as an increase in serum cortisol level of less than or equal to 9 μ g/dL 1 hour after administration of 250 μ g of adrenocorticotropic hormone, is associated with worse outcomes, including higher mortality rates and prolonged requirements for vasopressors compared to an adequate cortisol response to adrenocorticotropic hormone (54).

A few small single-center studies of septic shock prolonged administration of low doses of hydrocortisone could decrease requirements for vasopressors and improve survival (55, 56). In a French multicenter, randomized control trial by Annane et al (57), administration of corticosteroids (hydrocortisone 50 mg intravenously every 6 hours and the mineralocorticoid, 9α -fludrocortisone 50 µg, once daily by mouth for 7 days) resulted in a reduction of 28-day mortality rate in the treatment group among patients who did not respond appropriately to adrenocorticotropic hormone. In contrast, there was no improvement in survival when patients with an appropriate cortisol response to adrenocorticotropic hormone were treated with steroids. However, a recent large, European multicenter trial (CORTICUS) failed to demonstrate survival benefit with steroid therapy in septic shock (58), though administration of steroids was associated with a faster resolution of septic shock. It seems that it is more complicated than anticipated in terms of steroid use. In the meanwhile, intravenous steroid is recommended to be given *only* to adult septic shock patients after confirming that their blood pressure is poorly responsive to fluid resuscitation and vasopressor therapy (2).

2.9 Glucose Control and Insulin Therapy

Current guidelines recommend that patients with severe sepsis and hyperglycemia who are admitted to the ICU should receive intravenous insulin therapy to control blood glucose levels < 150 mg/dL (2). And also, all patients receiving intravenous insulin receive a glucose source and blood glucose values should be monitored every 1–2 hrs until glucose values and insulin infusion rates are stable and then every 4 hrs thereafter.

2.10 Other Management Guidelines

The updated guidelines of Surviving Sepsis Campaign in 2008 also provide other evidence-based recommendations that are of great value in clinical practice:

---- using either intermittent bolus sedation or continuous infusion sedation with daily interruptions or lightening

---- Prophylaxis for deep vein thrombosis

---- Use of stress ulcer prophylaxis to prevent upper gastrointestinal bleeding using H2 blockers or proton pump inhibitors

---- In the absence of tissue hypoperfusion, coronary artery disease, or acute hemorrhage, target a hemoglobin of 7–9 g/dL

---- Steroids only in children with suspected or proven adrenal Insufficiency

---- Equivalency of continuous veno-veno hemofiltration or intermittent hemodialysis Although much more insights are certainly required to improve the treatment of sepsis, numerous studies now indicate that improvements in outcomes are possible when clinical practices that incorporate all known beneficial therapies are applied in a timely fashion.

2.11 Further Perspective

The optimal treatment strategies to patients with severe sepsis and septic shock are still evolving. Exploration of new therapies and modification of current recommendations are applicable.

As evidence-directed medicine and early goal-directed therapies are getting more and more acceptance, one could expect a standard procedure or protocol can be used to improve outcome in near future, with the consideration of tailored treatment on individual patients. A comprehensive use of evidence-based effective treatments and supportive maneuvers are mandatory to improve patient prognosis and long-term life quality. Moreover, a broad collaboration between intensivists and researchers from different backgrounds in Medicine are of great importance to better delineate complex mechanisms and discover effective treatments to this life threatening clinical syndrome.

In author's opinion, some specific treatment aspects of should be highlighted that could lead to a great advance in sepsis management in future:

1) Improving the immune status in septic patients with various pathophysiological phases

It is more and more recognized that sepsis does not simply represent an aggressive inflammatory response in which uncontrolled mediator-induced host response results in cell and tissue injury. The failure of numerous clinical trials with anti-inflammatory-specific agents led people to realize that most septic or ICU patients with a prolonged pathophysiological course actually develop a hypo-inflammatory or immunosuppressive status, in which apoptosis-induced decrease in immune effector cells, combined with the immunosuppressive effect f apoptotic cells contributes to the high morbidity and mortality. Consequently, novel therapeutic insights are under way aiming to improve apoptotic-inflammatory imbalance, using modulators of caspases and other components of cell-death pathway like myeloid differentiation primary response gene 88 blocker and apoptosis 2 inhibitor. Some of these trials have already shown existing efficacy in animal studies. We can expect that some important findings in this field will open an entirely novel epoch and bring substantial improvements in the clinical treatment of sepsis.

2) Targeting the sepsis-induced microcirculatory disturbances

The emergence of new imaging techniques and evaluation methods help to identify microcirculation as playing a central role in the pathogenesis of sepsis. Microcirculatory dysfunction persisting for extended periods of time can act as a motor driving the pathogenic effects of sepsis leading to organ failure. Any recruitment maneuver or combination therapies that can ensure adequate microcirculatory perfusion, reduce pathological shunting and support pump function should ideally improve cellular metabolism and organ function. Certainly in near future, microcirculatory assay will be more and more utilized as an integrative part of clinical diagnosis and treatment of sepsis that must greatly result in a better outcome.

3) Using the therapeutic agents with anti-oxidant and pro-oxidant properties

Plasma concentrations of endogeneous antioxidants are depressed during critical illness and especially during sepsis. These result from losses with biological fluids, low intakes, dilution by resuscitation fluids, as well as systemic inflammatory response

syndrome-mediated redistribution of micronutrients from plasma to tissues. Many clinical trials have demonstrated beneficial effects of supplementation of antioxidant nutrients, like glutamine, glutathione and selenium, aiming to prevent ROS and inflammatory mediator-induced cellular injury and organ dysfunction. Interestingly, another trend of opinion by Dr. Forceville suggested a controlled use of oxidant effect of some compounds, especially sodium selenite to act against over-activated inflammatory responses, inspired by exciting results from preclinical studies and successful clinical trials. This may open a novel path forward for the clinical treatment of human sepsis, though many questions must be answered before taken into reality.

An optimal use of anti-oxidant or pro-oxidant compounds will greatly contribute to the improved manipulation of septic patients. Surely, controversies remain regarding the indications for this therapy, the combination of antioxidants, the doses, and the timing of supplementation.

4) Reconsidering animal models and its limitations

Although animal models remain essential for the development of new therapies in sepsis, they invariably fail to replicate all complexities of human illness. It is generally recognized that there is no single perfect model of sepsis, but a larger number of diverse models that highlight some features of this syndrome while minimizing others. The design and report of preclinical studies are also highly variable, thereby limiting the effective comparison and integration among studies. Furthermore, like human sepsis, heterogeneity is an intrinsic feature of animal models of sepsis. Biological responses are subjected to many impact factors like species, strains with a species, gender and age, time of intervention, nutritional status, and so on. Consequently, Successful translation from preclinical models to clinically effective therapy is not common. Sometimes, it can even mislead the subsequent design of clinical trials.

Therefore, successful preclinical studies warrant the use of complementary animal models intended to solve specific questions of relevance to the clinical setting. They may be unable to demonstrate promise for a novel intervention, but to refine decisions concerning optimal patient populations for trials and safety profile. The use of clinical relevant models holds premise for a successful translation study. For example, using a

post-treatment design plus source control and supportive intervention is certainly superior to a single dose endotoxin model devoid of routine managements. The results from such models are more persuasive and instructive to the design of subsequent clinical trials. Establishing strict model standardizations between animal studies can also serve to improve data interpretation and integrate results into systematic data syntheses.

5) Optimizing available interventions

The current international guidelines in Surviving Sepsis Campaign in 2008 provide broad and evidence-based measures that concern almost all aspects of clinical management in sepsis. However, many questions are still unsolved. New interventions will be proven and practiced. But more importantly, available interventions may require rectification. The examples in this regard are quite a lot, like the use of activated protein C, steroids and vasopressors. Many intensivists believe that the successful management of a specific case of severe sepsis and septic shock more likely results from an optimal and tailored use of generally accepted interventions than developing a novel therapy. The latter often takes long time to modify and verify.

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Chapter III: The Establishment of Clinically Relevant Sepsis Models: Their Characteristics and Evaluation

3.1 Sepsis Models: Ideality and Reality

Animal models play a fundamental role in the development of new therapies for severe sepsis and septic shock, because they provide abundant information about the pharmacokinetics, toxicity, and systemic responses to a potential therapeutic agent or *in vivo* implantation systems that cannot be obtained by other methods (1). The ideality of preclinical research is the expectation that a therapeutic approach that modifies pathophysiological course in an *in vivo* animal model will have a favorable impact on human sepsis in the same manner. However, in most cases, animal models fail to replicate the complexity of human sepsis due to their oversimplicity and poor clinical relevance (2). For example, unlike most clinical circumstances, preclinical studies are usually characterized by using young and healthy animals, with an acute onset of insult, early use of the intervention, and absence of supportive therapies. Extensive utilization of so many different sepsis and endotoxin models implies that no single "perfect" model exists (Table 3.1).

Hence, in reality, the evaluation of a novel therapeutic approach mandates the use of diverse models ranging from simple rodent models to more clinically relevant large animal models with supportive interventions, each of which focuses on a specific aspect of *in vivo* pathophysiology (3). The insights provided by these models are instrumental in determining whether a novel therapeutic strategy is eligible for further evaluation in clinical trials and, if so, in which patient population their effects may be optimal.

The failures of direct extrapolation from animal studies to patients also highlight the importance of standardizing models with explicit designs and appropriate statistical methods for data analysis. Randomization also plays a crucial role in maximizing comparability among study groups of known and unknown variables that might impact on the response to the intervention (4).

In accordance with the above principles, we developed several animal models to achieve various objectives in our research into severe sepsis and septic shock.

3.2 An autologous fecal peritonitis model in sheep

3.2.1 Introduction

As a tame species of mammal, sheep have considerable similarity to humans in many aspects of physiology (Tables 3.2, 3.3, 3.4). In severe sepsis and septic shock, resuscitated sheep models manifest hyperdynamic cardiovascular changes and profound myocardial depression similar to what is seen in humans (5, 6). Organ dysfunctions are consistently apparent in these models, including respiratory failure, acute renal failure, coagulopathy, and metabolic abnormality (5, 7). These models have also been used to study cytokine release in sepsis (8, 9) and to evaluate pulmonary transvascular fluid flux during sepsis (10).

In summary, a sheep model of sepsis has many advantages that mimic the clinical situation. This model provides reliable information that is instrumental in providing a better understanding of the intrinsic mechanisms of sepsis pathophysiology and can be used in the development of novel therapeutic agents in the treatment of sepsis. In addition, a number of innovations have been made to the model that will allow the measurement of specific organ function.

3.2.2 Methods and Materials

Animal Preparation

All studies comply with the guidelines established by the animal management committee of the Free University of Brussels. Care and handling of animals are in strict agreement with the National Institutes of Health guidelines. All the sheep are female, fasted for 24 hours prior to experiments with free access to tap water.

Anesthesia

The animals are weighed before the experiments and then placed in the supine position. Premedication is induced with intramuscular injection of midazolam 0.6 mg/kg (Dormicum, Roche, Germany) and ketamine hydrochloride 20 mg/kg (Ketalar, Pfizer, Ireland) initially, followed by continuous intravenous administration of a combination of ketamine 5 mg/kg/hr, morphine 0.5 mg/kg/hr, midazolam 0.5 mg/kg/hr for sedation and pancuronium bromide 0.1 mg/kg/hr (Esmeron, Organon, Netherlands) for muscular
blockade after insertion of a peripheral vein cannula (Surflo IV Catheter, Terumo, Belgium).

Intubation and Mechanical Ventilation

Immediately after an intravenous bolus injection of pancuronium bromide 0.3 mg/kg and fentanyl 20 μ g/kg (Janssen, Belgium), the animals are intubated endotracheally (Tracheal Tube, 7.5-8.0, Hi-Contour, Mallinckrodt Medical, Ireland) and their lungs are mechanically ventilated (Servo ventilator 900C, Siemens-Elema, Sweden) with the following settings throughout the experiments: tidal volume = 10 ml/kg, positive end-expiratory pressure (PEEP) = 5 cmH₂O, inspired oxygen fraction (FiO₂) = 50%, inspiratory /expiratory time (I/E) = 1:2, square wave pattern. The respiratory rate is adjusted to maintain end tidal carbon dioxide pressure (PetCO₂, 47210 A Capnometer, Boehlingen, Germany) between 35 and 45 mmHg during the surgical operation.

Instrumentation

A drainage tube is advanced into the stomach cavity to empty gastric contents and prevent rumen distension. A Foley catheter (14F, Beiersdorf AG, Germany) is inserted into the bladder to record urine output hourly. After strict procedures for sterilization, the right femoral artery and vein are surgically exposed and isolated. An arterial catheter (6-Fr Vygon, Cirencester, UK) is introduced into the femoral artery and connected to a pressure transducer for arterial pressure monitoring. A 7-Fr pulmonary artery catheter (Swan-Ganz catheter, Edwards life Sciences, Baxter, Irvine, CA) is advanced through the femoral vein under guidance of pressure wave until it reaches the pulmonary artery and the pulmonary artery occlusion pressure (PAOP) is identified. A midline laparotomy is performed and, after cecotomy, 1.5 g/kg body weight of autologous feces is collected. The incision is sutured gently and the surrounding area disinfected with iodine. A plastic tube with a large diameter is placed in the abdominal cavity for later feces injection. A combination of 1 ml/kg/hr hydroxyethyl starch solution (HES, Voluven, Fresenius) and 1 ml/kg/hr Ringer's lactate is infused during the surgical procedure.

After the surgical operation, the animals are turned back to the prone position and stabilized for a short period to ensure that heart rate, cardiac output and intravascular pressures remain stable. Prior to randomization, the following criteria must be met: Mean arterial pressure > 70 mmHg, hemoglobin concentration ≥ 8 g/dl, peak airway pressure \leq

30 cmH₂O and PaO₂/FiO₂ ratio > 300 with PEEP=5 cm H₂O. Sheep that fail to meet these criteria are excluded.

Induction of Sepsis

After stabilization, baseline measurements are obtained and 1.5 mg/kg of feces are then injected into the abdominal cavity through the drainage tube to induce bacterial peritonitis.

Fluid challenge is considered with supplementary infusion of 2 ml/kg HES plus 2 ml/kg RL over 3 min whenever hypovolemia is suspected by a fall in cardiac output by more than 10%; a decrease in cardiac filling pressure below the baseline level; a decrease in mean arterial pressure below 70 mmHg; a decrease in mixed venous oxygen saturation to below 70% or a decrease in urine output to below 0.5 ml/kg/hr. Incremental administration of both Ringer's lactate and HES solutions with a step of 2 ml/kg/hr for each is performed if there is a positive response to the fluid challenge.

No antibiotics or vasoactive agents are given during the experiments. All animals are observed until spontaneous death or up to 30 hours after baseline.

Changes in methods between the protocols

We have made some improvements in the animal model over time, for example, using a combination of colloid and crystalloid for fluid administration so as to limit fluid volumes and decrease intra-abdominal pressure, and changing the anesthesia regimens to avoid possible adverse cardiovascular effects of xylazine (Table 3.5). Moreover, surgical procedures are performed in a sterilized manner, and a stabilization period is allowed to obtain a relatively good baseline.

3.2.3 Variables for Measurement and Calculation

Hemodynamic variables Mean arterial pressure (MAP), pulmonary arterial pressure (MPAP), central venous pressure (CVP), and pulmonary artery occlusion pressure (PAOP) are recorded from zero-calibrated pressure transducers at the mid-thoracic line at end-exhalation at baseline and hourly intervals thereafter until the end. Heart rate, core temperature, and cardiac output are continuously monitored and recorded at the same time. Peak airway pressure, plateau airway pressure, expiratory gas flow, and FiO₂ are recorded hourly from the ventilator panel. Stroke volume, cardiac index, systemic

vascular resistance, pulmonary vascular resistance, oxygen delivery and oxygen consumption, respiratory compliance and resistance are calculated with standard formulas.

Microcirculatory assay The microvascular network of the sublingual mucosa is studied with a Cytoscan ARII (Cytometrics, Philadelphia, PA) with a \times 5 objective providing \times 167 magnification. The device probe is gently placed without pressure on the lateral side of the tongue, in an area roughly 1.5 to 4 cm from the tip of the tongue. Saliva and other secretions are removed with gauze. Five areas are recorded on disk, using a computer and a video card (MiroVideo; Pinnacle Systems, Mountain View, CA). Microcirculatory analysis is based on a scoring system developed by De Backer et al. (11) and the MFI score (12).

Gas analysis Blood gases are measured with an automated analyzer (ABL500 Radiometer, Copenhagen), which has an internalized temperature correction program. Ventilatory conditions are maintained constant during the whole experiment, except that FiO_2 is adjusted to maintain PaO_2 between 80 and 120 mmHg and respiratory rate is adjusted to maintain $PaCO_2$ between 35 and 45 mmHg. Hemoglobin concentration and oxygen saturation are measured with an analyzer adapted for ruminant animals (OSM3 Radiometer, Copenhagen, Demark), which adjusts hemoglobin measurements to specific species.

Hematology and coagulation Hematological variables like leukocyte counts and platelet counts and coagulation variables including of prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, protein C levels and anti-thrombin III are routinely measured in a hospital laboratory.

Hormone assays Plasma cortisol levels are determined by a two-step competitive immunoassay (Elecsys Cortisol Immunoassay, Roche Diagnostics, Indianapolis, IN), with streptavidin micro-particles and electro-chemiluminescence detection. Results are determined using a calibration curve that is generated specifically on each instrument by a 2 point calibration and a master curve provided with the reagent bar code and are expressed in ng/mL. Total thyroxine (TT4) and total triiodothyronine (TT3) are measured by chemiluminescence using Elecsys 2010 technology (Roche Molecular Biochemicals GmbH and Hitachi, Ltd., Indianapolis, IN). The results are expressed in µg/dL. Urinary catecholamines are isolated by cation exchange chromatography on a Biorex resin and

filtrated on alumina. They are quantified by HPLC (high pressure liquid chromatography) and electrochemical detection. Analysis is performed on a Waters system equipped with a W2465 amperometric detector. Separation is obtained on an Atlantis C-18 (150 x 4.6mm, 3 μ m) column eluted with a pH 3.0 buffer-acetonitrile mixture (1:0.083, (v/v) and detected at 420 mV. The original value is corrected with the urine creatinine concentration of the same sample, which is considered as a surrogate of urine volume.

Selenium measurements Plasma selenium concentrations are measured by atomic absorption spectrometry with Zeeman background correction (Perkin-Elmer Z3030; Perkin-Elmer, Uberlingen, Germany) (13). Generally, 300 μ l plasma sample is diluted with 900 μ l buffer solution (1.57 g copper acetate monohydrate, 10.55 g Mg(NO₃)₂. 6H₂O, 1.5 Triton X100 and 3.0 ml HNO₃ 65 % dissolved and adjusted to 1 L with milliQ water). The selenium concentration is measured with a slope obtained from a gradient of calibrated standard Seronorm Trace Element (83 ± 3 µg Se/l).

Interleukin-6 measurements Plasma interleukin (IL)-6 levels are measured using the ELISA technique. Mouse anti-ovine IL-6 monoclonal antibody (Serotec MCA1659, Kidlington, UK), rabbit anti-ovine IL-6 polyclonal antibody (Serotec AHP424), and sheep anti-rabbit horseradish peroxidase (HRP) conjugated antibody (Serotec STAR54) are used. The monoclonal antibody (MCA1659) is used as a coating antibody with a concentration of 1:200, diluted in phosphate-buffered saline (PBS) and incubated overnight on 96-well enzyme-linked immunosorbent assay plates (Nunclone) at 4°C. After discarding the coating solution, 250 µL of blocking buffer (PBS/2% BSA) is added for 2 hours at room temperature and then rinsed three times with PBS/0.05% Tween. A 50-µL serum sample is diluted with 50 µL of PBS/1% BSA, placed in plate wells, and incubated for another hour at room temperature. The plates are then washed three times before adding 100 µl solution of polyclonal antibody (AHP424) diluted in 1:500 into each well and incubating for 1 hour at room temperature. After washing three times with PBS/0.05% Tween, 100 µl conjugation HRP (STAR54) diluted 1:10,000 in PBS/1% BSA is added to each well and incubated for 1 hour before 100µl TMB reagent is added and allowed to react for 10 min. Stop solution (100 μ l) is then added to each well and the optical density of the plate-wells is read on an enzyme-linked immunosorbent assay plate reader at 450 nm within 5 mins. The optic absorbance values from the treatment group and the controls are directly compared because the standard purified IL-6 protein in sheep is not available, which make the quantitative analysis of IL-6 concentration impossible.

Bacterial count Serial blood samples (1.5 mL) are obtained from each animal at 0 (base line), 4, 9, 10, 12 and 18 hours for quantitative blood cultures (Isolator 1.5, Oxoid, England). Total bacteria are quantified by spiral-plating 100µL of the samples on 2 CS by using an autoplate 4000 (spiral biotech, USA). Plates are incubated (35°C) for 48h. Colony counts are performed at 24 and 48 hours using an automated method (Qcount, spiral Biotech). Standard aerobic and anaerobic blood cultures (Bactec Plus, Becton Dickinson, US) are also performed at 0 and 12 hours and incubated in a Bactec 9240 system. Cultures are considered as negative if no bacterial growth is detected after 5 days.

3.2.4 Statistical analysis

Statistical analyses are performed using three software packages: JMP 5.0 statistical software (SAS Institute Inc, Cary, NC, USA), GraphPad Prism 4 statistical software (GraphPad Software Inc, San Diego, CA) and SPSS 13.0 for Windows (2004, SPSS, Chicago, IL). A Kolmogorov-Smirnov test is used to verify the normality of distribution of continuous variables. Baseline parameters are compared using analysis of variance (one-way ANOVA). The significance of differences in the repeated measurements of physiological variables is analyzed with either two-way ANOVA (group and time) or mixed-effect model (time, group and subjects nested in group as factors), followed by a modified t-test with Bonferroni correction. For missing values in some animals that die early, we use a last-observation-carried-forward method of imputation. Survival curves are constructed using the Kaplan-Meier method and compared using the log-rank test. Statistical tests are two-tailed and a p value less than 0.05 is considered statistically significant.

3.2.5 Results

1) Hemodynamics

In this acute peritonitis model, arterial pressure drops progressively and hypotension develops after 10 to 12 hours (defined as systemic blood pressure<90 mmHg or mean arterial pressure <75 mmHg). Cardiac output increases dramatically in the early stages after fluid resuscitation and decreases progressively in later stages. Heart rate increases

and remains high throughout the experiments. Left ventricular stroke work declines significantly over time, indicating that cardiac dysfunction develops after induction of sepsis. Systemic vascular resistance decreases and pulmonary vascular resistance increases slightly over time (Figure 3.1).

2) Microcirculation OPS analysis

Sepsis induces marked microcirculatory alterations as indicated by significantly decreased total perfused vessels density and perfused capillary density. The proportion of perfused capillaries and the microcirculatory flow index are significantly reduced over time (Figure 3.2).

3) Hematology and coagulation

Sepsis induces marked leukopenia and thrombocytopenia in this model (Figure 2.3). Prothrombin time (PT) and activated partial thromboplastin time (APTT) increase over time, whereas anti-thrombin III, D-dimer and protein C concentration decrease progressively during the experiments (Figure 3.4).

4) Hormonology

The concentration of plasma cortisol increases at an early phase, followed by a decline over time. Total T3/T4 ratio increases progressively with the development of sepsis (Figure 3.5), suggesting an acute phase response.

Urine catecholamine constituents increase to varying degrees over time, suggesting that catecholamines are released and excreted in response to sepsis (Figure 3.6).

5) Bacteriology

In this acute peritonitis model, sepsis is associated with severe bacteremia (Figure 3.7). Infection is predominately caused by Gram-negative *E.coli* strains.

6) Survival time

In this lethal sepsis model, animals developed 100% mortality rate in the control group, with a median survival 18.6 hours (Figure 3.8, data from 14 individuals).

3.2.6 Evaluations of the experimental ovine model of septic shock

1) Advantages

Most of the clinical manifestations in severe sepsis and septic shock can be observed in this model, including a hyperdynamic hemodynamic response, lactic acidosis, coagulation abnormalities and organ failure

- The large animal model allows reliable hemodynamic monitoring and multiple blood samplings, which are crucial especially when obtaining pharmacodynamic and pharmacokinetic data for novel therapeutic agents
- > Peritoneal contamination with mixed flora resembles the clinical situation
- > Simple procedure and no need to prepare and quantify bacteria
- > Reproducible responses and low variability among individuals
- ➤ Use of survival time as the major observation

2) Disadvantages

- ➢ Small sample sizes
- > No supportive interventions except for fluid resuscitation
- Impossible to control the magnitude of the septic challenge when using autologous feces to induce peritonitis.

Indeed, it is difficult to quantity the magnitude of septic challenge in any CLP models. However, we once investigated about bacteria loads in feces samples from some experimental animals. It showed that bacteria load in feces was approximately in the same magnitude of order among individuals. Severe bacteremia is also comparably developed among septic animals in this model. Moreover, randomization can obviously partly help to cope with variability.

> The possible adverse cardiovascular effect of ketamine hydrochloride.

In this model, we use a relative large dose of ketamine for anesthesia, 5 mg/kg/hr. Although Ketamine hydrochloride was reported to have no significant adverse effects on cardiovascular response in animals (14, 15), the use of large dose could elicit an initial neuroadrenal stimulant effect (16) and subsequent depressed effects due to a depletion of catecholamine over an excess of stimulation (17). This adverse effect may be covered by sepsis-induced cardiovascular dysfunction and fluid resuscitation. We agree that high dose of ketamine administration may be suboptimal in this model. But it should be of no significant impact to the results because all the animals receive the same regimen of anesthesia.

Insufficient examination of immunology and molecular biology because of shortage of species-specific antibodies or reagents.

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Table 3.1 Widely Used Sepsis Models

Simple models with acute challenges		
Systemic challenges		
Endotoxins or intravenous microorganisms		
Local challenges		
Viable microorganisms (pneumonia, peritonitis and soft tissue infections)		
Complex acute challenges		
Cecal ligation and perforation		
Sequential or two-hit models		
Chronic model with intensive support and monitoring replacement		
In silico modeling		
Genetic modified rodent models		

Table 3.2 Normal values of hemodynamic variables in humans and sheep. Sheep values were obtained from 40 individual animals at baseline or from references.

Variables	Normal values in humans	Measured values in sheep	
Cardiac index	2.5-4.7 L/min/m ²	3.9±0.9 L/min/m ²	
Heart rate	58-86 beats/min	97±17 beats/min	
Mean arterial pressure	84-100 mmHg	100±15 mmHg	
Mean pulmonary arterial pressure	20-25 mmHg	16.5±0.6 mmHg	
Pulmonary arterial occlusion pressure	4 -12 mmHg	4±1 mmHg	
Central venous pressure	2-6 mmHg	2±1 mmHg	
Stroke volume index	40-50 ml/m²/beat	40.4±7.9 ml/m ² /beat	
Left ventricular stroke work index	43-61 g M/m ²	52.7±11.6 g M/m ²	
Systemic vascular resistance index	1800-2800 dynes*sec/cm ⁵ /m ²	1993±385 dynes*sec/cm ⁵ /m ²	
Pulmonary vascular resistance index	200-350 dynes*sec/cm ⁵ /m ²	199±29 dynes*sec/cm ⁵ /m ²	
Coronary perfusion pressure	60-80 mmHg		
Intra-abdominal pressure	2-10 mmHg	4±2 mmHg	
Oxygen consumption	200-250 ml/min	150±29 ml/min	
Oxygen extraction ratio	22-30%	22-30% 25.8±8.3%	

Table 3.3 Normal values of coagulation and hematology variables in humans and sheep. Sheep values were obtained from 40 individual animals at baseline or from references.

Variables	Normal values in humans Measured values in shee		
Prothrombin time	11-13 sec	15±2 sec	
Activated partial thromboplastin time	30-45 sec	33±4 sec	
Anti-thrombin III	70-130%	71±6 %	
Protein C concentration	>80%	32±4 %	
D-dimer concentration	0-1mg/L	0.22±0.016 mg/L	
Fibrinogen concentration	2-4 g/L	2.3±0.3 g/L	
White blood cell $(10^{3}/\mu l)$	4-10	4.8±1.3	
Neutrophil count (10 ³ /µl)	2-7	3.2±0.8	
Lymphocyte count $(10^{3}/\mu l)$	0.8-4	$1.9{\pm}0.5$	
Red blood cell (10 ⁶ /µl)	Male: 4.0-5.5	47.19	
	Female: 3.5-5	4./±1.8	
Platelet count $(10^3/\mu l)$	100-300	345±145	
Hemoglobin (g/dl)	Male: > 13.2 ¹⁸	12.1.0	
	Female: > 12.2 ¹⁸	13±1.0	

Table 3.4 Normal values of blood gas analysis and electrolytes in humans and sheep. Sheep values were obtained from 40 individual animals at baseline or from references.

Variables	Normal values in human Measured values in sheep	
Arterial pH	7.35-7.44	7.41±0.04
Arterial PCO ₂ (mmHg)	35-35	36±3
Arterial PO ₂ (mmHg)	80-120	111±12
HCO ₃ ⁻ (mmol/L)	23-27	23.3±4.3
Arterial oxygen saturation %	95-100	98.5±2.3
Na ⁺ (mmol/L)	135-145	138±8.7
K ⁺ (mmol/L)	4.1-5.6	4.3±0.8
Ca^{2+} (mmol/L)	2.25-2.75	1.23±0.2
CL ⁻ (mmol/L)	98-106	115±17
Glucose (mmol/L)	4.4-6.7	4.2 ± 1.4

	rhAPC protocol	Hypercapnia protocol Selenite protocol	
Pre-medication	Ketamine and xylazine	Ketamine and midazolam	
Anesthesia and muscular blockade	Fentanyl and midazolam Pancuronium bromide	Fentanyl, midazolam and morphine, pancuronium bromide	
Digestive drainage	No	Yes	
Sterilization operation	No	Yes	
Stabilization period	No	Yes	
Feces amounts	0.5 g/kg body weight	1.5 g/kg body weight	
Fluid types	Ringer's lactate	Ringer's lactate and voluven	
Fluid resuscitation goal	Baseline PAOP	Maintain CO, MAP, urine output and hemoglobin	
IAP control	no	yes	

Table 3.5 Changes in the animal model among the protocols



Figure 3.1 Changes in systemic mean arterial pressure, cardiac index, heart rate, left ventricular work index, systemic vascular resistance and pulmonary vascular resistance over time in control sepsis. Values are expressed mean \pm SD.



Figure 3.2 Changes in total vessel density, total capillary density, total perfused vessel density, perfused capillary density, proportion of perfused capillary and microcirculatory flow index over time in the control sepsis. Values are expressed mean \pm SD.





Figure 3.3 Changes in leukocyte count and platelet count over time. Values are expressed mean \pm SD.

Figure 3.4 Changes in prothrombin time (PT) and activated partial thromboplastin time (APTT), anti-thrombin III, D-dimer and protein C concentration over time. Values are expressed mean \pm SD.



Figure 3.5 Changes in plasma cortisol and total T3/T4 ratio over time. Values are expressed mean \pm SD.



Figure 3.6 Changes in concentrations of urine norepinephrine, epinephrine, dopamine and their metabolite over time. The results are corrected for urine creatinine concentrations. Values are expressed mean \pm SD.



Figure 3.7 Bacteremia evolution in the ovine model of acute peritonitis.



Figure 3.8: Kaplan-Meier survival plot in control peritonitis in sheep

Chapter IV Beneficial effects of recombinant human activated protein C in a ewe model of septic shock

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4.1 Introduction

Severe sepsis and septic shock remain serious problems worldwide, with resultant intensive care unit (ICU) mortality rates generally in excess of 30% (1). There is, therefore, a great need for new, effective therapies to be developed. Administration of recombinant human APC (rhAPC, drotrecogin (activated)) in patients with severe sepsis has been shown to reduce mortality and improve organ function in humans (2-4). Administration of APC has been shown to block the lethal effects of *Escherichia coli*-induced septic shock and multiple organ failure (5), improve capillary perfusion from lipopolysaccharide-mediated microcirculatory dysfunction (6), prevent apoptosis in a variety of cell lines (7), and attenuate intestinal ischemia/reperfusion-induced injury (8). Recent studies have further explored the complex actions of APC mediated by the endothelial protein C receptor (EPCR) and protease-activated receptor (PAR-1) (9). APC can improve lung endothelial permeability via sphingosine-1-phosphate (S1P) transactivation and Rac1 activation (10).

4.2 Objective

Doubts about the beneficial protective effects of APC have persisted and been refuelled by the recently published, negative trials in less severely ill patients (11) and in children (12). Infusion of rhAPC in human models of endotoxemia was also not shown to have any significant affect on proinflammatory responses, or on thrombin generation (13, 14). In the light of these ambiguities and uncertainties, the manufacturer of drotrecogin alfa (activated) has decided to conduct another phase III clinical trial to re-evaluate the drug's efficacy in patients with severe sepsis and septic shock.

On the basis of these observations and continuing controversy, it seems important to reexplore the effects of APC in relevant animal models. We, therefore, used a clinically relevant sepsis model to investigate whether APC could have beneficial therapeutic effects in septic shock.

4.3 Experimental protocol

After the surgical procedure, the sheep were turned back into the prone position and baseline measurements were obtained. After a two-hour observation period, the sheep were randomly divided into two groups to receive crystalloid fluid alone (control group, n=9), or in combination with APC (APC group, n=9, APC kindly provided by Eli Lilly and Co, Indianapolis, USA), which was continuously infused at a dose of 24 μ g/kg/hr throughout the experiment. Ringer's lactate solution was infused at a rate titrated to maintain the PAOP at baseline level. All animals were observed until spontaneous death (or a maximum of 28 hours) after the induction of peritonitis.

4.4 Results

There was no difference in the animal weights or in any other measured variable at baseline between the APC and the control group (Table 3.1).

The fall in arterial pressure over time was less in the APC group than in the control group (Figure 1). The increase in mean pulmonary artery pressure over time was less in the APC group than in the control group (p<0.05, Figure 1). Differences in cardiac index or vascular resistance did not reach statistical significance (Figure 3.1).

There were no significant differences between the groups in cardiac filling pressures or cumulative fluid intake (Figure 3.2). Urine output decreased over time in both groups, but less in the APC group than in the control group (p<0.05). The fluid balance, determined by subtracting hourly urine output from total fluid volume infused each hour, was less positive in the APC group than in the control group (p<0.05, Figure 3.3).

Increases in airway pressure and respiratory system resistance were lower in the APC than in the control group. Similarly, the thoracopulmonary compliance and lung PaO_2/FiO_2 ratio were better maintained in the APC than in the control group (p<0.05, Figure 3.4). At postmortem examination, the lung wet/dry ratio was significantly lower in the APC than in the control group (p<0.05).

Colloid oncotic pressure (COP) decreased over time in both groups, but less in the APC group than in the control group (p<0.05, Figure 3.5). Blood lactate concentrations increased markedly in the control group but much less in the APC group (p<0.05, Figure 3.6). There were no significant differences between the two groups in BUN, creatinine, AST, ALT, serum bilirubin concentrations, or urine analyses (data not shown).

PT, APTT, and D-dimer concentrations progressively increased in the control group, while anti-thrombin, protein C concentration, and platelet count progressively decreased. These changes were less notable in the APC treated animals (p<0.05, Figure 3.7).

Survival was significantly longer in the APC group than in the control group (median survival: 27 hrs vs. 20 hrs, p<0.05, Figure 3.8, shown in the Kaplan-Meier survival plots).

4.5 Discussion

The present study shows that APC administration exerted beneficial effects in a clinically relevant model of septic shock secondary to fecal peritonitis. APC administration postponed the occurrence of hypotension associated with septic shock, improved arterial oxygenation and pro-coagulant state, maintained renal function, and prolonged survival time. APC appeared to limit capillary leakage, as manifested indirectly by a maintained COP and a lower lung wet/dry ratio at postmortem examination.

The acute hemodynamic effects of APC have not been well studied. We observed a well-maintained arterial pressure in APC-treated animals. This was not due to any difference in cardiac preload as both groups received a similar amount of fluid and there were no differences in cardiac filling pressures. Indeed, Maybauer et al. (16) showed recently that APC significantly improved cardiac performance in an ovine model of septic shock after severe smoke inhalation. APC infusion was also shown to increase mean arterial pressure after endotoxin exposure in volunteers (13), and to decrease the norepinephrine dose required to maintain arterial pressure, in patients with septic shock (17). Although the exact mechanisms underlying these hemodynamic effects are not well defined, APC may have an inhibitory effect on nitric oxide (NO) production (18). In the absence of a significant impact on BUN and creatinine levels in this acute model, the better maintenance of diuresis in the APC-treated animals was probably the result of this hemodynamic improvement.

Acute lung injury in severe sepsis is due to a complex interaction between cytokines, oxygen free radicals, coagulation, and fibrinolysis (19). Decreased APC generation in the intra-alveolar space of patients with interstitial lung disease is proportional to the increased intra-alveolar activation of the coagulation system and enhanced deposition of collagen in the lung (20). APC administration may attenuate human endotoxin-induced pulmonary inflammation via inhibition of neutrophil accumulation and chemotaxis (21, 22). APC can also protect human lung endothelial cells after thrombin-induced injury (10). Maybauer et al. (16) recently reported that APC improved arterial oxygenation and lung mechanics in an ovine model of septic shock after severe smoke inhalation, and Waerhaug and colleagues reported similar findings in oleic acid- and endotoxin-induced lung injury in sheep (23, 24). APC-treated animals in our study had significant improvements in thoracopulmonary compliance and arterial oxygenation as a result of decreased capillary leakage and lung edema. The improvement in the endothelial permeability with APC treatment is suggested by the increased COP, the decrease in the postmortem lung wet/dry ratio, and the better maintenance of fluid balance in the APC treated animals. In this regard, it is worth mentioning the opposite findings in the study by Dr. Guery et al (44), in which the administration of supra-dose of APC in very early phase of bacterial lung injury was associated with increased alveolarcapillary permeability, lung edema formation and inflammatory response. The author concluded that APC impaired the initial favorable effect of coagulation activation at the alveolar levels and resulted in a loss of inflammation compartmentalization. However, several differences in experimental design may account for the apparent opposite findings. Compared to ours, this model is challenged with a large dose of bacterial intra-tracheal instillation, which logically results in a more acute and severe injury. Single bacterium action, short observation period and not a post-treatment design make this study less comparable to ours. Moreover, septic insults differ between the two studies. It logically speculates that local inflammatory response must be more severe when lung injury is caused by direct insult compared to non-pulmonary etiology. I note that coagulation

parameters are of no differences at systemic level in this study at 4 hours. But at alveolar level, APC inhibited thrombin formation dramatically. This may not the case in our study. Finally, I wonder if supra-dose of APC may have additionally adverse effects such as intra-alveolar hemorrhage, since that it is less clinical relevant.

Disseminated intravascular coagulation (DIC) is associated with increased mortality rates in septic patients (25, 26). Our model is characterized by severe coagulation abnormalities. APC administration was associated with a substantial improvement in these alterations, as indicated by shorter PT, shorter APTT, higher antithrombin activity, and lower D-dimer levels. These findings could be expected on the basis of APC's anti-thrombotic activity, inhibiting activated factors V and VIII, and its indirect fibrinolytic activity, inhibiting the action of plasminogen activator inhibitor (PAI-1) (22). The platelet count was also higher in the APC-treated than in the control animals, probably as a result of less severe DIC. It is worth mentioning that platelet count dropped early and dramatically in the septic animals before overt DIC occurs, indicating an acute inflammatory response. Platelet activation and aggregation are believed to result from sepsis-induced inflammation activation and be independent of coagulation process (42). Blood protein C concentrations fell less in the animals that received APC. The reduction in blood protein C levels in sepsis may be the result of several events, including reduced liver synthesis, shedding by the EPCR receptor, and conversion of protein C to APC. Low protein C levels are observed in the majority of patients with severe sepsis and are related to a poor prognosis (27). The reduction in blood D-dimer levels in the treated sheep in our study was also consistent with that observed in the PROWESS study (2), indicating its anti-thrombotic activity. Notely, a decreased D-dimer level observed in late stage indicated that fibrinolytic process is largely inhibited in septic animals. In sepsis, the early fibrinolytic response to acute inflammation is the release of plasminogen activators like t-PA and u-PA. However, this increase in plasminogen generation is neutralized by a delayed but sustained production of plasminogen activator inhibitor-1, resulting in an inhibition of fibrinolysis (43).

The protective mechanisms of action of APC have been only partially uncovered. APC has more beneficial effects than other natural anticoagulants, like antithrombin and tissue factor pathway inhibitor (28, 29). A number of in vitro and in vivo studies have indicated that APC also has anti-inflammatory and anti-apoptotic properties (30, 31). APC can exhibit direct anti-inflammatory properties by inhibiting tumor necrosis factoralpha (TNF- α) and macrophage migration inhibitory factor (MIF) production, blocking leukocyte adhesion to selectins, and decreasing nuclear factor-kB (NF-kB) activation in target cells (30, 32). APC can improve the visceral microcirculation by attenuating leukocyte-endothelial interactions and leukocyte rolling (6, 33); the underlying mechanism seems to be independent of thrombin inhibition (34). It appears that most of APC's anti-inflammatory activities are related to the EPCR-mediated pathway (35, 36). EPCR is involved in the translocation of APC into the nucleus to affect gene regulation and then downregulate inflammatory cytokine formation, such as TNF- α and interleukin (IL)-6 (37). The soluble form of EPCR, shed mainly from cell surfaces of endothelial cells or monocytes into the circulation by metalloproteinase-mediated cleavage, can bind to proteinase 3 from activated neutrophils, which can then bind to the neutrophil integrin, Mac-1, and this interaction decreases tight adhesion of neutrophils to the activated endothelium (38). Moreover, a novel mechanism of EPCR-mediated protection via transactivation of S1P has been demonstrated to be involved in APC's effects on preventing increased lung endothelial cell permeability in vitro and maintaining vascular integrity (10). Unfortunately, measurements of most inflammatory mediators are presently impossible in sheep.

Moreover in this study, we did not find that administration of recombinant human APC was associated with allergic response. rhAPC was shown to have protective effects in diverse species including rats (18, 39), mouse (40), rabbits (41), and indeed sheep (16, 23, 24). There is no specific report of immunologic problem of APC on animals in literatures.

There are some limitations to this study. First, it was conducted in previously healthy animals under highly controlled circumstances, in contrast to the clinical situation where patients often have underlying illness and other risk factors. Second, the fact that our model is acute and lethal makes it difficult to comment on the longer-term effects of APC. Third, the variability of a septic challenge associated with autologous feces is an intrinsic drawback in any CLP model, although the approximate bacterial load among sheep in this experimental model is relatively constant.

In summary, the beneficial effects of APC in this sheep model were quite dramatic when compared to those observed in clinical trials. Although data from sheep cannot be immediately extrapolated to humans, these observations do support a role for APC administration in septic shock.

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4.6 References

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Table 4.1 The physiological variables at baseline

Variable	APC group	Control group	P value
Body weight (kg)	30.7±1.4	31.0 ± 3.1	0.35
Temperature (°C)	38.5±0.5	38.5 ± 0.6	0.94
Mean Airway Pressure (cmH ₂ O)	16.3 ± 1.6	16.7 ± 1.8	0.6
HR (beats/min)	104. ±14	95±18	0.23
CI (l/min/m²)	4.4±1.2	4.1 ± 0.6	0.59
MAP (mmHg)	115 ± 10	114 ± 15	0.85
MPAP (mmHg)	17.4 ± 0.9	15.8 ± 1.1	0.23
lactate (mmol/l)	1.2 ± 0.3	1.1 ± 0.2	0.18
PaO ₂ /FiO ₂	348 ± 43	354 ± 62	0.81
Arterial pH	7.39 ± 0.05	7.42±0.03	0.22
PaCO ₂ (mmHg)	38.8±5.6	37.8±5.9	0.7



Figure 4.1 Changes in systemic mean arterial pressure (*MAP*), mean pulmonary arterial pressure, cardiac index, and left ventricular stroke work index (*LVSWI*) over time. Values are mean \pm SD. *Circles*, control group; *triangles*, activated protein C group. **p*<0.05 between activated protein C and control groups. Data are truncated at 20 hrs in the control group due to the small number of animals after this time point.



Figure 4.2 Changes in right atrial pressure (*RAP*), pulmonary artery occlusion pressure (*PAOP*), and cumulative fluid volume over time. Values are mean ± SD. *Circles*, control group; *triangles*, activated protein C group. There were no statistically significant differences between groups for these variables.



Figure 4.3 Changes in fluid balance per hour and urine output over time. Values are mean \pm SD. *Circles*, control group; *triangles*, activated protein C group. **p*<0.05 between activated protein C and control groups. Data are truncated at 20 hrs in the control group due to the small number of animals after this time point.



Figure 4.4 Changes in respiratory variables over time. Values are mean \pm SD. *Circles*, control group; *triangles*, activated protein C group. *p< 0.05 between activated protein C and control groups. Data are truncated at 20 hrs in the control group due to the small number of animals after this time point.



Figure 4.5 Changes in colloid oncotic pressure (*COP*) between two groups. Values are mean \pm SD. Values are mean \pm SD. *Circles*, control group; *triangles*, activated protein C group.**p* <.05 between activated protein C and control groups.



Figure 4.6 Changes in blood lactate concentration in relation to time. Values are mean \pm SD. *Circles*, control group; *triangles*, activated protein C group. **p*<0.05 between activated protein C and control groups. Data are truncated at 20 hrs in the control group due to the small number of animals after this time point.



Figure 4.7 Changes in coagulation variables over time. *Circles*, control group; *triangles*, activated protein C group. *PT*, prothrombin time; *APTT*, activated partial thromboplastin time; *AT*, antithrombin; *p < .05 between activated protein C and control groups.



Figure 4.8 Kaplan-Meier graphs of survival in the two groups. *Solid line*, activated protein C group; *dashed line*, control group. Treatment with activated protein C was associated with a significantly longer survival (p = 0.002 by stratified log-rank test).
Chapter V Acute hypercapnia improves indices of tissue oxygenation more than dobutamine in Septic Shock

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5.1 Introduction

Hypercapnia is commonly accepted as a consequence of protective ventilation strategies, in which tidal volume is reduced to minimize the excessive lung stretch in patients with acute lung injury (hence the term "permissive hypercapnia"). Unlike severe and uncompensated hypercapnia, which can be associated with harmful events, such as the development of pulmonary hypertension, elevation of intracranial pressure (1), impairment of myocardial contractility in vitro (2), and prolonged muscular weakness (3), permissive hypercapnia is usually moderate and relatively well tolerated, and may be associated with an independent survival benefit (4). Hypercapnia and respiratory acidosis have been shown to have beneficial effects in diverse pathophysiological settings, including ischemia-reperfusion (5, 6), ventilator-induced lung injury (7, 8), and sepsis (9). Hypercapnia can exert multiple beneficial effects at the systemic, cellular, and molecular levels that may improve ventilation perfusion matching in the injured lungs (10, 11), attenuate cytokine release and free radical production (12), and suppress lactic acidosis (13). Hence, one may speculate that acute hypercapnia, whether induced by carbon dioxide (CO_2) retention following a reduction in minute ventilation or by adding CO_2 to the inspired gas, may be beneficial.

Acute cardiovascular responses to hypercapnia have been well studied in physiological and pathophysiological conditions. Hypercapnia has direct inhibitory effects on myocardial contractility in normal and failing hearts (14-16), but these direct effects are counteracted by neuroadrenal stimulation (17, 18), so that the overall effects of hypercapnic acidosis are characterized as increased heart rate and cardiac output, increased pulmonary and decreased systemic vascular tone, and possible venoconstriction (14, 19-21). Based on these findings, one may suppose that the acute cardiovascular

effects of hypercapnia would be quite similar to those induced by dobutamine, a predominantly β -adrenergic agent. This may be particularly relevant in the presence of hemodynamic instability associated with respiratory failure. However, no study has really addressed this issue.

5.2 Objective

We'd like to investigate the pulmonary and systemic effects of hypercapnia and compared them to those of dobutamine in the condition of septic shock. We selected a large animal model to facilitate vascular catheterization and, hence, hemodynamic measurements. Blood sampling is also more convenient and reliable in such animals. We chose to add CO_2 to the inspiratory gas mixture to induce arterial hypercapnia in order to avoid the additional effects of a change in ventilatory settings. Thus, the effects of hypercapnia could be separated from those of protective ventilation strategies. Some of the results of these studies have been previously reported in the form of an abstract (22).

5.3 Experimental protocol

After the surgical procedure, the sheep were turned back to the prone position and stabilized for a short period to ensure that heart rate, cardiac output, and intravascular pressures remained stable. Feces (1.5 mg/kg) were then injected into the abdominal cavity through the drainage tube to induce bacterial peritonitis and the animals were randomized to one of three groups: hypercapnia, dobutamine, and control. In the hypercapnia group, PaCO2 was increased to a level between 55 and 65 mmHg by adding CO_2 at various concentrations from 2 hours after the induction of peritonitis until the end of the experiment. After the desired PaCO2 was reached, the FiCO2 was unchanged for the rest of the experiment. In the dobutamine group, dobutamine was administered at a fixed dose of 7 µg/kg/min from 2 hours after the induction of peritonitis until the end of the experiment. In the dobutamine group and the control group, PaCO2 was maintained between 35 and 45 mmHg throughout the experiment. In all three groups, PaCO2 was maintained in the desired range by altering the respiratory rate. No antibiotics or antipyretic agents were administered during the experiments.

5.4 Results

There were no significant differences in baseline body weight, vital signs, or other physiological variables among the groups (Table 4.1).

The mean FiCO₂ was 4.2% in the hypercapnic group. PaCO₂ was significantly higher and arterial pH significantly lower in the hypercapnia group than in the other groups (Figure 4.1). Animals in the hypercapnia and dobutamine groups had higher heart rates and cardiac outputs than the control group (p<0.05). Tachycardia was more pronounced in the dobutamine group than in the hypercapnia group (p<0.05) (Figure 4.2). There were no significant differences in arterial pressure, pulmonary artery pressure, systemic and pulmonary vascular resistance, or left ventricular stroke work at any time points among the three groups.

PaO₂/FiO₂ was higher in the hypercapnic group than in the control group (p<0.05); the alveolar-arterial oxygen partial pressure difference $P(A-a)O_2$ and the venous admixture (Qs/Qt) were lower in the hypercapnic group than in the other two groups (p< 0.05) (Figure 4.3). However, the changes in airway pressure, respiratory system compliance and resistance were similar in all groups. There were no differences in core temperature among groups. There were no differences in fluid infusion rates over time among groups, but postmortem lung wet/dry ratio was lower in the hypercapnia group than in the control group.

The PvO₂ was significantly higher in the hypercapnic than in the other two groups (p< 0.05); there was no difference in the SvO₂ among groups (Figure 4.4). The arterial lactate concentration was significantly lower in the hypercapnia and dobutamine groups than in the control group (p<0.05). Arterial IL-6 levels were lower in the hypercapnic than in the control group (p<0.05) (Figure 4.5).

There was no significant difference in survival time among the three groups (19.2 \pm 2.2 hr in the hypercapnic group vs. 18.1 \pm 3.1 hr in the dobutamine group vs.18.0 \pm 2.3 hr in the control group, p=0.36).

5.5 Discussion

Our study is the first to investigate the independent effects of acute hypercapnia in experimental septic shock. As hypercapnia is known to cause neuroadrenal stimulation and vasodilatation, we can expect that these effects would be comparable to those of dobutamine, a commonly used inotropic agent with predominant β 1-receptor agonist properties. Indeed, in this acute model of septic shock, hypercapnia induced similar hemodynamic responses to dobutamine, and improved systemic oxygen delivery and suppressed hyperlactatemia, even though these potentially beneficial effects did not translate into a prolonged survival time. Given that all groups were ventilated with the same tidal volume and PEEP, this limits any potential influence of lung stretch or atelectasis as viable explanations for the observed differences. We kept FiCO2 around 4.1% to obtain the desired PaCO₂ in the range of 55-65 mmHg.

Hemodynamic Effects

Both hypercapnia and dobutamine induced similar elevations in cardiac output and heart rate. Unlike the inotropic action exerted by a β -receptor agonist, the direct effect of hypercapnia on the heart is to reduce contractility (14-16). However, this effect can be negated by a combination of several mechanisms: First, hypercapnia can initiate a sympathetically mediated release of catecholamines due to neuroadrenal stimulation (14, 23, 24); second, hypercaphic acidosis induces ATP-sensitive K^+ channel-mediated vasodilation (25); third, preload may be increased via venoconstriction in acidemia (14, 17). Therefore, cardiac output may increase as a result of increased preload, decreased afterload, and increased contractility. Although some studies have suggested that acidosis rather than increased CO_2 concentration may have the predominant beneficial role (14, 15, 26), the evidence indicates that respiratory acidosis is associated with more beneficial effects than a similar degree of metabolic acidosis (27, 28). In our study, systemic vascular resistance decreased more rapidly in the two treatment groups than in the control group, but also decreased late in the control group because decreased vascular tone is one important characteristic of our model of fluid-resuscitated septic shock. Although acute hypercapnic acidosis can increase pulmonary vascular tone by vasoconstriction (6, 15), we did not observe this effect, probably because the hypercapnia was only moderate. Two recent studies (19, 29) showed that hypercapnic acidosis may have beneficial effects on pulmonary hypertension and vascular remodeling induced by chronic hypoxia via antioxidant properties and consequent decreased generation of oxidant-induced pulmonary vasoconstrictors, such as peroxynitrite and endothelin-1. Whether or not these protective mechanisms can develop more acutely could not be explored in this study.

Gas exchange and oxygenation

Hypercapnic acidosis obtained by adding CO_2 to the inspired gases can have advantages over a reduction in tidal volume, since it can cause a more homogenous hypercapnia and acidotic environment in the lung alveoli (30). Lower tidal volumes may impair gas exchange and increase intrapulmonary shunt in critically ill patients (31, 32). In contrast, inspiring CO₂ may improve ventilation perfusion matching and arterial oxygenation (10, 11, 33, 34) in normal or injured lungs. The lower alveolar-to-arterial oxygen gradient and venous admixture in our study reflect an improvement in ventilation/perfusion matching and less lung edema formation. Our findings differ from those by O'Croinin et al., in which hypercapnia did not reduce the magnitude of the lung injury induced by intratracheal instillation of *Escherichia coli* (35). It is possible that in their study, the shorter observation period, the high severity of lung injury induced, and the lack of fluid resuscitation may have limited the beneficial effects of hypercapnia from occurring or being observed. Indeed, hypercapnic acidosis has been demonstrated to enhance hypoxic pulmonary vasoconstriction (17) and to decrease regional pulmonary blood flow heterogeneity (36). Although hypercapnia in our experiments did not influence lung compliance or airway pressure globally, we cannot rule out the possibility that CO_2 could have more local bronchodilating effects (37, 38) on dependent areas with altered ventilation/perfusion matching and regional bronchoconstriction.

Another interesting finding was the higher PvO2 but not SvO2 in the hypercapnia group than in the other two groups, probably related to a facilitated release of oxygen in the acidotic environment (Bohr Effect), improved arterial oxygenation due to CO_2 induced amelioration in ventilation/perfusion matching, and decreased oxygen demands of the tissues in the acidotic environment. By suppressing hypoxic pulmonary vasoconstriction, an increased PvO₂ associated with a high cardiac output can increase the shunt fraction and impair oxygen exchange (32), but dobutamine administration induced similar effects.

In addition to these effects on the lung, some studies (21, 39) have reported that hypercapnia may improve cerebral blood flow and tissue oxygenation. Thus, hypercapnia may be beneficial to the central nervous system in the absence of intracranial hypertension.

Anti-inflammatory and anti-oxidant effects

In this study, serum IL-6 concentrations were significantly lower in the hypercapnic animals than the controls, suggesting an anti-inflammatory effect of hypercapnic acidosis. Hypercapnia has been shown to have anti-inflammatory effects on the lung (5, 7), the myocardium (40) and other tissues (41) in a context of acute inflammation. Indeed, acidosis can block the activation of nuclear factor-kappa B (NF- κ B), by decreasing the phosphorylation and degradation of the inhibitor of NF- κ B (I κ -B) (12). Hypercapnia can attenuate the release of tumor necrosis factor (TNF)- α and IL-8 (12) by alveolar macrophages, decrease lung neutrophil infiltration (42, 43), and blunt the expression of intercellular adhesion molecule-1 (ICAM-1).

Hypercapnia may also attenuate oxidant-induced injury (13) and lipid peroxidation (44). In this regard, CO2 may have some advantage over other anti-oxidants because of its amphiphilic characteristics. CO_2 can rapidly diffuse through all physiological compartments and permeate into the cells, in which it suppresses the enzymes involved in the generation of free radicals (17). Hence, the anti-inflammatory properties of hypercapnic acidosis may have contributed to the reduction in lung edema.

The effect on hyperlactatemia

In this study, acute hypercapnia, like dobutamine administration, resulted in lower blood lactate concentrations than in the control group. Dobutamine administration in septic shock has been found to decrease lactate levels by improving cellular oxygen delivery and facilitating the removal of lactate accumulated in the tissues, including the splanchnic region (45, 46). Furthermore, the lower pH can facilitate oxygen unloading from hemoglobin and thus contribute to reduce cellular hypoxia (47). In addition to the hemodynamic effects, acidosis can decrease cellular lactate production by a decrease in cell metabolism through the inhibition of the glycolytic enzyme, phosphofructokinase, and an increase in its intracellular uptake by muscle and liver (48).

In sepsis, increased blood lactate concentration is usually considered to be a reflection of tissue hypoperfusion, cellular hypoxia, increased pyruvate dehydrogenase activity, microcirculatory defect and mitochondria dysfunction (Kruse JA, 1999; Vary TC, 1996; Ince 2005; Schaefer CF, 1991). However, in many cases especially in early stage of illness, lactate acidosis may develop without hypoxia or obvious defect in the

oxidative metabolism (Hotchkiss RS, 1991, 1992). Many studies observed a correlation between increased Na⁺-K⁺ ATPase and aerobic glycolysis in endotoxic or septic animals (Jacob DO, 1988, 1991; Mizobata Y, 1995). In several cell types, increased activity of Na⁺-K⁺ ATPase leads to increased glycolysis, 6-phosphofructo-1-kinase activity and lactate production under fully oxygenated conditions (Lynch RM, 1987; Erecinska MF, 1991; Pellerin L, 1994). It was observed that ATP production by glycolysis in smooth muscle occurs, in the presence of O₂, to support ATP consumption by ion pumps located in the plasma membrane or the sarcoplasmic reticulum, such as the Na⁺-K⁺ ATPase and the Ca²⁺-ATPase (Lynch RM, 1983). This can contribute a lot to overall lactate metabolism, taking into consideration that skeletal muscles take up a large fraction of body mass. High levels of CO₂ independent of acidosis were observed to inhibit Na⁺-K⁺ ATPase in lung epithelial cells, causing it to endocytose from the plasma membrane into intracellular pools (Briva A, 2007). We can speculate the similar effect could also take place in muscular cells due to its highly permeability to biology membrane.

In this study, we found that arterial lactate concentration increased in all the three groups, partly due to increased catecholamine production in the sepsis and inflammation state. Catecholamines are known to be involved in glucose metabolism by stimulating glycogen breakdown in liver and muscle. Lactate levels rise as a result of pyruvate accumulation, which might not be oxidized in cells that lack available oxygen in sepsis (Day NPJ, 1996; Levy B,1997). The suppressive effect of two treatment groups on lactic acidosis is certainly not related to catecholamines.

Limitations

Our study has several limitations. First, we compared only one dose of dobutamine and one level of hypercapnia at a fixed $FiCO_2$, so that dose/effect relationships for either dobutamine or CO_2 were not established. Hypercapnia with a $PaCO_2$ of around 60 mmHg seemed clinically relevant, and a $FiCO_2$ of 4.1% seems reasonable. Indeed, an FiCO2 above 5% may have less beneficial and possibly harmful effects (6, 49, 50). Second, the significant difference in arterial pH between hypercapnic animals and the others could not be maintained throughout the experiments, since metabolic acidosis develops in the late stages of this model, and this may have biased the

results. Finally, as the major objective of the study was to distinguish the effects of hypercapnia from other potentially confounding factors, such as antibiotic or vasopressor treatment, therapeutic interventions which would be used clinically were not included in our model.

Conclusion

In summary, this study provides evidence of a potentially beneficial effect of hypercapnia in a clinically relevant model of septic shock due to peritonitis. We show that acute hypercapnia can induce similar hemodynamic effects to the inotropic agent, dobutamine, and may even have superior effects to dobutamine. These observations are reassuring in terms of the effects of permissive hypercapnia in septic shock and further suggest that acute hypercapnia may be a safe and promising intervention in these conditions.

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Variables	Hypercapnia	Dobutamine	Control
Body weight (kg)	24.3±3.4	26.4±2.7	26±3.7
Core temperature (°C)	39.8±0.4	39.5±0.7	39.7±0.7
Heart rate (beats/min)	99±12	101±20.9	97±17
Cardiac index (L/min/m ²)	4.4 ± 0.8	4.3±0.5	3.9±0.9
MAP (mmHg)	99±14.1	106 ± 8.7	100 ± 14.7
MPAP (mmHg)	13.5±3.3	16.6±4.5	16.5±3.6
Arterial lactate (mmol/)	1.0 ± 0.2	1.1±0.3	1.1±0.2
PaO ₂ /FiO ₂	345±53	382±62	326±52
Mean airway pressure (cmH ₂ O)	9.8±0.5	10.4 ± 0.7	10.2±0.5
PaCO2 (mmHg)	40.7 ± 2.6	40.7 ± 2.2	36.3±3.1
Arterial pH	7.41±0.04	7.39±0.06	7.45±0.02

Table 5.1 Physiological variables in the three groups at baseline



Figure 5.1 Changes in arterial partial pressure of carbon dioxide (PaCO₂) and pH over time in the normocapnic animals (control, solid circle), normocapnic animals treated with dobutamine at 7 μ g/kg/hr (dobutamine, open diamond) and hypercapnic animals (hypercapnia, open square). *p<0.05 hypercapnia vs. control; # p<0.05 hypercapnia vs. dobutamine. Data are truncated at 18 hours due to the small number of animals after this time point.



Figure 5.2 Changes in cardiac index and heart rate over time over time in the normocapnic animals (control, solid circle), normocapnic animals treated with dobutamine at 7 μ g/kg/hr (dobutamine, open diamond) and hypercapnic animals (hypercapnia, open square). *p<0.05 hypercapnia vs. control; # p<0.05 hypercapnia vs. dobutamine; \$ p<0.05 dobutamine vs. control. Data are truncated at 18 hours due to the small number of animals after this time point.



Figure 5.3 Changes in PaO₂/FiO₂, alveolar-to-arterial oxygen tension (P(A-a)O₂), and shunt fraction (Qs/Qt) over time in the normocapnic animals (control, solid circle), normocapnic animals treated with dobutamine at 7 μ g/kg/hr (dobutamine, open diamond) and hypercapnic animals (hypercapnia, open square). *p<0.05 hypercapnia vs. control; # p<0.05 hypercapnia vs. dobutamine. Data are truncated at 18 hours due to the small number of animals after this time point.



Figure 5.4 Changes in mixed venous oxygen tension (PO₂) and oxygen saturation (SO₂) over time in the normocapnic animals (control, solid circle), normocapnic animals treated with dobutamine at 7 μ g/kg/hr (dobutamine, open diamond) and hypercapnic animals (hypercapnia, open square). *p<0.05 hypercapnia vs. control; # p<0.05 hypercapnia vs. dobutamine. Data are truncated at 18 hours due to the small number of animals after this time point.



Figure 5.5 Upper panel: Changes in arterial lactate concentration over time in the normocapnic animals (control, solid circle), normocapnic animals treated with dobutamine at 7 μ g/kg/hr (dobutamine, open diamond) and hypercapnic animals (hypercapnia, open square). *Lower panel:* Changes in serum interleukin (IL)-6 levels (presented as the optical density value at 450 nm) over time in the normocapnic animals (control, open circle) and hypercapnic animals (hypercapnia, open square). *p<0.05 hypercapnia vs. control; \$ p<0.05 dobutamine vs. control. Data are truncated at 18 hours due to the small number of animals after this time point.

Chapter VI A large dose bolus, but not a continuous infusion, of sodium selenite improves outcome in peritonitis

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6.1 Introduction

Severe sepsis and septic shock are the most common causes of death among patients hospitalized in non-coronary intensive care units (ICUs) (1). It is generally considered that septic complications result from an overwhelming systemic inflammatory host response to infection (2, 3), leading to widespread endothelial activation and microcirculatory dysfunction, coagulation abnormalities, and ultimately circulatory shock and multiple organ failure (2, 4, 5). There is increasing evidence that reactive oxygen species (ROS) play a major role in microcirculatory dysfunction (6, 7). The production and actions of ROS are complex and mainly related to the interaction between polymorphonuclear cells (PMN) and endothelium (8). One could stress that an effective treatment on microvasculatures should be acute and have multi-site actions in order to reduce the sepsis-induced organ injury (9).

Selenocompounds, especially selenite, was historically known to cause animal poisoning by selenium rich plants (10). Their toxicity has been extensively studied in livestock and laboratory animals (11). The mechanism of toxicity is thought to relate to the interaction between Se and sulfur, especially on thiol groups and disulfide bridges (12), resulting in ROS production (13, 14). These oxidative effects of seleno-compounds are broadly studied for tumor research due to its direct pro-apoptotic effects (15). The reported lethal plasma concentrations of selenium in humans are around 25-30 μ mol/L but the toxicity depends on the compound used (16, 17).

However, at the early stage of severe inflammatory diseases such as septic shock, there could be - paradoxically – a great interest in a controlled use of oxidative effect of Se compounds on thiol groups. In vitro, large concentration of selenite could result in : (i) reversible inhibition of NF- κ B to DNA binding through direct oxidation of the thiol groups of this transcription factor (18, 19); (ii) a transient pro-apoptotic action on proinflammatory circulating cells (15, 20). All of these effects are linked to direct actions of selenite on thiol groups-containing proteins at multi-target sites.

Recently, two multi-center, randomized, double-blind clinical trials investigated the effects of sodium selenite administration in septic shock, one with a bolus injection in the SIC study (Selenium in Intensive Care, 21) and the other using only continuous administration in the SERENITE study (22). These two studies yielded opposite results with a seemingly lower 28-day mortality rate with a bolus injection and no change in mortality with continuous infusion. Up to now, the most impressive results in sepsis studies using selenite administration have been obtained by using bolus administration (23, 24) and attempt to reproduce beneficial effects using only continuous administration often failed in published studies (25, 26).

6.2 Objective

We, therefore, hypothesized that the administration of a large bolus dose of sodium selenite firstly, may allow to obtain transient peak concentration of plasma selenium at least 5 μ mol/L, which could not be obtained by the continuous administration. Secondly, this peak concentration would be associated with a beneficial effect on sepsis-induced microvascular alterations, organ failure, hemodynamic and finally, survival. We conducted a prospective, randomized and controlled study in a clinically relevant ovine model of septic shock to test these two hypotheses.

6.3 Experimental Protocol

The study complied with the guidelines established by the animal management committee of the Université libre de Bruxelles. Care and management of animals was in strict agreement with the National Institutes of Health guidelines. Complete methodological details regarding anesthesia, mechanical ventilation, surgical procedure, and measurements in this model have been described previously (27).

All sheep were female, with a weight between 23 and 28 kg, fasted for 24 hours prior to experiments with free access to tap water. A midline laparotomy was performed and, after cecotomy, 1.5 g/kg body weight of feces was collected. The animals were

mechanically ventilated with a tidal volume of 10 ml/kg and PEEP of 5cmH₂O throughout experiments. FiO₂ was adjusted to maintain PaO₂ between 80 and 120mmHg. Respiratory rate was adjusted to maintain PaCO₂ between 35 and 45 mmHg. A plastic tube with a large diameter was placed in the abdominal cavity for later feces injection. A combination of 1 ml/kg/hr hydroxyethyl starch solution (HES, Voluven, Fresenius, Bad Homburg, Germany) and 1 ml/kg/hr Ringer's lactate was infused during the surgical procedure.

After the surgical operation, the sheep was turned back to the prone position and stabilized for a short period to ensure that heart rate, cardiac output and intravascular pressures remained stable. Prior to induction of sepsis, the following criteria were verified: Mean arterial pressure > 70 mmHg, hemoglobin concentration ≥ 8 g/dl, peak airway pressure ≤ 30 cmH₂O and PaO₂/FiO₂ ratio > 300 with PEEP=5 cm H₂O. Three sheep that did not meet these criteria were excluded. As a result, 21 animals were formally recruited into the study, with 7 in each group.

After stabilization, physiological variables including of hemodynamics, respiratory mechanics and blood gas analysis were recorded as baseline (designated as T0) and then 1.5 mg/kg of feces was injected into abdominal cavity through the disposed drainage tube to induce bacterial peritonitis. All the above variables were recorded at an hourly interval until the end (designated as T1, T2, T3 and so on). Nine hours after induction of sepsis (T9), the animals were randomized into one of three experimental groups: 1) bolus injection group: a single bolus of 2 mg selenium as sodium selenite (sodium selenite pentahydrate, Sigma-Aldrich, Basel, Switzerland) diluted with standard sterilized water was injected into the right atrium through the proximal lumen of the pulmonary artery catheter, followed by a continuous intravenous infusion at a dose of 0.6 µg/kg/hour selenium until the end of the study; 2) continuous infusion alone group: sodium selenite in the same dilution as above was given as a continuous intravenous infusion, without bolus, at a dose of 4 μ g/kg/h selenium until the end of the study. We selected this dose so that the two treatment groups would receive roughly equal amounts of sodium selenite in total if they had a similar survival time after selenite administration; 3) control group: no treatment. No antibiotics or anti-pyretic agents were administered during the experiments.

Fluid challenge with additional infusion of 2 ml/kg HES plus 2 ml/kg Ringer's lactate over 3 min was considered whenever hypovolemia was suspected in the presence of a decrease in cardiac output by more than 10%, a decrease in cardiac filling pressure to below the baseline level, a decrease in mean arterial pressure to less than 70 mmHg, a decrease in mixed venous oxygen saturation (SvO₂) to less than 70%, or a decrease in urine output to below 0.5 ml/kg/h. Incremental administration of both Ringer's lactate and HES solutions with a step for each of 2 ml/kg/hr was given if a positive response to the fluid challenge was observed.

Microcirculatory assay The microvascular network of the sublingual mucosa was analyzed at T0 (baseline), T4 (4 hours after the baseline measurements, and so on), T11 and T17 with a Cytoscan ARII (Cytometrics, Philadelphia, PA) with a \times 5 objective providing \times 167 magnification. The device probe was placed gently without pressure on the lateral side of the tongue, in an area roughly 1.5 to 4 cm from the tip of the tongue. Saliva and other secretions were removed with gauze. Five areas were recorded on disk, using a computer and a video card (MiroVideo; Pinnacle Systems, Mountain View, CA). Microcirculatory analysis was based on a scoring system developed by De Backer et al. (5) and the microvascular flow index (MFI) score (28).

Selenium measurements Arterial blood samples for selenium concentration were collected at T0, T4, T9, T10, T12, T14 and T18. Plasma selenium concentrations were additionally measured 1, 5, 10, 30, 90 and 120 min after administration of the bolus and at T11 in the continuous infusion alone group. Plasma selenium was measured by atomic absorption spectrometry with Zeeman background correction (Perkin-Elmer Z3030; Perkin-Elmer, Uberlingen, Germany). Briefly, a 300 µl plasma sample was diluted with 900 µl buffer solution (1.57 g copper acetate monohydrate, 10.55 g Mg(NO₃)₂. 6H₂O, 1.5 Triton X100 and 3.0 ml HNO₃ 65% dissolved and adjusted to 1 L with milliQ water). The selenium concentration was measured from a slope obtained from a gradient of calibrated standard Seronorm Trace Element ($83 \pm 3 \mu g$ selenium/1).

Plasma lipid peroxidation Lipid peroxidation was measured according to the technique proposed by Yagi et al (29), by adding 4.0 ml of sulfuric acid (N/12) and 500 μ l of phosphotungstic acid (10 %) to 100 μ l of plasma. After 5 min., the tubes were centrifuged at 1600 g during 10 min. and the supernatant discarded. The residue was

suspended in sulfuric acid (2.0 ml) and phosphotungstic acid (300 μ l) during 10 min. The tubes were centrifuged at 1600 g during 10 min. and the supernatant discarded again. The residue was weighed and dissolved in 4.0 ml of water and 1.0 ml of a thiobarbituric acid reagent in acetic acid (335 mg in 50.0 ml of water diluted 50:50 in acetic acid 99%). The tubes were incubated 1 h at 95 °C. The solutions were cooled and the product of the reaction was extracted by 5.0 ml of n-butanol. The fluorescence of the organic layer was measured at 553 nm with an excitation at 515 nm. The concentration of the total lipid peroxide (expressed in nmol per mg of residue) was calculated with the slope obtained from a gradient concentration of tetramethoxypropane pure standard diluted in 4.0 ml of water and 1.0 ml of the thiobarbituric acid reagent.

Hematology and coagulation tests Blood leukocyte count, platelet count, prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured at T0, T4, T9, T12, T15, and T18.

Interleukin-6 measurements Serum interleukin (IL)-6 concentrations were measured at T0, T4, T9, T10, T12, T15, and T18 using an enzyme-linked immunosorbent assay (ELISA) technique as previously described (27). Mouse anti-ovine IL-6 monoclonal antibody (Serotec MCA1659, Kidlington, UK), rabbit anti-ovine IL-6 polyclonal antibody (Serotec AHP424), and sheep anti-rabbit horseradish peroxidase (HRP) conjugated antibody (Serotec STAR54) were used. The monoclonal antibody (MCA1659) was used as a coating antibody with a concentration of 1:200, diluted in phosphate-buffered saline (PBS) and incubated overnight on 96-well ELISA plates (Nunclone) at 4°C. After discarding the coating solution, 250 μ L of blocking buffer (PBS/2% BSA) was added for 2 hours at room temperature and the plates then rinsed three times with PBS/0.05% Tween. A 50- μ L serum sample was diluted with 50 μ L of PBS/1% BSA, placed in plate wells, and incubated for another hour at room temperature. The plates were then washed three times before adding 100 μ l solution of polyclonal antibody (AHP424) diluted in 1:500 into each well and incubating for 1 hour at room temperature.

After washing three times with PBS/0.05%Tween, 100 μ l conjugation HRP (STAR54) diluted 1:10,000 in PBS/1%BSA was added to each well and the plate incubated for 1 hour before 100 μ l tetramethyl benzidine (TMB) reagent was added and allowed to react for 10 min. Stop solution (100 μ l) was then added to each well and the

optical density of the plate-wells was read on an ELISA plate reader at 450 nm within 5 mins. The optic absorbance values from animals in each group were directly compared because a standard purified IL-6 protein is not available for sheep, which makes the quantitative analysis of IL-6 concentration impossible.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4 statistical software (GraphPad Software Inc, San Diego, CA). A Kolmogorov-Smirnov test was used to verify the normality of distribution of continuous variables. Baseline parameters were compared using analysis of variance (ANOVA) with subsequent pairwise comparisons using Student's t-test. The significance of differences in the repeated measurements of physiological variables was analyzed with a mixed-model (taking into account time and group), followed by a modified t-test with Bonferroni correction. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Statistical tests were two-tailed and a p-value less than 0.05 was considered statistically significant.

6.4 Results

There were no significant differences in baseline body weight, vital signs, or other physiological variables among the groups (Table 6.1).

Bolus injection of sodium selenite induced a peak plasma selenium concentration of $7.7 \pm 2.7 \mu \text{mol/L}$ (4-14 $\mu \text{mol/L}$, Figure 6.1). Selenium concentrations were initially much higher in the bolus injection group than in the continuous infusion group (at H10 and H12) (p<0.05), but they were similar at later time points (at H14 and H18) (Figure 6.2).

Arterial hypotension developed 5 hours later in the bolus injection group than in the continuous infusion group, and cardiac index, stroke volume index and left ventricular stroke work index were maintained better in the bolus injection group than in the other two groups (p<0.05) (Figure 6.3). There were no significant differences among the groups in heart rate, pulmonary artery pressure, or vascular resistances at any time.

Bolus injected animals were more responsive to fluid resuscitation and received more fluid volume than the animals in the other two groups (p<0.05). Urine output was similar in the three groups. Pulmonary artery occlusion pressure and central venous pressure were of no differences among the groups (Figure 6.4).

At H17 we observed a significantly higher perfused capillary density 3.93/mm [3.04-4.82] in the bolus injection group vs. 2.78/mm [2.31-3.25] in the continuous infusion alone group and 3.01/mm [2.34-3.68] in the control group; larger perfused capillary proportion 81% [77%-85%]) in the bolus group vs. 66% [60%-72%] in the continuous infusion alone group and 68% [62%-74%] in the control group; higher microcirculatory flow index (MFI) 1.97 [1.63-2.31] in the bolus group, 1.05 [0.65-1.55] in the continuous infusion group and 1.12 [0.75-1.49] in the control group (p<0.05) (Figure 6.5).

Lactic acidosis appeared 5 hours later in the bolus injection group than in the other two groups. Serum IL-6 concentrations were significantly lower in the bolus injection group than in the control group at T13 and T17 (p<0.05) (Figure 6.6, 6.7).

There were no statistical differences in lung mechanics or PaO₂/FiO₂ over time among the three groups. Likewise, there was no difference in PT, APTT, leukocyte or platelet counts.

Lipid peroxidation was measured at the same time. There were no significant differences among the groups. In the first nine hours, lipid peroxidation decreased consecutively to the decrease of lipid mass in the plasma. After selenite administration, no peak was observed and the lipid peroxidation was under the limit of detection (Figure 6.8).

Survival was significantly longer in the bolus group than in the continuous and control group (median survival time: 21.9 hours, versus 18.4 hours and 18.3 hours, respectively, p=0.043) (Figure 6.9).

6.5 Discussion

This study in a sheep peritonitis model has demonstrated that firstly only bolus but not continuous administration of sodium selenite allowed to obtain a peak plasma concentration of selenium, and secondly, only bolus administration was associated with a beneficial effect in septic shock. We observed an improvement in microcirculatory perfusion and cardiac function, a delayed development of hypotension, a delayed and smaller increase in blood lactate concentrations, and, importantly, a significant prolongation of survival in the bolus-injected animals. In the continuous infusion group, despite a similarly large dose and simultaneous initiation of selenium administration, results were of no difference to those of the control group. Notably, there were no signs of toxicity specifically associated with high dose selenite administration in the both treatment groups in terms of cardiac, lung or hematological function.

Large dose sodium selenite and acute intoxication

Historically, selenocompounds are known to be highly toxic before recognition of antioxidant effect of selenium through its incorporation into selenoenzymes (9). As previously mentioned, the toxicity is at least in part due to their oxidant properites, resulting from the interaction of selenium with reduced glutathione (GSH) (14, 15). In a sheep model of sodium selenite acute intoxication, the minimum lethal dose (mini LD) was 0.7 mg/kg with a narrow margin: from 100% survival at 0.6 mg/kg to 100% mortality at 0.8 mg/kg (30), time to death being more rapid for the higher doses. The minimum lethal doses are even higher, in the range of 1.5 to 3.0 mg/kg, in rabbits, rats, cats and dogs (11). In humans, toxic effects have been reported at blood concentrations from 0.179–7.5 μ g/ml (31) and documented lethal selenium concentrations in cases of acute poisoning are around 25-30 μ mol/L (16, 17). Poisoned people display sepsis-like characteristics, with gastrointestinal discomfort and muscle weakness, followed by pulmonary edema, hypotension and shock. The adverse effects of selenium have not yet been fully elucidated, especially in the condition of septic shock.

Although clinical signs of selenocompound intoxication and septic shock are quite similar, pulmonary toxicity may be particularly relevant (32). In the current study, we selected a dose of 2 mg selenium as sodium selenite for the bolus injection. This dose corresponds to 0.08 mg/kg body weight. We do not observe any significant pulmonary toxicity (as reflected by PaO_2/FiO_2 ratio), pulmonary hypertension or other adverse effects obviously associated with the administration of sodium selenite.

The interest of induction of a peak of selenium in sepsis

As shown in figure 1 we obtained huge peak plasma concentrations of selenium only after bolus injection. The first several measurements were performed only minutes after injection, so that selenium measured into the plasma was likely to be selenite or an immediate metabolite.

It has been observed that, at high concentrations, selenite also directly inhibits the DNAbinding activity of many transcription factors, like NF- \Box B and AP-1, by reacting with essential thiol groups (14, 33). In an in vitro study, Kim et al. reported that addition of sodium selenite at concentrations greater than 3 to 5 µmol/L (comparable to those achieved in our study) to cell media that pre-treated with tumor necrosis factor (TNF)- α or lipopolysaccharide (LPS), reversibly inhibited NF- \Box B binding activity to DNA in jurkat T cells or alveolar epithelial cells (18, 19). Likewise, sodium selenite has been shown to inhibit cellular adhesion and to induce a reversible pro-apoptotic effect at high concentrations, which is attributed to an oxidative mechanism leading to the opening of the mitochondrial permeability transition pore and cytochrome c release (15, 20, 35).

we speculate that it may paradoxically be beneficial in septic shock, which is characterized by an overwhelming inflammatory state with high levels of circulating cytokines and ROS, phagocytotic PMN hyperactivity due to a delayed apoptosis and prolonged NF- \Box B activation (2, 4, 35).

In the current study, selenite administration was started 9 hours after the induction of peritonitis, at which time leukopenia and coagulation abnormalities were already present. Arterial hypotension and lactate acidosis were also about to occur. Plasma selenium concentrations declined approximately 60% of baseline levels, as observed in patients with severe sepsis and septic shock (36). We speculate that under this acute inflammatory condition, an anti-inflammatory effect of selenium may be induced in response to the oxidative concentrations achieved. This hypothesis was also suggested by a significantly lower plasma IL-6 concentration in bolus-injected animals compared to the controls.

We initially speculated on a possible lipid peroxidation induced by the injection of selenite. However in this study, no differences between groups were observed after the injection of the selenite. In fact, we observed a decrease of the lipid peroxidation that was

expected in view of a concomitant decrease of the lipid mass in the plasma samples. Selenite may alter redox state and cause oxidative damage by other cellular mechanisms (9, 35).

The improvement of microcirculation and cardiac function

Sepsis-induced microcirculatory alterations are characterized by heterogeneous abnormalities in blood flow and pathological shunt, in which the heterogeneous expression of inducible nitric oxide synthase (iNOS) and NO production in different areas of organ beds plays an important role (4). In the study by Kim et al. (18), the authors observed a dose-dependent inhibition of iNOS activity by sodium selenite in Jurkat T cells activated by LPS, which is related to the inhibition of NF- \Box B activity. We speculate that a similar mechanism could account for the microcirculatory improvements seen in our study, as well as for decreased leukocyte activation and adhesion molecules. A decrease in blood lactate concentrations can be the result of improved capillary perfusion and tissue oxygenation at the microcirculatory level. Interestingly, an amelioration of lactic acidosis has also been observed in endotoxic rats that received a high dose of selenite intraperitoneally, compared to control rats (37).

Bolus selenium administration was associated with an impressive improvement of cardiac function, as reflected by a better maintained ventricular stroke work and fluid loading responsiveness. This improvement may be caused by a decreased activity of circulating inflammatory cells leading to improved microcirculation in the myocardium and by inhibition of iNOS activity and NO production (38). Further studies are mandatory to clarify the underlying mechanisms.

Limitations of the study

First, as in all studies with large animals, we recruited a limited number of young, healthy animals. Only female animals were selected, which made this study unable to clarify the impact of gender on the response to selenium administration. Although they received sedation, ventilation and fluid resuscitation, there was no surgical procedure or antibiotics to treat the peritonitis, and no other supportive care, such as vasopressor therapy, was given. However, this allowed us to obtain a very predictable model with fewer confounding factors. Secondly, we noted that sheep have plasma selenium concentrations at baseline lower than humans. This may influence the applications of our observation to humans. But consistent with human sepsis, plasma selenium concentrations decline rapidly and dramatically in sepsis. In addition, this study was not designed to explore the precise mechanisms induced by selenite peak, the optimal administration regimen, or the toxicity limit of sodium selenite in sepsis.

Conclusion

In summary, there currently exist discrepant results regarding administration of sodium selenite with or without bolus in patients of severe sepsis and septic shock. Our study in a peritonitis model of sheep has demonstrated that only bolus but not continuous administration of selenite allowed to obtain a transient peak plasma concentration of selenium and clinical improvements at the early phase of septic shock. This effect was absent in the group of continuous administration despite of a similarly large quantity of selenium administered. This favors the hypothesis that selenite could be used as a multisite drug than providing selenium supplementation. If this is the case its use and development in septic shock should be in line with the same rule of precaution as other drug. Better knowledge of selenite toxicity and efficiency is required before acute intravenous administration of sodium selenite, or other selenocompounds, can be recommended in septic patients. However, the possibility of a novel therapeutic strategy in the management of severe sepsis and septic shock based on the paradoxical use of a transient oxidative effect of selenocompounds needs to be explored further.

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Variables	Bolus injection group	Continuous infusion group	Control group	P value
Body weight (kg)	26.5±3.6	25.3±2.8	27.2±2.9	0.58
Core temperature (°C)	39.5±0.4	39.3±0.7	39.3±0.5	0.76
Heart rate (beats/min)	102±12	95±13	111±22	0.32
Cardiac index (l/min/m ²)	4.9±1.3	5.4±0.5	6.2±0.9	0.18
MAP (mmHg)	92±8	95±5	94±7	0.89
MPAP (mmHg)	12.7±3.4	13.9±4.3	16.3±3.9	0.23
Lactate concentration				
(mmol/l)	0.9±0.3	0.9 ± 0.2	0.9 ± 0.4	0.87
PaO2/FiO2	395±75	394±70	396±69	0.78
Mean airway pressure				
(cmH ₂ O)	10.1±1.0	9.9±0.9	10.1±0.6	0.76

Table 6.1 Physiological variables at baseline



Figure 6.1 Changes in plasma selenium concentration in sodium selenite bolus-injected animals in the first 60min after bolus injection.



Figure 6.2 Changes in plasma total selenium concentration over time in the control animals (solid circles), animals treated with just a continuous infusion of sodium selenite (continuous, open diamonds), and animals treated with a bolus injection plus a

continuous infusion of sodium selenite (bolus, open squares). *p<0.05 bolus vs control; # p<0.05 bolus vs continuous; \$ p<0.05 continuous vs control.



Figure 6.3 Changes in mean arterial pressure, cardiac index, stroke volume index and left ventricular stroke work index over time in the control animals (solid circles), animals treated with just a continuous infusion of sodium selenite (continuous, open diamonds) and animals treated with a bolus injection plus continuous infusion of sodium selenite (bolus, open squares); *p<0.05 bolus vs. control; # p<0.05 bolus vs. continuous. The numbers in the parentheses besides the time points on the abscissa indicate the remaining numbers of surviving animals at the corresponding time point.


Figure 6.4 Changes in urine output, fluid infusion rate, pulmonary artery occlusion pressure and central venous pressure over time in the control animals (solid circles), animals treated with just a continuous infusion of sodium selenite (continuous, open diamonds) and animals treated with a bolus injection plus continuous infusion of sodium selenite (bolus, open squares); *p<0.05 bolus vs. control; # p<0.05 bolus vs. continuous. The numbers in the parentheses besides the time points on the abscissa indicate the remaining numbers of surviving animals at the corresponding time point.



Figure 6.5 Changes in total capillary density, perfused capillary density, proportion of perfused capillaries and microcirculatory flow index over time in the control animals (solid circles), animals treated with just a continuous infusion of sodium selenite (continuous, open diamonds) and animals treated with a bolus injection plus continuous infusion of sodium selenite (bolus, open squares); *p<0.05 bolus vs. control; # p<0.05 bolus vs. continuous.



Figure 6.6 Changes in arterial lactate concentration over time in the control animals (solid circles), animals treated with just a continuous infusion of sodium selenite (continuous, open diamonds) and animals treated with a bolus injection plus continuous infusion of sodium selenite (bolus, open squares). The numbers in the parentheses besides the time points on the abscissa indicate the remaining numbers of surviving animals at the corresponding time point. *p<0.05 bolus vs. control; # p<0.05 bolus vs. continuous.



Figure 6.7 Changes in serum interleukin (IL)-6 concentrations (presented as the optical density value at 450 nm) over time in the control animals (control, solid circles), animals with continuous infusion alone of sodium selenite (continuous, open diamonds) and animals with bolus injection plus continuous infusion of sodium selenite (bolus, open squares). *p<0.05 bolus vs. control; # p<0.05 bolus vs. continuous.



Figure 6.8 Changes in lipid peroxidation levels and lipid mass in plasma (presented as the optical density value at 450 nm) over time in the control animals (control, solid circles), animals with continuous infusion alone of sodium selenite (continuous, open diamonds) and animals with bolus injection plus continuous infusion of sodium selenite (bolus, open squares).



Figure 6.9 Kaplan-Meier survival plots for the control animals (solid circles), animals treated with just a continuous infusion of sodium selenite (open diamonds), and animals treated with a bolus injection plus continuous infusion of sodium selenite (open squares).

Chapter VII Ongoing Studies and Future Perspective

7.1 Ongoing Studies

7.1.1 Anti-thrombotic and anti-inflammatory effects of a tick saliva-derived serine protease inhibitor in sepsis

Blood-sucking arthropods, like ticks, produce a wide range of bio-active substances in their saliva during blood meals, many of which are anti-hemostatic and immuno-suppressive (1-5). Among them, Iris (named after <u>*I*</u>. <u>*ricinus*</u> <u>immunos</u>uppressor) was found to possess serpin (<u>ser</u>ine <u>protease</u> <u>in</u>hibitor) motifs and leukocyte elastase inhibition (6). Like many other serpins, the structure of Iris is usually characterized by three β -sheets (A, B, and C), eight or nine α -helices, and one typical reactive centre loop (RCL) (7). The RCL motif is involved in the interaction between serpins and serine proteases and assumes many functions of Iris like anti-coagulation, fibrinolysis and immunomodulation.

Numerous serine proteases are involved in blood coagulation pathways. *In vitro* enzymatic assays showed that Iris inhibited several of these proteases (*e.g.* factor Xa or thrombin) suggesting that Iris could act as an inhibitor of coagulation (7). Pre-treatment of Iris in a rat LPS model and in vitro cell culture showed dose-dependent inhibition of pro-inflammatory cytokines, like TNF- α , Il-1 β , Il-6, Il-10, IFN- γ , etc (unpublished data). This effect may be exerted by one or more subunits other than the RCL.

In this study, we would like to investigate whether pre- or post-treatment with Iris can have a beneficial effect on mortality rates in a murine cecal ligation and puncture model of sepsis, and, if so, the underlying mechanisms of its efficacy will be further explored.

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7.1.2 Early administration of arginine vasopressin and a selective V1a agonist (FE 202158) in an ovine model of septic shock

A relative vasopressin insufficiency has been observed in septic shock and vasopressin is commonly used as an adjunct to catecholamines to support blood pressure in refractory septic shock (1). Randomized controlled studies have shown that vasopressin infusion can increase systemic vascular resistance, decrease need for norepinephrine, and improve renal function and SOFA score (2-4). In the Surviving Sepsis Campaign Guidelines, it is suggested that vasopressin may be added to norepinephrine in patients with refractory shock despite adequate fluid resuscitation, at a dose of 0.03 units/min (5).

A biphasic response of vasopressin has been observed in septic animals and patients, with high levels in the early phase and low levels in later stages. However, we hypothesize that even an early increased secretion may not meet the host's demands. The early administration of low dose vasopressin in sepsis may have beneficial effects, especially on mortality. Additionally, a V1a agonist may limit edema formation by attenuating capillary leakage. A recent clinical trial has also suggested that there is a synergic effect of vasopressin and glucocorticoids.

Therefore, in a clinically relevant ovine model of septic shock, we will investigate whether early application of low dose vasopressin can have beneficial effects, and whether a combined use of vasopressin and glucocorticoid is associated with superior effects. Moreover, the efficacy of a selective V1a receptor agonist will also be studied.

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7.2 Future Study

7.2.1 Skeletal muscular dysfunction in sepsis

Acute medical problems associated with the development of skeletal muscle weakness include pneumonia, generalized infections causing bacteremia, acute respiratory distress syndrome (ARDS), burns, uremia, and trauma ^{1, 2}. Weakness in these patients can manifest as severe limb muscle weakness (even to the point of virtual paralysis), respiratory muscle weakness requiring mechanical ventilatory support, and/or some combination of these phenomena. It is likely that inflammation-induced respiratory skeletal muscle dysfunction is a major factor contributing to the morbidity and mortality of patients admitted to intensive care units³.

While factors such as nutritional deficiency and disuse may contribute to the development of muscle weakness in these common acute medical conditions, studies have found evidence of systemic inflammation in all of the disease processes listed above, and systemic inflammation may be the key factor in producing skeletal muscle dysfunction. Administration of cytokines or induction of cytokine production in animals has been shown to produce muscle wasting and muscle weakness ⁴. Incubation of muscle cell line cultures with cytokines has been shown to produce loss of cellular proteins ⁵. Other reports have shown that in animal models of sepsis significant alterations in skeletal muscle mitochondrial function occur, with significant reductions in state 3 respiration rates, decreases in maximal skeletal muscle mitochondrial ATP generation rates, and profound reductions in the activity of one of the major transmitochondrial transporters of high-energy phosphate compounds, the sarcomeric mitochondrially specific creatine kinase ^{6,7,8}. Moreover, free radical species (superoxide, hydroxyl radicals, nitric oxide, peroxynitrite, and the free radical-derived product, hydrogen peroxide) play an important role in triggering activation of a number of downstream processes that collectively act to impair muscle function and lead to reductions in muscle strength and mass ^{9,10}.

A complete elucidation is needed of the pathophysiological sequences by which cytokines and free radicals alter muscle function and of the local metabolic alterations during skeletal muscle dysfunction. Moreover, we believe it would be useful to differentiate the hemodynamic factors, intrinsic cellular mechanism and neurogenic factors that may account for skeletal muscle functions.

For this study, we are using an anesthetized rat model, with sepsis induced by a large dose of intraperitoneal LPS. Heart rate and rectal temperature are recorded. Blood pressure is invasively measured by femoral artery catheterization. Local gastrocnemius muscle metabolites are analyzed by the microdialysis technique, and local muscle blood flow by laser flow probe. Preliminary trials have shown significant changes in terms of mRNA expression of key-enzymes for muscle function, including hexokinase and NOS isoforms.

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7.2.2. Cardiac Effects of Sodium Selenite

Selenium (Se) is an essential element for all the selenoproteins with antioxidant functions in which it is incorporated as selenocysteine. Se deficiency has been established As an etiological factor for endemic human dilated cardiomyopathy (DCM) known as Keshan disease, which is found in areas of China with Se-poor soils¹. Epidemiological studies in Europe have linked low serum selenium levels to cardiac diseases² and selenium supplementation has a protective effect during myocardial ischemia³.

The beneficial effects of sodium selenite on heart function were associated with an NOrelated increase in the inotropic response of adult cardiac muscle to beta-adrenergic stimulation, reducing ROS-induced pro-oxidant injury, and restoring the diminished K^+ currents ^{4, 5, 6}. However, the exact mechanism by which selenium deficiency or selenium administration impact on the cardiac function is unknown.

In future studies we would like to investigate the direct effect of sodium selenite on myocardium and its intra-cellular regulating pathway in sepsis.

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Chapter VIII Conclusions

In this clinically relevant ovine peritonitis model, we have investigated a series of adjunctive therapies in severe sepsis and septic shock and conclude:

- administration of activated protein C had beneficial effects on hemodynamic variables, gas exchange, lactic acidosis, and coagulation abnormalities. Higher colloid oncotic pressures and lower lung wet/dry ratios at autopsy suggest preserved endothelial integrity. Administration of activated protein C resulted in prolonged survival.
- hypercapnia had similar effects to dobutamine on hemodynamic variables and lactic acidosis. Hypercapnia improved tissue oxygenation and reduced lung edema formation more than dobutamine administration.
- the administration of a large bolus of sodium selenite (rather than a continuous administration) resulted in beneficial effects, perhaps by a transient oxidative effect of sodium selenite