uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Yang Song
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbiology and Immunology)
GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Rodent Model to Study Severe Sepsis

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. Gary Garber
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. Syed Sattar

Dr. Alain Stintzi

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

RODENT MODEL TO STUDY SEVERE SEPSIS

A Thesis Submitted to the

Faculty of Graduate and Postdoctoral Studies

University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

By

Yang Song

© Yang Song, Ottawa, Canada, 2007.

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-49279-6
Our file Notre référence
ISBN: 978-0-494-49279-6

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

Background:

The sepsis syndrome is characterized by such clinical changes as hypotension, hypothermia or fever, metabolic acidosis, pulmonary hemorrhage, and death within several hours to days. Antibiotics are a mainstay of treatment and have been repeatedly demonstrated to improve survival in both human studies and animal models of sepsis. Appropriate fluid resuscitation is an important component of the initial therapy for severe sepsis. The antibody against TNF-α has improved survival in certain models of sepsis (Reinhart et al. 2001).

Hypothesis:

Timing of antibiotics is pivotal in survival outcome of sepsis and early fluid resuscitation is crucial in the maintenance of survival rates.

Materials & Methods:

Male Balb/c mice (23-25g) received the cecal ligation and puncture. During the surgery, 1/3 cecum was ligated and 2 18G needle punctures were made from which feces were expressed. During antibiotic treatment strategies, cefotaxime was firstly given at different time points (0, 3, 6 & 12h post-surgery) and thereafter every 6 hours for up to 72 hours. All the mice were given 1ml 0.9% saline fluid at 0 hour post-surgery and continuously every 6 hours for up to 72 hours. For fluid resuscitation 1ml 0.9% saline fluid was started at 0, 3, 6, 9 or 12 hours post-surgery respectively by subcutaneous injection and continuously every 6 hours for up to 72 hours. All the mice were given

cefotaxime at 0 hour post-surgery and continuously every 6 hours. Finally, we repeated cefotaxime 12 hours (Group 1) and fluid 12 hours post-surgery (Group 2) groups. Anti-TNF-α monoclonal antibody was given at 6 hours post-surgery for both groups.

Results:

After surgery, untreated mice showed signs of sepsis, such as anorexia, hypothermia and dehydration, and the mortality was 100% within the first 30 hours. The mortalities in cefotaxime 6 & 12 hours post-surgery groups were nearly 100% while cefotaxime 0 & 3 hours post-surgery groups were 0% and 25% respectively. The mortality in fluid resuscitation 12 hours post-surgery group was nearly 100% whereas the other groups (0, 3, 6 & 9 hours) were 12.5% respectively. The mortality of group 1 decreased significantly (Fisher's exact test) compared to the same treatment group without anti-TNF-α while the mortality of group 2 did not.

Discussion:

This model provides consistent results of sepsis, which could enable us to control for treatment factors that are not controllable in human sepsis. In this model we can clearly show the impact of timing of antibiotics on survival. Despite early antibiotics administration (time 0), mortality of this model is still high when fluid resuscitation is delayed. Anti-TNF-α can decrease the mortality under condition that fluid resuscitation are given at an early time point.

Acknowledgement

Firstly, I owe my grateful thanks to Dr. Gary Garber for providing me the opportunity to pursue my Master of Science degree in his lab. Without his guidance and support, this study would have been impossible. Along with his well-established career, he built such a great research environment that my working in this lab has been a great experience.

I would also like to thank the members of my thesis advisory committee, Dr. Lauralyn McIntyre and Dr. Bill Cameron for their advice and suggestions on the thesis.

I want to thank Cathy Moreau for all she has done. Thanks are also extended to the staff of the Animal Care Department, Dr. Jennifer Keyte, Dr. Michelle Groleau and Dr. Mark Liepmann, especially technician Kim Yates, for all the advice, ethical considerations, technical support and the use of their facilities. I also want to thank Dr. Marc Desjardins for showing how to handle various bacterial cultures and Dr. Dean Fergusson for teaching me how to use the statistical analyse.

Thanks to Dr. Garber's lab, particularly the technician Julie Zhu, and fellow graduate students, Melissa, Shahed, Sarah, Kelly, Angela and Cathy. They have all been wonderful sources of support, both technical and personal, and have just made life a lot more fun.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENT iv

TABLE OF CONTENTS v

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF ABBREVIATIONS xii

1. INTRODUCTION 1

1.1 History of Sepsis 1

1.2. Definition 1

1.3. Cause of Sepsis 2

1.4. Epidemiology 5

1.5. Pathophysiology 6

1.6. Organ Dysfunction in Sepsis 8

1.7. Blood Cells 16

1.8. Immune System 23

1.9. Current Therapy in Sepsis 30

1.10. Animal Model 37

1.11. Hypothesis 43

2. MATERIAL & METHODS 44

2.1. Materials & Regular Procedures 44

2.1.1. Animals 44

2.1.2. Intraperitoneal Sepsis Model 44

2.1.3. Potential Mortality Endpoint 45

2.1.4. Blood Sampling 46

2.2. Model Development & Parameter Determination 46

2.2.1. Pathological Examination 48

2.2.2. Blood Glucose Assay 48

2.2.3. Lactate Assay 48

2.2.4. ALT Assay 49

2.3. Treatment Strategies 51

2.3.1. Timing of Antibiotic Administration 51

2.3.1.1. Bacterial Culture Swab 53

2.3.1.2. Blood Culture 53

2.3.1.3. WBC Counts 54

2.3.2. Fluid Resuscitation 54

2.3.3. Anti-TNF-α Monoclonal Antibody Adjunctive Treatment 55

2.3.3.1. Anti-TNF-α Treatment for Late Administration

(12 hours Post-surgery) of Cefotaxime ········ 56

2.3.3.2. Anti-TNF-α Treatment for Late Fluid (12 hours Post-surgery) Resuscitation ········ 56

2.4. Cytokines (TNF-α & IL-6) Kinetics during Late Intervention Strategies ········ 57

2.4.1. Cefotaxime Administration 12 hours Post-surgery Group ········ 57

2.4.2. Fluid Resuscitation 12 hours Post-surgery Group ········ 58

2.5. TNF-α Assay ········ 58

2.5.1. ELISA Technique ········ 59

2.6. IL-6 Assay ········ 60

2.6.1. ELISA Technique ········ 60

2.7. Statistics Analysis ········ 61

2.8. Animal Care ········ 62

3. RESULTS ········ 63

3.1. Model Development & Parameter Determination ········ 63

3.1.1. Pathological Examination ········ 64

3.1.2. Blood Test ········ 65

3.1.2.1. ALT Assay ········ 65

3.1.2.2. Blood Glucose Assay ········ 65

3.1.2.3. Lactate Assay ········ 66

3.1.2.4. TNF-α & IL-6 Assays ········ 66

3.2. Treatment Strategies 67

3.2.1. Timing of Antibiotic Administration 67

3.2.2. Fluid Resuscitation 70

3.2.3. Anti-TNF-α Adjunctive Treatment 71

3.2.3.1. Anti-TNF-α Treatment for Late Administration (12 hours Post-surgery) of Cefotaxime 71

3.2.3.2. Anti-TNF-α Treatment for Late Fluid (12 hours Post-surgery) Resuscitation 72

3.3. Cytokines (TNF-α & IL-6) Kinetics during Late Intervention Strategies 73

4. DISSCUSSION 75

4.1. Model Development & Parameter Determination. 75

4.2. Treatment Strategies 79

4.2.1. Timing of Antibiotic Administration 79

4.2.2. Fluid Resuscitation 83

4.2.3. Anti-TNF-α Adjunctive Treatment 85

4.3. Limitation of Mouse Model 88

LIST OF REFERENCES 118

APPENDIX I 135

APPENDIX II 136

LIST OF TABLES

1. Model Development & Parameter Determination 89

2. ALT Assay Procedures 90

3. Treatment Strategies 91

4. Bacterial Cultures of CLP Mice with & without Cefotaxime and Negative Control Mice 92

LIST OF FIGURES

1. Mortalities of CLP Mice with Different Surgical Procedures within First 24 Hours Post-surgery ··· 93

2. Body Temperatures of Positive Control Mice & Negative Control Mice ··· 94

3. Images of Hepatic Tissue ··· 95

4. Images of Pulmonary Tissue ··· 95

5. ALT Assay of Positive Control Mice & Negative Control Mice at Different Time Points Post-surgery ··· 96

6. Blood Glucose Assay of Positive Control Mice & Negative Control Mice ··· 97

7. Lactate Assay of Positive Control Mice at Different Time Points Post-surgery ··· 98

8. TNF-α Assay of Positive & Negative Control Mice from Different Time Points ··· 99

9. IL-6 Assay of Positive & Negative Mice at Different Time points ··· 100

10. Mortalities of CLP Mice with Cefotaxime at Different Starting Time Points ··· 101

11. Body Temperatures of CLP Mice with Cefotaxime at Different Time Points ··· 102

12. WBC Count (Pre-surgery vs. Post-surgery) of CLP Mice with Cefotaxime at Different Time Point (Including Positive & Negative Control) ··· 103

13. TNF-α Assay of CLP Mice with Cefotaxime at Different Starting Time Points · 104

14. IL-6 Assay of CLP Mice with Cefotaxime Starting at Different Time Points ··· 105

15. Mortalities of CLP Mice with Fluid Resuscitation at Different Starting Time Points ··· 106

16. Body Temperatures of CLP Mice with Fluid Resuscitation Starting at Different Time Points 107

17. TNF-α Assay of CLP Mice with Fluid Resuscitation at Different Starting Time Points 108

18. IL-6 Assay of CLP Mice with Fluid Resuscitation at Different Starting Time Points 109

19. Mortalities of CLP Mice with Anti-TNF-α Adjunctive Treatment in Different Groups 110

20. Body Temperatures of CLP Mice with Anti-TNF-α Adjunctive Treatment in Different Groups 111

21. TNF-α Assay of CLP Mice with Anti-TNF-α Adjunctive Treatments 112

22. IL-6 Assay of CLP Mice with Anti-TNF-α Adjunctive Treatment 113

23. TNF-α Assay of CLP Mice with Cefotaxime 12h Post-surgery at Different Time Points 114

24. TNF-α Assay of CLP Mice with Fluid Resuscitation 12h Post-surgery at DifferentTimePoints 115

25. IL-6 Assay of CLP Mice with Cefotaxime 12h Post-surgery at Different Time Points 116

26. IL-6 Assay of CLP Mice with Fluid Resuscitation 12h Post-surgery at Different Time Points 117

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AIDS	acquired immune deficiency syndrome
ALT	alanine aminotransferase
AM	adrenomedullin
aPC	activated protein C
APP	acute phase protein
APR	acute phase response
ARDS	acute respiratory distress syndrome
ARF	acute renal failure
BPI	bactericidal/permeability increasing protein
CLP	cecal ligation and puncture
cm	centimeter
CRH	corticotropin releasing hormone
CVP	central venous pressure
DIC	disseminated intravascular coagulation
ecNOS	endothelial constitutive nitric oxide synthase
EDTA	ethylene diamine tetracetic acid
EGDT	early goal directed therapy

ELISA	enzyme linked immunosorbent assay
ESR	erythrocyte sedimentation rate
fl	femtoliter
g	gram
G	gauge
G-CSF	granulocyte colony-stimulating factor
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
HIV	human immunodeficiency virus
HTS	hypertonic saline
ICU	intensive care unit
IFN	interferon
IL-6	interleukin-6
IP	intraperitoneal
IV	intravenous
LPS	lipopolysaccharide
MAP	mean arterial pressure
min	minute
MODS	multi-organs dysfunction syndrome

mRNA	Messenger Ribonucleic Acid
NADPH	β-Nicotinamide adenine dinucleotide
NETs	neutrophil extracellular traps
ng	nanogram
nm	nanometer
NO	nitric oxide
PAMPs	pathogen associated molecular patterns
PARs	protease activated receptors
pg	picogram
PGE2	prostaglandin E2
PMNs	polymorphonuclear cells
PROWESS	Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis
PYR	pyrrolidonyl-beta-naphthylaminde hydrolysis
RAAS	renin-angiotensin aldosterone system
r-APC	r-activated protein C
RAI	relative adrenal insufficiency
RBC	red blood cell
rpm	revolutions per minute
S.C.	subcutaneous

ScvO2	central venous oxygen saturation
SIRS	systemic inflammatory response syndrome
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLR	toll-like receptor
TNF	tumor necrosis factor
TSA	tryptic soy agar
µL	microlitre
µm	micrometer
UTIs	urinary tract infections
VEGF	vascular endothelial cell growth factor
WBC	white blood cell

1. Introduction

1.1. History of Sepsis

Sepsis originates from the Greek word "σήψις". Its meaning is "decomposition, decay", which has been observed and described since the dawn of medicine. There are a number of landmarks in our understanding of sepsis: A surgeon named John Pringle first used the term "antiseptic" in 1752. Richard Pfeiffer identified "endotoxin" in 1892 and John Stenflo discovered protein C in 1975. In recent years, considerable advances have been made in understanding the pathogenesis and management of sepsis.

1.2. Definition

Sepsis is a complex syndrome that is difficult to define, diagnose and treat. Because the symptoms and signs of sepsis may occur without a definite infection, the concept of systemic inflammatory response syndrome (SIRS) was created in 1995 (Bone 1996). A patient with SIRS has two or more symptoms: body temperature < 36℃ or >38℃, heart rate > 90/min, respiratory rate > 20 or white blood cell (WBC) 12000/mL or > 10% immature cells (Liolios 2003).

Sepsis is SIRS caused by overwhelming infection, which can be caused by any infectious agent from viruses, bacteria, fungi or parasites. If associated with a blood infection, the term bacteremia is used. Clinical presentation can be due to bacteria and/or bacterial products, such as cell wall, lipopolysaccharide (LPS) or toxins.

Severe sepsis is defined as sepsis with organ dysfunction, such as hypotension or hypoperfusion (lactic acidosis), oliguria and mental status changes. If sepsis induces hypotension (<90mmHg) that does not respond to 500mL intravenous (IV) fluid replacement, this stage of sepsis is called septic shock. If the septic shock lasts for more than one hour and does not respond to fluid and vasopressor administration (Tiwari et al. 2004), it is called refractory septic shock.

Sepsis is a range of clinical conditions caused by the body's systemic response to an infection, which is accompanied by single or multiple organ dysfunction or failure (septic shock) leading to death if it develops into severe sepsis. Although this much is known, there is no clear clinical definition that can be easily communicated and adopted globally. Its absence makes the diagnosis and management of sepsis a clinical challenge. Some of the symptoms of sepsis, such as body temperature, rapid pulse and respiratory difficulty are very general and are present in many other disorders. In a recent survey conducted among physicians, 87% felt that the symptoms of sepsis could easily be attributed to other conditions, creating problems of late or misdiagnosis (Levy et al. 2003) and most realized that a lack of a common definition could potentially lead to delays in treatment and additional complications and death of the patient (Levy et al. 2003).

1.3. Cause of Sepsis

The most common cause of sepsis is bacterial infection, which can originate anywhere

in the body. The most usual areas the infections originate from are: kidneys, liver, gall bladder, bowel, lungs and even skin. In hospital, patients can also get bacterial infection from intravenous lines, surgical wounds, surgical drains and sites of skin ulcers or bedsores.

Sepsis can also be triggered by events such as pneumonia, trauma, surgery and burn injury, or by conditions such as cancer or acquired immune deficiency syndrome (AIDS).

Intra-abdominal sepsis is the focus of my project. The common cause of this sepsis is infection with bacteria such as *Enterbacteraciae, Streptococcus pneumoniae and Anaerobes.*

In the early 1980s, Dr. Edwin A. Deitch and Dr. Rodney Berg indicated that gut bacteria and their products could be an important trigger for the development of sepsis and multi-organ dysfunction syndrome (MODS) in critically injured or burned patients (Deitch 2004). Their concept is that during shock or stress states, the body's response was to decrease blood flow to the intestines, thereby ensuring sufficient blood flow to critical organs such as the heart and brain (Deitch 2004). This decrease in intestinal blood flow, if sufficient in magnitude or depth, would lead to gut injury and loss of normal gut barrier function, causing the gut to become vulnerable. This gut barrier failure in turn would allow bacteria and their toxic products, such as endotoxin, to escape from the gut and enter the systemic circulation, thereby causing systemic sepsis

and leading to MODS (Deitch 2004). Because the normal gut contains enough bacteria and endotoxin to kill the host thousands of times over, therefore even small increase in gut permeability could cause profound physiological consequences (Deitch 2004). Their concept explains the clinical paradox of how MODS patients without a clinical focus of infection could have bacteria recovered from their blood or have a septic picture in the absence of microbial evidence of infection (Deitch 2004).

Enterococcus species: A large number of bacteria could be described for the cause of sepsis. I have chosen enterococcus as a representative organism due to its prevalence in the model I developed. Enterococcus genus is Gram-positive cocci and facultative anaerobe. Most commonly isolated species are *Enterococcus faecalis* and *Enterococcus faecium.* It is normal flora of the intestinal tract in humans and animals. It is responsible for about 10% of all nosocomial infections and most common infections are urinary tract infections (UTIs) and bacteremia. Other infections include endocarditis as a complication of bacteremia and wound infections; infections within the peritoneal cavity including intra-abdominal abscesses (usually polymicrobial) especially following penetrating trauma such as gunshot wounds, knife wounds, and surgical wounds; kidney infections as a complication of UTIs, prostate infections, and infections of damaged or compromised skin, such as in diabetic or decubitus ulcers, burns, and surgical wounds (often polymicrobial). The enterococci have become the second most common bacterium isolated from nosocomial urinary and wound

infections, and the third most common cause of nosocomial bacteremia (Facklam et al. 1989). The predisposing factors include urinary and intravascular catheters and long-term hospitalization while receiving broad-spectrum antibiotics. The treatment is ampicillin or vancomycin, depending upon the results of susceptibility testing.

1.4. Epidemiology

Sepsis is associated with significant mortality, killing approximately 1,400 people worldwide every day (Bone et al. 1992). Sepsis is one of the leading causes of death in the intensive care unit (ICU). Due to its aggressive, multifactorial nature, sepsis is a rapid killer. Death is common among sepsis patients, about 30% of patients dying within the first month of diagnosis, and 50% dying within 6 months (Natanson et al. 1998; Rivers et al. 2001; Matthay 2001). The 28-day mortality in sepsis patients is comparable to the 1960s hospital mortality rate in patients of acute myocardial infarction (Julian et al. 1996). In recent years, there has been an improvement in the awareness and management of acute myocardial infarction, resulting in a decline in mortality, while sepsis remains with a high mortality (Julian et al. 1996).

Moreover, the number of severe sepsis cases is set to increase at a rate of 1.5% per year (Angus et al. 2001) and currently the burden of severe sepsis was estimated to be $36.4 to $72.9 million per year in Quebec (Letarte et al. 2002). This will increase total mortality and increase the burden on healthcare resources. The increase is mainly due to the growing use of invasive surgical procedures and increasing numbers of elderly

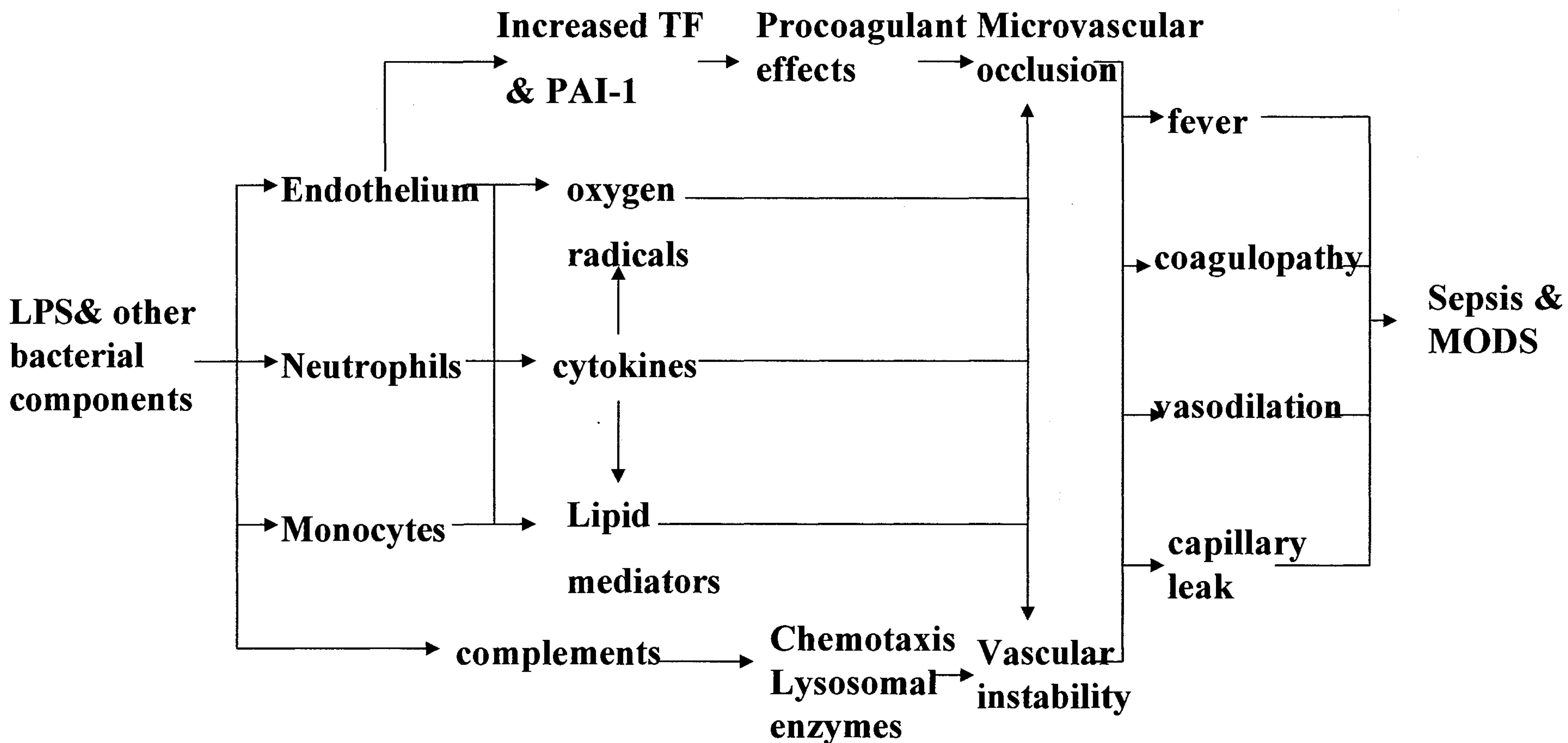
and high-risk individuals such as human immunodeficiency virus (HIV) patients (Letarte et al. 2002). Older people are at increased risk of sepsis as they are more vulnerable to infections due to aging, co-morbidities, weaker immune system, use of invasive surgical techniques and problems associated with institutionalization.

1.5. Pathophysiology

Because of the complexity of the septic process, an appropriate and necessary immune response to infection or insult can become excessive and detrimental if its control is lost. The uncontrolled host response may lead, on a downward spiral, to tissue damage and MODS.

Infectious insults (e.g. endotoxins, exotoxins, cell wall components of Gram-positive bacteria, viruses, fungi) and non-infectious insults (e.g. cellular debris, complements, immune complex) are able to stimulate the host immune system to produce a number of important mediators such as cytokines, complements, coagulation components, platelet activating factor that are capable of causing profound effects on vascular tone and permeability, resulting in microcirculatory disturbances and finally shock and organ dysfunction (Badr 1994; Koch 1998).

Pathophysiology of Sepsis



Jonathan Cohen The Immunopathogenesis of Sepsis. *Nature* 420, 885-891(19 Dec. 2002)

Sepsis develops by way of a complex cascade of inflammatory responses triggered by certain initiating factors and involving multiple mediators and elaborates feedback loops and interactions.

After an infectious insult, endothelial damage occurs with subsequent activation of neutrophils. This causes increased vascular permeability that results in tissue edema and release of oxidants by the neutrophil. Tissue factor (TF) is expressed by monocytes and damaged vascular endothelium. Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are secreted by the monocytes. The coagulation cascade is activated with release of thrombin and the formation of the fibrin clot and its purpose is to "wall off" the infection. Recent research recognized that immune system overstimulation is not the key to sepsis, while the cytokines, such as TNF-α, IL-6 (interleukin-6), IL-1, may actually be beneficial in it (Williams et al. 2003). The innate immune system is stimulated via the Toll Like Receptors (TLR) and initiates the immune response. TLR 4 is part of a recognition complex for bacterial LPS and it has been shown that upregulation of tissue TLRs during early stages of sepsis increases mortality (Williams et al. 2003). Then, nuclear factor kappaB, a transcription factor involved in immediate early gene activation during inflammation is activated and cytokines inducing an inflammatory response are released (Williams et al. 2003). The role of host genetic factors has been recently

elucidated: polymorphisms in TNF, IL-1, Fc, and TLR4 may influence the response to infection (Homels et al. 2003; Mira et al. 1999). This suggests that there could be a targeted therapy of sepsis and septic shock based on genetic variability in the future. Inflammation promotes coagulation and coagulation induces inflammation by the release of inflammatory cytokines. Coagulation factor Xa was found to produce a proinflammatory response in endothelial cells and activated protein C (aPC) inhibits the production of inflammatory cytokines. As the inflammatory responses go on, the enhancing vascular permeability may result in a generalized capillary leakage with interstitial edema. Persistent systemic hypotension due to microcirculatory disturbance and capillary leak, results in tissue hypoperfusion and hypoxia. If the cascade is not interrupted, it will result in acute renal failure (ARF), cardiovascular and pulmonary insufficiency and finally MODS (Noris 1998; Koch 1998).

1.6. Organ Dysfunction in Sepsis

After local and systemic responses are initiated by tissue damage, the failure of respiratory system is common in the first 72 hours after the original insult. Following this, hepatic failure may be seen within 5 to 7 days, then gastrointestinal (GI) bleeding within 10 to 15 days, then renal failure within 11 to 17 days (Bone 1996).

Liver: The liver plays a major role in metabolism and has a number of functions in the

body including glycogen storage, plasma protein synthesis, and drug detoxification. It performs several roles in gluconeogenesis, glycogenolysis and glycogenesis, produces coagulation factors and protein C, converts ammonia to urea. It is also responsible for immunological effects as the reticuloendothelial system of the liver contains many immunologically active cells, acting as a 'sieve' for antigens carried to it via the portal system (Stark et al. 1992).

In sepsis, the liver has a significant role in host defense mechanisms. Kupffer cells are responsible for bacterial scavenging, bacterial products inactivation, and clearance and production of inflammatory mediators. Hepatocytes, via receptors for many proinflammatory cytokines, modify their metabolic pathway toward gluconeogenesis, amino-acid uptake, and increased synthesis of coagulant and complement factors and protease inhibitors via pro-inflammation cytokine receptor (Dhainaut et al. 2001). The acute-phase protein (APP) response also contributes to the procoagulant state, especially by enhancing the inhibition of protein C and by decreasing liver synthesis of protein C and antithrombin. Elevated C-reactive protein levels (positive APPs) promote the expression of tissue factor by mononuclear cells. Increased liver production of thrombin-activatable fibrinolytic inhibitor (positive APPs) enhances fibrinolysis inhibition (Póvoa 2002). Conversely, these hepatic inflammatory and coagulation processes seen in sepsis may alter the function of liver. Previous research indicates that there is a correlation between hepatic leukocyte recruitment and hepatic

dysfunction. Indeed, activated Kupffer cells that release chemokines, attract blood neutrophils into the liver, and activate them (Molnar et al. 1997). Neutrophils upregulate their surface adhesion molecules, tissue factor, and Kupffer cells, whereas tissue factor pathway inhibitor and thrombomodulin are almost undetectable in endothelial cells. This may lead to microcirculatory disturbances, fibrin deposition, hepatocyte injury, endotoxin and bacteria spillover, and multiple organ failure in sepsis (Molnar et al. 1997).

Alanine aminotransferase (ALT) is measured to see if the liver is damaged or diseased. Low levels of ALT are normally found in the blood. However, when the liver is damaged or diseased, it releases ALT into the bloodstream, which makes ALT levels go up. Most increases in ALT levels are caused by liver damage (Molnar et al. 1997). The ALT level is usually high in patients suffering from sepsis-induced hepatic dysfunction (Molnar et al. 1997).

Kidney: ARF is the most common renal manifestation of sepsis, which is often a component of MODS that complicates sepsis. Most knowledge related to the pathophysiology of ARF has been derived from animal models.

During severe sepsis, there is activation of sympathico-adrenal axis with increased plasma levels of epinephrine or norepinephrine; of renin-angiotensin aldosterone system (RAAS) with elevated levels of angiotensin II and a rise in vasopressin levels are often part of host response (Tiwari et al. 2004). These mechanisms are largely

responsible for the clinical manifestations of sepsis, including the haemodynamic alterations that are characterized by vasodilation, hyperdynamic circulation and microcirculatory changes contributing to inefficient oxygen extraction (Thijs et al. 1998).

Lung: The lung is the most frequently identified organ to fail in sepsis and is also the primary site of infection (Costa et al. 2006). The development of acute respiratory distress syndrome (ARDS) is common in septic patients. The current research of pathogenesis of ARDS indicated that the degree of inflammatory response and its sustained leukocyte activation of sepsis might determine the clinical evolution of ARDS. On one hand, due to the loss of alveolar compartmentalization in ARDS, the proinflammatory cascade activation can reach the bloodstream and induce MODS. On the other hand, the loss of alveolar compartmentalization allows the passage of cytokines to be released by the other organs to affect the pulmonary endothelium during sepsis (Costa et al. 2006). These cytokines, especially TNF-α, have important roles in the lung dysfunction. Experimental and clinical studies have shown that ventilation strategies using low tidal volumes and limitation of airway pressures can reduce cytokines levels and mortality of patients with respiratory failure (Costa et al. 2006).

Cardiovascular Response: The cardiovascular response in sepsis is the result of subcellular dysfunction and impaired metabolism from the complex interaction of

cytokine and mediators with cellular involvement (Porembka 1993). The typical cardiovascular abnormalities are tachycardia, hypotension, increased cardiac index, decrease in left ventricular stroke work index, decrease in ejection fraction and an apparent decrease in contractility. The mostly typical cardiovascular response to polymicrobial sepsis is characterized by an early, hyperdynamic phase followed by a late, hypodynamic phase (Wang 2001).

Previous research suggests the reduction of NO derived from endothelial constitutive nitric oxide synthase (ecNOS) is significant in the progression of sepsis and associated with damage to the vascular endothelial cells (Beckman et al. 1990). Although the factors and/or mediators responsible for producing the transition from the hyperdynamic to the hypodynamic stage are not fully understood, recent studies have suggested that adrenomedullin (AM), a potent vasodilatory peptide, appears to play an important role in initiating the hyperdynamic response following the onset of sepsis (Fowler et al. 2002). In addition, the reduced vascular responsiveness to AM may result in the transition from the early, hyperdynamic phase to the late, hypodynamic phase of sepsis (Fowler et al. 2002). The mechanism of AM gene actually has not been elucidated but can be modified by pharmacologic agents, which reduce sepsis-induced mortality. Thus reduced AM receptor responsiveness may be crucial factors responsible for the transition from the hyperdynamic phase to the hypodynamic phase of sepsis (Fowler et al. 2002).

Spleen: Animal models have demonstrated that apoptosis in the spleen contributes to sepsis severity during its process (Dear et al. 2006).

The critical importance of the spleen in host defense against bacteremia is suggested by the occurrence of overwhelming pneumococcal sepsis in patients who are anatomically or functionally asplenic (Bisno et al. 1970; Eraklis et al. 1967; Singer 1973). Experimental models of pneumococcal infection have also demonstrated increased mortality and prolonged bacteremia in asplenic animals (Bogart et al. 1972; Leung et al. 1972; Shinefield et al. 1966; Whitaker 1968). Animal models indicate that the normal spleen plays a unique role in the clearance of bacteremia (Brown et al. 1981). When the spleen is removed, the liver is unable to compensate for the loss of phagocytic acitvity and complement production of the spleen (Brown et al. 1981).

Intravascular Coagulation Pathway: Disseminated intravascular coagulation (DIC), is common in patients with severe sepsis along with microvascular thrombosis (Hinsaw 1996). The ensuing tissue damage may have an important role in the MODS during sepsis. In the circulatory system of sepsis patients, hemodynamic alterations and microcirculatory disturbance are associated and previous research showed that cardiac and/or vascular effects play a role in these changes during sepsis (Ferguson et al. 1996; Hinsaw 1996).

Virtually all patients with sepsis have an activated coagulation system, which may range from minor changes in highly sensitive molecular markers for activation of

coagulation to full-blown DIC, with intravascular fibrin deposition and consumption of platelets and clotting factors (Levi et al. 2003). In recent years the importance of coagulation system changes in the pathogenesis of sepsis has become clear (Levi et al. 1993). Ongoing systemic activation of coagulation does not only lead to microvascular failure due to obstruction of the blood supply to various organs, thereby contributing to organ failure, but also plays a central role in the inflammatory response to severe infection and sepsis (Esmon et al. 1999). Various mechanisms that are important for the derangement of coagulation in patients with sepsis have been elucidated in the last 10–15 years. The pivotal role of tissue factor, impairment of physiological anticoagulant pathways, and depression of fibrinolytic response have been extensively studied (Esmon 2000). Also, the in vivo differential effect of inflammatory cytokines on each of these processes has been delineated and the tight interaction and cross-talk between activation of coagulation and inflammatory activity has been extensively studied (Esmon 2000; Levi et al. 2004).

Firstly, inflammation induces coagulation by tissue factor. Blocking tissue factor activity completely abrogates inflammation-induced coagulation activation in models of experimental endotoxemia or bacteremia, whereas antibodies that inhibit the contact system have no effect on thrombin formation (Levi et al. 1993; Pixley et al. 1993). In severe sepsis, circulating mononuclear factor, stimulated by proinflammatory cytokines, express tissue factor, which leads to systemic activation of coagulation

(Levi et al. 2004), even low-dose endotoxemia in healthy subjects results in a 125-fold increase in tissue factor mRNA levels in blood monocytes (Franco et al. 2000). Although many cytokines are capable of inducing tissue factor expression on mononuclear cells in vitro, the in vivo expression of tissue factor appears to be mostly dependent on IL-6 (Levi et al. 2004). Studies show that inhibition of IL-6 by monoclonal antibodies completely blocks tissue factor-dependent thrombin generation in experimental endotoxemia, whereas specific inhibition of other proinflammatory cytokines had less effect or no effect (Levi et al. 1997). Inflammation-induced activation of coagulation can also downregulate the three major anticoagulation pathways: antithrombin, the protein C system and tissue factor pathway inhibitor (TFPI), which regulate activation of coagulation (Levi et al. 2004).

Secondly, coagulation affects inflammation through protease-activated cell receptors and activation of platelets (Levi et al. 2004). Coagulation activation yields proteases that not only interact with coagulation protein but also with specific cell receptors to induce signaling pathways that mediate inflammatory responses (Levi et al. 2004). Many in vitro observations point to a role of coagulation proteases in upregulating the expression of proinflammatory mediators (Jones et al. 1990; van der et al. 2001). The most important mechanism by which coagulation proteases influence inflammation is by binding to protease activated receptors (PARs). The binding results in upregulation of inflammatory responses in macrophages and was shown to affect neutrophil

infiltration and proinflammatory cytokines expressions, such as TNF-α or IL-1β (Cunningham et al. 1999). In vivo evidence for a role of coagulation-protease stimulation of inflammation comes from recent experiments showing that administration of recombinant factor VIIa to healthy human subjects causes a 3- to 4-fold rise in plasma levels of IL-6 and IL-8 (Esmon 2002).

Thirdly, apart from its central role in regulation of coagulation activation, there is evidence that the protein C system also has an important function in modulating inflammation (Okajima 2001). Activated protein C has been found to inhibit endotoxin-induced production of TNF-α, IL-1β, IL-6 and IL-8 by cultured monocytes/macrophages (Okajima 2001). Furthermore, activated protein C abrogates endotoxin-induced cytokine release and leukocyte activation in rats in vivo (Murakami et al. 1996). Blocking the protein C pathway in septic baboons exacerbates the inflammatory response, whereas administration of activated protein C ameliorates the inflammatory activation in various models of severe systemic inflammation (Okajima 2001; Taylor et al. 1987). Mice with a heterozygous protein C deficiency not only have a more severe coagulation response to endotoxin but also demonstrate significant differences in inflammatory responses (Levi et al. 2003).

1.7. Blood Cells

Red Blood Cell (RBC): Red blood cells are the most common type of blood cell and the vertebrate body's principal means of delivering oxygen from the lungs or gills to

body tissues via the blood.

Increased erythrocyte sedimentation rate (ESR) is a common finding in sepsis, and it is used as a criterion in the diagnosis of the sepsis syndrome (Koppensteiner 1996; Philip 1982; Schulak et al. 1982). Sepsis-related increases in ESR are associated with acute phase reaction (Lowe 1988). Several studies have shown that decreased RBC deformability in sepsis (Astiz et al. 1995; Machiedo et al. 1989; Powell et al. 1991) that may contributed to the observed hemodynamic alternations. RBC deformability refers to the ability of the entire erythrocyte to change its shape in response to applied forces and has been assessed by several experimental methods (Baskurt et al. 1998). Clinical observation in humans also shows that sepsis induces a significant increase in free cytosolic concentrations of erythrocyte intracellular calcium (Todd et al. 1995). These altered biochemical and biomechanical properties of RBC in sepsis are partially related to increased RBC oxidative stress mediated by activated white blood cells (Machiedo et al. 1989; Powell et al. 1991). In addition, sepsis can induce increased RBC aggregation (Bedell et al. 1985), which play an important role in tissue perfusion problems encountered in sepsis (Astiz et al. 1995; Lam et al. 1994).

White Blood Cell (WBC) : White blood cells or leukocytes are cells which form a component of the blood. They are produced in the bone marrow and help to defend the body against infectious disease and foreign materials as part of the immune system.

The physical properties of leukocytes (volume, conductivity, light scatter, etc.) may change due to activation and presence of immature cells.

There are many different types of white blood cells. One primary technique to classify them is to look for the presence of granules.

Granulocytes are a category of white blood cells, characterized by the fact that all types have differently staining granules in their cytoplasm on light microscopy. These granules are related to lysosomes found in some regular cells and primarily act in the digestion of engulfed invaders. There are three types of granulocytes: neutrophils, basophils, and eosinophils (named according to their staining properties).

Besides granulocytes, there are two other types of white blood cells: lymphocytes and monocytes, which are characterized by the absence of granules in their cytoplasm. Monocytes are also known as macrophages after they migrate from the bloodstream and enter tissue.

Neutrophils are the most abundant type of white blood cells and form an integral part of the immune system, constituting approximately 65% of human's white blood cells (Lichanska et al. 2000). These phagocytes are normally found in the blood stream. However, during the acute phase of inflammation, particularly as a result of bacterial infection, neutrophils leave the vasculature and migrate toward the site of

inflammation in a process called chemotaxis. They are the predominant cells in pus, accounting for its whitish appearance. Neutrophils react within an hour of insult (Sampson 2000).

Neutrophil granulocytes have an average volume of 330 femtoliters (fl) and a diameter of 12-15 micrometres (μm) in peripheral blood smears.With the eosinophil and the basophil, they form the class of *polymorphonuclear cells* (PMNs), named for the nucleus's characteristic multilobulated shape (as compared to lymphocytes and monocytes, the other types of white cells). Neutrophils are the most abundant white blood cells; they account for 70% of all white blood cells (leukocytes). The stated normal range for blood counts varies between laboratories, but a neutrophil count of 2.5-7.5 x 10^9/L is a standard normal range. The average $t^{1/2}$ of a non-activated neutrophil in the circulation is about 4-10 hours. Upon activation, they marginate (position themselves adjacent to the blood vessel endothelium), undergo selectin dependent capture followed by integrin dependent adhesion after which they migrate into tissues, where they survive for 1-2 days. Cell surface receptors are able to detect chemical gradients of molecules such as IL-8, interferon gamma (IFN-gamma), and C5a that these cells use to direct the path of their migration (Sampson 2000).

Neutrophils are active phagocytes, capable of ingesting microorganisms or particles. However, they can only execute one phagocytic event, expending all of their glucose

reserves in an extremely vigorous "respiratory burst" (Hughes et al. 1997).

The respiratory burst involves the activation of a β-Nicotinamide adenine dinucleotide (NADPH) oxidase enzyme, which produces large quantities of superoxide, a reactive oxygen species, which breaks down to H_2O_2 by superoxide dismutation (Hughes et al. 1997). H_2O_2 then converted to hypochlorous acid (HOCl) by the green heme enzyme myeloperoxidase. It is thought that the bactericidal properties of HOCl are enough to kill bacteria phagocytosed by the neutrophil, but this has not been proven conclusively (Hughes et al. 1997).

Neutrophils also release an assortment of proteins in three types of granules: first, specific granules which help kill the ingested microbe by a variety of oxygen-independent mechanisms, such as defensins; second, azurophilic granules: myeloperoxidase, bactericidal/permeability increasing protein (BPI), serine proteases, neutrophil elastase and cathepsin G. Third, tertiary granules: cathepsin and gelatinase (Gutiérrez-Peña et al. 1996). Neutrophils can also extrude neutrophil extracellular traps (NETs), a web of fibers composed of chromatin and serine proteases that trap and kill microbes extracellularly. NETs provide for a high local concentration of antimicrobial components and bind, disarm, and kill microbes independent of phagocytic uptake. In addition to their antimicrobial properties, NETs may serve as a physical barrier that prevents further spread of pathogens. Furthermore, delivering the

granule proteins into NETs may keep potentially injurious proteins like proteases from diffusing away and inducing damage in tissue adjacent to the site of inflammation. Recently, NETs have been shown to play a role in inflammatory diseases, as NETs could be detected in preeclampsia, a pregnancy related inflammatory disorder in which neutrophils are known to be activated (Urban et al. 2006).

The biochemical behavior of WBC also changes in sepsis (Drost et al. 1995), primarily triggered by the activation process (Buttrum et al. 1994). Recent studies showed that WBC mechanical behavior has a significant role in microcirculation hemodynamic (Eppihimer et al. 1994).

The role of neutrophils in producing hepatocellular dysfunction during the hyperdynamic stage of sepsis has been shown in an animal model (Molnar et al. 1997). The results indicate that although circulating levels of serum alanine aminotransferase (ALT) were not elevated, hepatocellular function was significantly depressed during early sepsis, which can be prevented by neutrophil depletion. The results also suggest an integral role of the neutrophils in depressing hepatocellular function under certain conditions and prudent modulation of neutrophil function during the early stage of polymicrobial sepsis may be beneficial for preventing or delaying the occurrence of hepatocellular dysfunction. However, the neutrophils also play a protective role in

bacterial induced acute lung injury sepsis model (Ueda et al. 2001).

Neutrophils play a key role in the pathophysiology of septic MODS through excessive release of toxic granule components and reactive oxygen metabolites with the resulting tissue destruction. The increase in the number of senescent neutrophils during sepsis indicates a potential breakdown of autoregulatory mechanisms including apoptotic processes to remove activated neutrophils from inflammatory sites (Keel et al. 1997). Spontaneous neutrophil apoptosis in patients with severe sepsis is significantly reduced in comparison to healthy individuals. Cytokines detected in the circulation during sepsis (TNF-α, interferon-γ [IFN-γ], granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF]) all inhibited neutrophil apoptosis in both severe sepsis patients and healthy human, though the effect was more distinct in neutrophils from healthy humans (Keel et al. 1997). The addition of LPS to neutrophils from healthy humans markedly reduced apoptosis, which was partially restored through addition of anti-TNF-antibody (Keel et al. 1997).

Platelet: Platelets or thrombocytes are the cell component circulating in the blood that are involved in the mechanisms of primary hemostasis leading to the formation of blood clots. Platelet dysfunction and low platelet number of platelets predispose an individual to bleeding, while high levels, although usually asymptomatic, may increase the risk of thrombosis.

Platelets are involved in the pathogenesis of coagulation abnormalities seen in sepsis. Marked thrombocytopenia is a common feature of sepsis with an incidence of (platelet count <$150*10^9$/ L) critically ill medical patients at 35-44% (Baughman et al. 1993; Strauss et al. 2002; Vanderschueren et al. 2000). Typically, the platelet count in patients with sepsis decreases during the first 4 days in a critical care unit (Akca et al. 2002). Sepsis is a clear risk factor for thrombocytopenia in critically ill patients and the severity of sepsis correlates with the decrease in platelet count (Mavrommatis et al. 2000). It has been observed that platelets collected from patients with sepsis have reduced aggregability and decrease in platelets aggregation was related to the severity of sepsis. This is explained because increased platelet aggregation activity in patients with sepsis will lead to circulating platelets that are already activated and will not aggregate in an ex vivo setting (Yaguchi et al. 2004). As well there is a differential release of growth factors from platelets in patients with sepsis (Yaguchi et al. 2004), particularly the release of vascular endothelial cell growth factor (VEGF) along with the adherence of platelets to leukocytes and endothelial cells in the previous studies (Shebuski et al. 2002), all suggest that platelets have an essential role in the inflammation and coagulation pathways of sepsis.

1.8. Immune System

The innate immune system is the first line of defense against infection and is activated when a pathogen crosses the host's natural defence barrier (Janeway et al. 2002). It

consists of soluble elements (the alternative and mannan-binding lectin pathways of complement system, acute phase protein [APP] and cytokines) and cellular elements (monocytes, macrophages, neutrophils, dendritic cells and natural killer cells). Innate immune responses must be tightly regulated as unbalanced inflammatory and immune reactions that can result in either uncontrolled microbial growth or devastating inflammatory responses with tissue injury, vascular collapse and MODS (Bochud et al. 2003).

Detection of invading microorganisms is mediated by pattern recognition receptors expressed on the surface of innate immune cells, such as Toll-like Receptors (TLRs) on macrophage (Bochud et al. 2003). Pattern recognition receptors recognize the common structures of each pathogen, such as LPS, peptidoglycan, lipoteichoic acid, lipopeptides, flagellin, mannan and viral RNA, which are essential for survival of pathogen and avoid mutations.

When a pathogen associated molecular pattern binds to a pattern recognition receptor, it activates several intracellular signaling pathways resulting in the activation of transcription factors. The transcription factors control the expression of immune responses genes and the release of cytokines. Cytokines have an essential role in orchestrating the innate and acquired immune responses to an invading pathogen.

Sepsis is always associated with upregulation of proinflammatory cytokines, such as TNF-α, IL-6 and IL-1.

IL-1: IL-1 is composed of two distinct proteins: IL-1α and IL-1β. Both molecules activate the same IL-1 receptors and therefore share various biological activities. IL-1 is synthesized by mononuclear phagocytes, polymorphonuclear leukocytes and other cell types (Dinarello et al. 1991). IL-1β is predominant form of this mediator produced by endotoxin-stimulated human monocytes and detected in the plasma of septic animals (Smith et al. 1990). Both IL-1α and IL-1β mimic many of the biological activities of TNF-α. Infusion of either form of IL-1 into humans causes fever, haemodynamic abnormalities, anorexia, malaise, arthralgia, headache and neutrophilia (Cannon et al. 1990; Dinarello et al. 1991). IL-1β is increased in humans after infusion of endotoxin, although at lower concentration than TNF-α (Cannon et al. 1990; Hesse et al. 1988). Like TNF-α, IL-1β activates the production of other cytokines, including IL-6, IL-8 and TNF-α. Other evidence showing a primary role of IL-1β in sepsis syndrome is provided by using IL-1ra to block the biological activity of exogenous and endogenous IL-1β (Dinarello et al. 1991). For example, prior treatment with IL-1ra decreased the mortality of rabbits treated with endotoxin (Ohlsson et al. 1990). It is also reported that IL-1ra treatment reduced the production of IL-1, IL-6 and IL-8 (but not TNF-α) and improved survival after *E. coli* infusion in baboons (Fischer et al. 1992). IL-1β is not normally present in human plasma, but has been detected in the plasma of patients with sepsis. The results indicate initial plasma IL-1β concentration

may correlate with severity of sepsis, but no convincing independent association with mortality has been found (Casey et al. 1993; Endo et al .1992; Goldie et al. 1995).

TNF-α: Tumor necrosis factor α (TNF-α) is a member of a group of cytokines that stimulate the acute phase reaction. It is a 185 amino acid glycoprotein peptide hormone, cleaved from a 212 amino acid-long propeptide on the surface of macrophages (Goldie et al. 1995).

TNF-α is released by white blood cells, the vascular endothelium and several other damaged tissues, for example, by infection. Its release can also be stimulated by several other mediators, such as IL-1 and bacterial endotoxin. It has a number of actions on various organ systems, generally together with interleukins 1 and 6.

TNF-α can stimulate the hypothalamic-pituitary-adrenal axis by stimulating the release of corticotropin releasing hormone (CRH), suppress the appetite and cause hyperthermia due to its effect on the hypothalamus. It is also able to stimulate the acute phase response (APR), leading to an increase in C-reactive protein and a number of other mediators made by the liver. TNF-α attracts neutrophils very potently, and helps them to stick to the endothelial cell. It also stimulates phagocytosis, and the production of IL-1 oxidants and the inflammatory lipid prostaglandin E2 (PGE2). As well, it increases insulin resistance on other tissues. In addition, a local increased

concentration of TNF-α can cause the cardinal signs of inflammation to occur: heat, swelling, redness and pain.

Infusion of recombinant TNF-α into humans results in SIRS with fever, haemodynamic abnormalities, leucopenia, elevated liver enzymes and coagulopathy (Champman et al. 1987; Selby et al. 1987; Sherman et al. 1988; van der et al. 1990). In human and animal models of sepsis induced by injection of bacterial endotoxin, TNF-α can be quickly detected in plasma. Several studies show that the peak concentration of TNF-α can be detected 60-90 min after endotoxin infusion (Cannon et al. 1990; Hesse et al. 1988; Suffredini et al. 1989). In vitro, TNF-α can induce production of IL-1β, IL-6 and IL-8. In baboons injected with *E. coli*, treatment with TNF-α antibodies decreased the production of IL-1β, IL-6 and IL-8, in addition to reducing morbidity and mortality (Fong et al. 1989; Emerson et al. 1992; Hinshaw et al. 1992; Tracey et al. 1987; Redl et al. 1993). Clinical reports (Casey et al. 1993; Endo et al. 1992; Waage et al. 1987) indicate that TNF-α can be detected in the plasma of many patients with sepsis, and concentrations correlate with severity of illness and outcome.

IL-6: IL-6 is a proinflammatory cytokine secreted by T cells and macrophages to stimulate an immune response to trauma, especially burns or other tissue damage leading to inflammation (van der et al. 1997). IL-6 is one of the most important mediators of fever and of the APR. In the muscle and fatty tissue IL-6 stimulates

energy mobilization which leads to increased body temperature. IL-6 can be secreted by macrophages in response to pathogen associated molecular patterns (PAMPs) binding the TLR present on an active macrophage. (Kishimoto et al. 1995). IL-6 can also activate the coagulation system, induce APP production in the liver and modulate haematopoiesis (Borden et al. 1994; van der et al. 1994). Additionally, osteoblasts secrete IL-6 to stimulate osteoclast formation. Inhibitors of IL-6 (including estrogen) are used to treat postmenopausal osteoporosis (Febbraio et al. 2005).

In vitro, IL-6 suppresses the production of TNF-α and IL-1β (Aderka et al. 1989; Schindler et al. 1990). Experimental injections of endotoxin or bacteria result in detectable plasma IL-6, with peak concentrations occuring subsequent to peak TNF-α and IL-1 concentrations (Casey et al. 1993; Kuhns et al. 1995; Van Zee et al. 1991). In mice, IL-1 and TNF-α synergize to increase IL-6 production and the production of IL-6 after endotoxin injection can be inhibited by prior treatment with anti-TNF-α antibodies (Shalaby et al. 1989). In addition, TNF-α antibodies and IL-1ra can attenuate IL-6 production in bacteremic baboons (Fischer et al. 1992; Fong et al. 1989). All these indicate that the appearance of plasma IL-6 in sepsis may be related directly to TNF-α and IL-1 production. Although the role of IL-6 in sepsis is not well defined, several clinical reports show IL-6 concentrations correlate more closely than other cytokines with severity and outcome of human sepsis (Endo et al. 1992; Goldie

et al. 1995; Hack et al. 1989; Damas et al. 1992), which suggest that the IL-6 concentration in human sepsis may be a marker for activation of cytokine cascade and can predict MODS.

From an immunologic perspective of sepsis research, IL-6 and TNF-α. have been implicated as key mediators of inflammation, morbidity and mortality associated with sepsis. Hepatic APP synthesis is indirectly regulated by TNF-α. Several reports support the role of TNF-α and IL-6 in the sepsis syndrome, such as hypotension, hypothermia or fever, metabolic acidosis, pulmonary hemorrhage and even sudden death (Bone et al. 1989). From the rodent model of sepsis, the administration of TNF-α or IL-6 into mice induces an APR that consists of sepsis like symptoms (Bauss et al. 1987). Secondly, high circulating concentrations of TNF-α and IL-6 have been measured during septic shock and are negatively correlated with survival (Mózes et al. 1991). Thirdly, cachexia (loss of weight, muscle atrophy, fatigue, weakness and significant loss of appetite in someone who is not actively trying to lose weight) did not differ between wild type and IL-6 or TNF-α knockout mice, whereas anorexia was prolonged in TNF-α knockout mouse. Finally, survival was significantly enhanced in TNF-α knockout mice compared with the wild type controls, whereas the lack of IL-6 did not affect the lethality (Rivers et al. 2001). Clinical reports suggest that TNF-α and IL-6 concentrations were higher in septic shock than non-septic shock patients and the

persistence of TNF-α and IL-6 in serum predicted a poor outcome in patients with shock (Pinksy et al. 1993).

1.9. Current Therapy In Sepsis

The therapy for sepsis depends on antibiotics, surgical drainage of infected fluid collections, fluid resuscitation and appropriate support for organ dysfunction. This may include hemodialysis in kidney failure, mechanical ventilation in pulmonary dysfunction, transfusion of blood products, and drug and fluid therapy for circulatory failure. Ensuring adequate nutrition, if necessary by parenteral nutrition, is important during prolonged illness (Annane et al. 2002).

Antibiotics: Bacteria are the most common cause of sepsis, therefore antibiotics are absolutely the first line of therapy.

Regardless of the source of bacterial sepsis, antibiotics have been shown to impact cytokine levels. Antimicrobial agents can cause increases in LPS in humans and animals with sepsis (Newcomb et al. 1998; Shenep et al. 1984). Increased LPS, in turn, can lead to the production of multiple cytokines, including TNF-α and IL-6, both of which have been associated with increased mortality in sepsis (Hack et al. 1989; Negussie et al. 1992; Remick et al. 2002; Waage et al. 1989). An example of the impact of antimicrobial agents on cytokines is the Jarisch-Herxheimer reaction, which manifests fevers, rigors, and hypotension after treatment with antibiotics for louse-

borne relapsing fever, is associated with increased plasma levels of TNF-α, IL-6 and IL-1 (Bickell et al. 1994).

Broad spectrum antibiotics are used until the source of infection has been identified. There are a variety of antibiotics, as an example, cefotaxime is a third-generation cephalosporin. Like other third-generation cephalosporins, it has broad spectrum activity against aerobic Gram positive and Gram negative bacteria as well as some anaerobic organisms. In most cases, it is considered to be equivalent to ceftriaxone in terms of safety and efficacy but it is used to treat pneumonia and various infections such as intra-abdominal infection or sepsis.

Early and appropriate administration of antibiotics with decreased morbidity and mortality has been established in the ICU setting (Kollef et al. 1999; Houck et al. 2005). Clinical studies (Houck et al. 2004; Meehan et al. 1997) indicate that administration of antibiotics within 4 and 8 hours (Houck et al. 2004; Meehan et al. 1997) of hospital presentation can reduce mortality significantly ($P<0.01$).

Fluid Resuscitation: Fluid resuscitation is the first line therapy for patients suffering hypotension during septic shock in ICU and it can improve hemodynamic variables as well as the effect of vasopressor agent.

It may be a lifesaving procedure before the bleeding is controlled in hemorrhagic shock. However, the timing of fluid resuscitation on patients with septic shock is very complicated, which depends on the original disease, the cause of shock and the health

or morbidity of the patients. For example, delay of aggressive fluid resuscitation until operative intervention improves the survival of hypotensive patients with penetrating torso injuries compared to early fluid resuscitation that improves the survival of patients with endotoxin-induced septic shock (Pascual et al. 2003).

In animal models of fluid resuscitation, researchers found that hypertonic saline (HTS) resuscitation attenuates neutrophil lung sequestration and transmigration by diminishing leukocyte-endothelial interactions in the model of hemorrhagic shock, but not in endotoxin shock (Wilson et al. 1996). Other research has shown that fluid administration immediately after cecal ligation and puncture (CLP) in rats is essential for producing the hyperdynamic response during the early stages of sepsis in the rat model, but whether continuous fluid resuscitation prolongs the hyperdynamic response during sepsis is not clear (Wilson et al. 1996). However, much less is known about the relation between timing of fluid resuscitation and the mortality rate of sepsis in mice (Pascual et al. 2003).

CLP mice without fluid resuscitation resulted in significant increases in hepatic TNF-α mRNA, IL-1β mRNA and intestinal 1L-1β mRNA (Pascual et al. 2003). Volume administration attenuated cytokine expression at both 3 and 6 hours, and saline seemed to have more potent effects than serum. The volume of resuscitation correlated with survival at 18 hours. Survival in the saline (1 mL) + serum group was 90% at 18 hours compared with 20 to 40% in the groups with little or no resuscitation (Pascual et al.

2003). Therefore it is confirmed that fluid resuscitation (amount, composition, and timing) is an important consideration in the utilization of experimental infection models. Furthermore, optimization of a patient's intravascular volume status during sepsis may have an important impact on the immune response, in addition to improving the hemodynamic variables (Pascual et al. 2003).

However there are no survivors at 30 hour in the experiment described above because antibiotic treatment was not combined with fluid.

Early Goal Directed Therapy (EGDT): developed at Henry Ford Hospital by Rivers, is a systematic approach to resuscitation that has been validated in the treatment of severe sepsis and septic shock. The theory is that one should use a step-wise approach, having the patient meet physiologic goals, to optimize cardiac preload, afterload, and contractility, thus optimizing oxygen delivery to the tissues (Rivers et al. 2001). In EGDT, fluids are administered until the central venous pressure (CVP), as measured by a central venous catheter reachs 8-12 centimeter (cm) of water (or 10-15cm of water in mechanically ventilated patients). If the mean arterial pressure (MAP) is less than 65 mmHg or greater than 90 mmHg, vasopressors or vasodilators are given as needed to reach the goal. Once these goals are met the central venous oxygen saturation ($ScvO_2$), i.e. the oxgyen saturation of venous blood as it returns to the heart as measured at the superior vena cava, is optimized. If the $ScvO_2$ is less than 70%, blood is given to reach a hemoglobin of 10 g/dl and then inotropes are added until the

$ScvO_2$ is optimized. Elective intubation may be performed to reduce oxygen demand if the ScVO2 remains low despite optimization of hemodynamics. Urine output is also monitored, with a goal of 0.5 mL/kg/h. In the original trial, mortality was cut from 46.5% in the control group to 30.5% in the intervention group (Dellinger et al. 2004). The Surviving Sepsis Campaign guidelines recommends EGDT for the initial resuscitation of the septic patient with a level B strength of evidence (single randomized control trial) (Bernard et al. 2001).

Adjunctive Therapies: Most therapies aimed at the inflammatory process itself have failed to improve outcome. However, drotrecogin alfa (activated protein C, one of the coagulation factors) has been shown to decrease mortality from about 31% to about 25% in severe sepsis and a protective effect by reducing IL-6 and TNF-α on sepsis lethality has also been demonstrated (Baker et al. 1983). Low dose hydrocortisone treatment has shown promise for septic shock patients with relative adrenal insufficiency (RAI) as defined by adrenocorticotropic hormone (ACTH) stimulation (Annane et al. 2002).

A number of adjunctive treatments for severe sepsis and septic shock have been tested in clinical trials (Bochud et al. 2003). These include neutralisation of microbial toxins such as LPS, non-specific anti-inflammatory and immunosuppressive drugs, neutralisation of proinflammatory cytokines, and correction of abnormalities in

coagulation. The results have been mixed (Vincent et al. 2002), although several recent clinical trials have given encouraging results.

The profibrinolytic, anti-inflammatory anti-apoptotic effects of protein C may improve microcirclatory flow (Tugrul et al. 2002). According to the study of Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS), a multicentre randomized controlled trial showed that the administration of r-APC (drotecogin alfa), which started within 24 hours after the criteria for severe sepsis were met, reduced mortality from severe sepsis or septic shock by 6% compared with placebo (Bernard et al. 2001). Several other studies also suggest that early administration of r-APC may improve morbidity and mortality (McLeay 2004).

In the neurohumoral response to septic shock, many patients have manifestly inadequate adrenal reserve or relative adrenal insufficiency (Chadda et al. 2002; Lamberts et al. 1997; Rothwell et al. 1991; Soni et al. 1995). Thus glucocorticoids, which exert broad metabolic and immunomodulating effects, have been used to treat many inflammatory diseases. Although high dose of steroids have no clinical benefit in sepsis (Vincent et al. 2002), a recent multicentred trial found that a seven day course of low doses of hydrocortisone and fludrocortisone reduced mortality in patients with septic shock and relative adrenal insufficiency (Annane et al. 2002). Corticosteroids (hydrocortisone 50mg intravenously every 6 hours and fludrocortisone 50 μ g by mouth once daily) were started within 8 hours after the diagnosis of septic

shock and continued for 7 days. There was a 10% absolute reduction in 28-day mortality in the group that received hydrocortisone and fludrocortisone and who were diagnosed with RAI at randomization (Annane et al. 2002).

Meticulous control of glucose with insulin has also shown to reduce mortality in patients with sepsis (Rivers et al. 2001; van den et al. 2001).

Anti-TNF-α Monoclonal Antibody: TNF-α plays a central role in the pathogenesis of endotoxemia and septic shock (Debets et al. 1989). Elevated plasma TNF levels correlate with mortality in human with sepsis (Beutler et al. 1985; Waage et al. 1987), and the parenteral administration of large doses of TNF-α causes clinical symptoms indistinguishable from those in septic shock (Tracey et al. 1987). Antibody against TNF-α has improved survival in certain model of sepsis, but it only shows a small, nonsignificant survival benefit in clinical studies (Reinhart et al. 2001). To be effective, antibody must be administrated shortly before or after the septic challenge (Bagby et al. 1991; Eskandari et al. 1992; Hinshaw et al. 1992; Windsor et al. 1994). The administration of anti-TNF prior to septic challenge has not been shown to be of benefit in some models of sepsis (Echtenacher et al. 1990; Havell 1989) and indeed TNF-α itself may offer some protection against sepsis (Koch 1998). A complete neutralization of TNF-α may be ultimately detrimental to the host (Koch 1998; Badr 1994)

1.10. Animal Model

Two milestones in the history of antimicrobial chemotherapy are directly attributed to experiments conducted in animal models of infection. The first resulted from the surprising observation, sulphonamidocrysoidine (Prontosil), although devoid of antibacterial activity *in vitro*, was effective against a pneumococcal infection in mice (Domagk 1986). This finding furnished the very first proof that (Abraham 1980) systemic bacterial infections could be cured by drugs and (Barza 1978) *in vivo* testing was an indispensable component of antimicrobial drug research. The second was penicillin. In retrospect, it seems that this antibiotic could have been available to patients much earlier; Fleming only used penicillin in differentiating cultures and in a few cases as a local antisepsis. He apparently had no thought of the possibility of its systemic clinical effect. Not until the 1940s, was the substance partially purified and then demonstrated its potent therapeutic activity, first in the mouse and afterward in humans (Abraham, 1980).

Since 1960, more than 1000 different animal models for experimental chemotherapy have been described. Depending on their purpose, nature and predictive value, they can be assigned to various categories (Zak, 1980); each has its justification at some point in the development of an antibiotic or therapeutic strategy.

The basic antimicrobial screening models are *in-vivo* test systems frequently used in the early evaluation of new antibiotics. The best known model in this category is that of acute septicemia in mice, and it is, so far, the simplest and most inexpensive test, allowing a rough estimate of whether or not an antibiotics is effective *in vivo*. The clinical relevance of the results obtained in this model is limited by its many inherent drawbacks. For example, the rapidly fatal course of infection in mice is not usually a characteristic of human disease. As well, the sensitivity of the test to the size of the inoculum, the prophylactic rather than therapeutic regimens used in this test may yield indicative but not necessarily predictive of human outcome.

Ex-vivo models appear to simulate human infections somewhat more closely than do screening tests. This category comprises models in which foreign bodies, e.g. fibrin clots or dialysis sacks, are implanted into animals and infected. Other variants use perforated rubber or plastic tubing, small spiral springs or perforated balls, which are infected after they have been surrounded by granulation tissue and filled with edematous fluid. The effects of antibiotics in these tests are then evaluated *in vitro*, e.g. by determination of the antibiotic concentrations reached or the bacterial counts in samples of the foreign bodies or the fluid.

A group of models used in the evaluation of antibiotics belong to the category described as monoparametric/polyparametric models. Their difference from the

screening models is that, instead of waiting for the ultimate therapeutic effects to appear (cure or death), only one single, or preferably many, parameter (s) is/are examined during the experiment. The determination of serial bacterial counts and other substances in the blood or tissue at different time point during infection, or studies of how bacteria or agents change the immune system throughout treatment, can be concluded in this category.

Both *ex-vivo* and monoparametric models are frequently used during the more advanced secondary phase of the evaluation of a new treatment. In general they are very helpful in differentiating the properties of various drugs, although their main merit is in providing data on the effects of drugs that would be impossible to study in humans, whether for ethical or technical reasons, e.g. the effects of subtherapeutic doses or very short treatment regimens. Many of the drawbacks of screening models, however, also apply to the *ex-vivo* and monoparametric test systems and especially to their use in small rodents.

Experimental infections which belong to the category of discriminative models are designed to simulate human infection as closely as possible. They permit the potential therapeutic effects of new or already established drugs and drug combinations to be differentiated and define the indications in which these drugs might be effective in humans. They are also applied for the research in interactions between drugs and

pathogen(s). Here the ideal model is able to exhibit some features that connect between animal and human clinical trials. Firstly, the route of entry and spread of causative organism should be identical or at least similar with the situation in human. Secondly, the involved tissue and severity, course and duration of disease should be predictable, reproducible and amenable to analysis. Thirdly, the susceptibility of the model to the drugs should be measurable and reproducible.

When we design sepsis experiments using an animal model, one may focus on the similarities of the animal model to the human disease; in the mean time, one must also be aware of the inherent differences between animal models and human.

Studies of sepsis in humans are difficult because the seriousness of the disease mandates immediate intervention and because the heterogeneity of patient presentations imposes substantial limitations on clinical trials. Thus, animal models have been used extensively to explore the pathogenesis of sepsis and to generate preclinical data for therapeutic interventions. Translation of findings in these models into therapeutic strategies has been difficult, in part because of limitations in preclinical models and in part to imperfect understanding of the pathophysiology of sepsis. It is important to use an animal model that reproduces the relevant physiologic parameters present in patients with septic shock. Mouse models have been particularly

useful for understanding molecular mechanisms of disease because of the potential of transgenic strains.

Before studying early signals of sepsis and introducing the treatment strategies, we needed to establish a reproducible and reliable mouse model for sepsis. The CLP model, which is a widely used experimental model to produce polymicrobial sepsis created by Baker in 1980, was chosen and Balb/c mice (average weight 25 gram) were used for the model.

The CLP model was selected because it mimics clinically encountered peritonitis in the following ways. Indigenous gut flora are seeded into the systemic circulation from a remote septic focus, blood cultures are positive, and the insult is polymicrobial (McCallum et al. 1986). Also, the CLP model has been well characterized and has been used to investigate the protective effects of endotoxin, IL-1 and TNF without treatment in lethality studies and to characterize the bacteria found in the blood and peritoneal fluid of septic mice (Munford et al. 1984; Urbaschek et al. 1984; Urbaschek et al. 1987).

The establishment of this model can help us observe the septic process, measure the immune system response and identify early signals and effective intervention strategies of sepsis. Since the combinations of antibiotic administration and fluid

resuscitation has not been studied well, our treatment strategies will focus on them after the establishment of the model. Also, we will try to introduce anti-TNF-α monoclonal antibody as an adjunctive treatment for the two other main treatment strategies.

The reasons of choosing a mouse model are: 1) Its low cost enables us to have a large sample size which is enough for the study. 2) We can collaborate with expertise outside University of Ottawa who have also established the same mouse model as there was no local expertise. 3) We can study relevant clinical questions that have not been previously investigated. Finally, although the pig may be more appropriate for the model because its body constitution is more similar with human than mouse, it is unaffordable as a pilot study.

1.11. Hypothesis

Timing of antibiotic administration and fluid resuscitation are crucial to the survival in severe sepsis. Early administration of antibiotics and fluid resuscitation will result in the best survival advantage.

The goal of our project was to indicate:

a. Hepatic dysfunction occurs in the early stage of sepsis and has an impact on MODS
b. Timing of antibiotic administration is important in survival outcome of septic mice.
c. Early fluid resuscitation is crucial in the maintenance of the survival in septic mice.
d. The administration of anti-cytokines and other adjunctive treatments after antibiotics may mitigate the septic process.

2. Material & Methods

2.1. Materials & Regular Procedures

2.1.1. Animals:

Male balb/c mice employed in this study were obtained from Charles River Company, Montreal, Canada. They were housed in plastic cages and food and water were provided. Mice used in this experiment were 23-25g in weight.

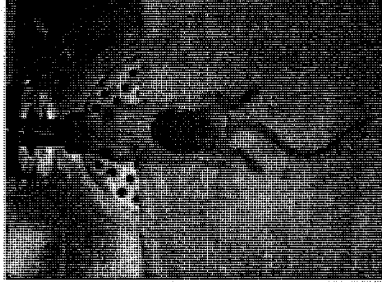
2.1.2. Intraperitoneal Sepsis model:

Pre-surgery: 1mL 0.9% saline fluid and 0.03mL buprenorphine[1] (sedative) were given by subcutaneous(S.C.) injection one hour pre-surgery. The weight and body temperature of the mouse were also recorded at the same time.

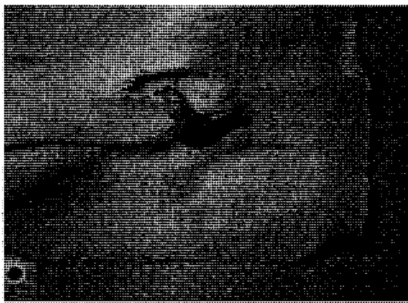
Surgery/Cecal Ligation and Puncture (CLP): A one centimeter cut was made on the midline of abdominal wall. The cecum was then pulled out and a ligation was made at one centimeter from the end of cecum. Then a through and through puncture was made by an 18 gauge (G) needle and a little feces was expressed from the holes to cause a bacterial infection in abdominal cavity.

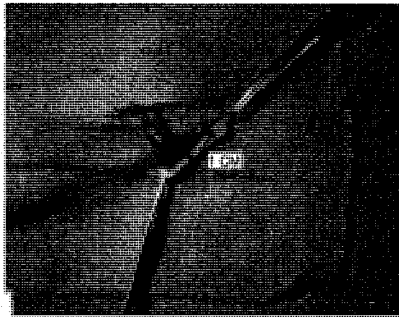
Post-surgery: All the mice were observed and given food and water for 72 hours. Body temperature, weight and other vital signs were recorded every three hours. Other

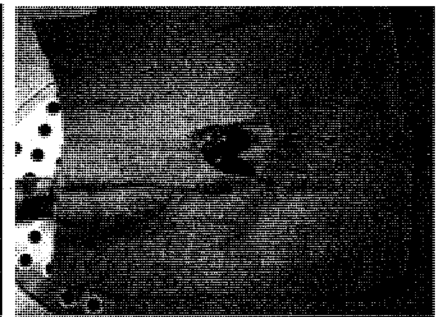
Picture 1

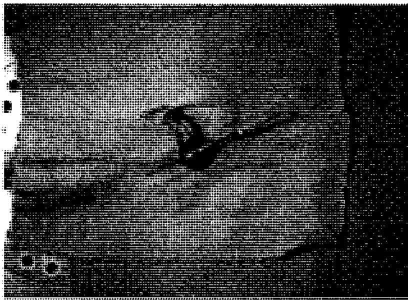

Picture 2

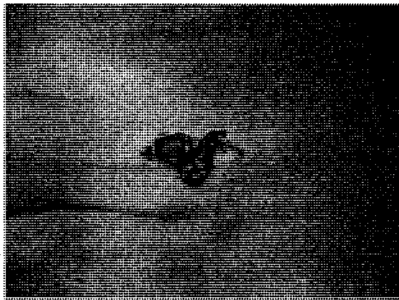
Picture 3


Picture 4


Picture 5

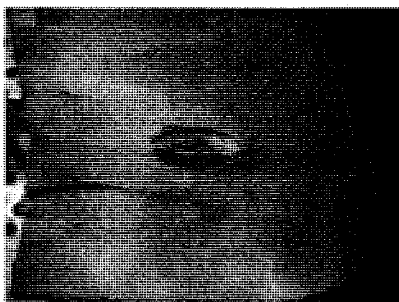

Picture 6

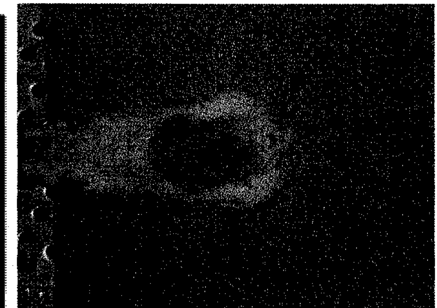

Picture 7


Picture 8

Picture 9

Picture 10


Picture11


Picture 12

clinical signs observed included dehydration, behavior and demeanour, abdominal distention, respiratory distress, incision site and diarrhea. 0.03mL buprenorphine were given every 6 hours for 72 hours to alleviate any discomfort.

2.1.3. Potential Mortality Endpoint:

As death was not an acceptable endpoint for the animal model according to University of Ottawa Animal Care Policy, we adopted a surrogate measure for death approved by the Committee, and had defined our own potential mortality endpoint. The rule of potential mortality endpoint in Animal Care is that the mouse should be euthanized immediately when its body temperature drops below 32℃ and remains low for one hour. Based on the observation of our model and discussion with Animal Care, we agreed that this rule is not appropriate for our project because some mice did not die when their body temperature dropped below 32℃, thus we defined our own potential mortality endpoint with other clinical signs: if a mouse's body temperature drops below 32℃ for more than one hour along with other abnormal clinical signs, such as drowsiness (no response to touching), groggy (unable to walk straightly), high dehydration, respiratory distress, GI tract shut-down and diarrhea, it reaches potential mortality endpoint and will be euthanized immediately. The Committee approved this endpoint after reviewing all the data.

1. Buprenorphine is an opioid drug with partial agonist and antagonist actions. According to animal care, the drug must be used 1h before surgery and every 6h after surgery until the euthanasia to relieve the pain of mice.

2.1.4. Blood Sampling:

24 hour pre-surgery, 0.05mL blood was collected from the hind leg of each CLP mouse in the "Model Development & Parameter Determination" group by a veno-puncture collected in an EDTA tube for WBC count. At the euthanasia time point, 1mL blood was collected by cardiac puncture from all the mice in this project. The post-surgery blood was used for multiple tests, including WBC count, lactate, ALT, and cytokines (TNF-α, IL-6) levels measure. In some mice, blood samples were used for culture. Due to limited quantity of blood from each mouse, each measurement was done on selected mice.

2.2. Model Development & Parameter Determination:

During this experiment we were trying to establish an appropriate sepsis model for administration of different treatment strategies. 1mL 0.9% saline fluid was given to each mouse in this experiment every 6 hours from 0 hour post-surgery to maintain the metabolic system and prevent dehydration. We initially used a standard ligation (1cm) and a standard needle (18G); we tested one vs two punctures in different mice

respectively to compare the difference of mortality between them. Then we ligated different length of the cecum from 1/3 cecum (about 1cm), 1/2 cecum and the whole cecum in different mice in order to achieve a reliable consistent level of inflammatory response and potential mortality. All the ligated ceca were punctured twice by an 18G needle (table 1).

The influence of the size of the cecal puncture on mortality, bacteremia, endotoxemia and plasma TNF-α levels has been previously studied. It showed that bacteremia was present in all the animals with different size of needles (from 13G to 22G) or blade incision of the cecum (Shenep et al. 1984). Endotoxemia and plasma TNF levels tended to increase along with the diameter of the cecal puncture. Mortality gradually increased with the puncture size, from 27% with a 22G needle to 95% with the blade incision (Shenep et al. 1984). Therefore, we tested the 16G and 18G needles with two puncture respectively, in order to achieve a moderate level of inflammatory response and potential mortality. All the ceca were ligated 1cm from the end.

The goal of above experiment was to achieve consistency of inflammation and potential mortality endpoint in order to study treatment option. After choosing 1cm ligation of cecum and two punctures with 18G needle as our regular surgical procedure, the CLP mice were kept for 18 hours, 24 hours and 30 hours. There were 5

mice in each group with different time points (18h, 24h and 30h). These included four CLP mice and one negative control mouse. All three experiments were repeated twice.

2.2.1. Pathological Examination:

After the euthanasia, autopsies were done on all the CLP mice and negative control mice. The main organs (liver: 34 mice, kidney: 10 mice, heart: 9 mice, lung: 9 mice, spleen: 6 mice and intestine: one mice) were harvested and sent for pathological examination.

2.2.2. Blood Glucose Assay:

We randomly chose 10 CLP mice and 2 negative control mice from the pilot group and tested their blood glucose level every 6 hours for up to 30 hours from 6 hours post-surgery. 0.02ml blood was collected from the hind leg of the examined mice for the test. The blood glucose meter (produced by Bayer HealthCare, Toronto, Ontario) was provided by Animal Care.

2.2.3. Lactate Assay Test:

Since the CLP mice may suffer from decreased tissue perforation post-operatively, we measured the serum lactate from 6 CLP mice at different time points after surgery. The lactate assay kit was purchased from Biochemistry Research Service Center,

University of Buffalo (New York, U. S. A.). Sera were stored at –70℃ in the freezer for 24 hours.

Technique:

1mM lactate standard was diluted 1:4 in ice-cold distilled water to obtain a 250μM lactate concentration. Then additional dilutions were performed to obtain 200, 150, 100 and 50μmol/L lactate standards. The sera were diluted 1:20 in distilled water. 20μL diluted sera and lactate standards were added to a 96-well microplate, including 20μL distilled water as a sample blank. Enzymatic reaction was initiated by addition of 50μL Lactate Assay Solution to each well. The plate was incubated in a humidified 37℃ incubator for 30 minutes. The reaction was stopped by adding 50μL 3% acetic acid per well. Then the optical density of each well was read by a microplate reader at 492 nanometer (nm).

2.2.4. ALT Assay:

To measure hepatic dysfunction in CLP mice, we also tested the alanine aminotransferase (ALT) serum level of 7 CLP mice and 2 negative control mice from 18, 24 and 30 hours post-surgery groups separately. The method used was described in "Biochemical Experiment" published by Science Press (Beijing, China) in 1986.

Technique:

We used a manual method for the assay. The materials include: 2μmol/L pyruvic acid solvent, 0.1mol/L phosphate buffer (pH 7.4), ALT substrate (α-ketoglutaric acid + phosphate buffer+ sodium hydroxide, pH 7.4), 2, 4-M nitrobenzene and 0.4mol/L sodium hydroxide.

Six tubes with different volumes of pyruvic acid solvent, ALT substrate, phosphate buffer and 2, 4-M nitrobenzene were used to make a standard curve (table 2) after they were read by spectrophotometer. Then the ALT substrate was replaced by 0.1mL serum from the mice, read by spectrophotometer (table 2) and the ALT level was deduced by applying the established standard curve (Chen et al. 1986).

2.3. Treatment Strategies (Table 3):

After the development of the mouse model that reproduces the timing and degree of mortality seen in patients with septic shock, early intervention pathways were introduced into the CLP mice. Three treatment strategies were evaluated: timing of antibiotic administration, timing of fluid resuscitation and adjunctive anti-cytokine treatment. Antibiotics are a mainstay of sepsis treatment and have been repeatedly demonstrated to improve survival in both human studies and animal research models of sepsis. Rapid fluid resuscitation is an important component of the initial therapy for severe sepsis. The antibody against TNF-α has improved survival in certain models of sepsis.

2.3.1. Timing of Antibiotics Administration:

Cefotaxime, a third generation cephalosporin, was given by intraperitioneal (IP) injection at various start time points after surgery every 6 hours over a 72 hour period. The cefotaxime was purchased from the pharmacy of the Ottawa Hospital (Ottawa, Canada). The dose was calculated with the assistance of Dr. M. Lippeman of Animal Care. The calculation of the dose for Balb/c mice was adopted from Formula for Laboratory Animals (Appendix I). We diluted the powder in 30mL sterile water and gave each mouse 0.05mL (33.3mg/mL) by IP injection. Antibiotic administration in

humans is every 12 hours. However, administration in mice is every 6 hours due to their high metabolic rate.

All the mice in this treatment strategy were given 1mL 0.9% saline fluid by subcutaneous injection 0 hour post-surgery which is at the time of abdominal incision closure. Then the same amount of fluid was given to each mouse every 6 hours after surgery for up to 72 hours.

Cefotaxime Post-surgery Treatment: There were 6 mice in the experiment. Four CLP mice were given 0.05mL diluted cefotaxime (1g cefotaxime : 30ml sterile water) by intraperitoneal injection at 0 hour post-surgery and then the same amount of cefotaxime was continually given to each of them every 6 hours for up to 72 hours. One CLP mouse without cefotaxime was used as a positive control. One mouse with a sham operation was used as a negative control. The experiment was repeated twice.

This design was repeated for cefotaxime starting at the following time points: 3 hours, 6 hours and 12 hours post-surgery. In all cases the antibiotic was continued every 6 hours for up to 72 hours with a group of 6 mice including one positive control and one negative control mice. Each time point was repeated twice.

All the mice were observed and given food and water for up to 72 hours. Mice that reached the potential mortality endpoint were euthanized, as previously described. The

blood samples of all the mice, which were taken at euthanasia, were tested for TNF-α (53 mice) and IL-6 levels (32 mice). Some blood samples were tested for WBC count (22 mice) or used for bacterial culture (5 mice).

2.3.1.1. Bacterial Culture Swab:

During the surgery, some CLP mice and negative control mice had their abdominal cavity swabbed for culturing. It was also performed during autopsy at the euthanasia time-point. The swabs were sent to the Microbiology Laboratory at the Ottawa Hospital for tests of the identification of bacteria.

2.3.1.2. Blood Culture:

1mL blood from each selected mouse was injected into thiologycate broth tube (10mL, OXOID, Nepean, Ontario) under sterile conditions. The culture was then kept in the incubator (35℃, 5% CO_2) for 4 days. On day 2, the tube was shaken to make sure the fluid is mixed. At day 4, 100μL mixed fluid was cultured on each tryptic soy agar (TSA) plate and the plates were kept in the incubator (35℃, 5% CO_2) for 48 hours.

Two days later, the different colonies on the plates were subcultured onto Chocolate, TSA, MacConkey and CBC-Control (Anaerobic Bacteria Agar) plates to separate the different types of bacteria. All the subcultured plates were put into the CO_2 incubator

for 24 hours except MacConkey, which was put in the O_2 incubator, and CBC-Control, which was put in the anaerobic glove box. Subculture of each colony was plated on TSA plates, incubated for 24 hours to get pure organism for identification.

Pastorex staph-plus was used for the identification of *Staphylococcus aureus*, PYR (Pyrrolidonyl-beta-naphthylaminde hydrolysis) for the identification of *Enterococcus* and the API120 STREP for identification of other types of bacteria, such as *Streptococcus*.

2.3.1.3. WBC Counts:

0.38mL 10% acetic acid was mixed with 0.02mL anticoagulated mouse blood, then one drop of crystal violet was added to the mixture. The number of WBC was counted on the hemocytometer under the microscope.

2.3.2. Fluid Resuscitation:

All the mice in this intervention strategy were given 0.05mL diluted cefotaxime by intraperitoneal injection every 6 hours for up to 72 hours, which started from 0 hour post-surgery. Because of the consistency of the model, positive and negative controls were no longer included in the following studies.

The same study design was used with 8 mice with varying starting point for 0.9% saline fluid: 3 hours, 6 hours, 9 hours and 12 hours post-surgery and then fluid continued every 6 hours for up to 72 hours. The reason we adopted 9 hours time mark is because the mortality at 12 hours was 100% while at 6 hours it was 12.5%. Thus we wanted to find out the mortality between the two time points.

All the mice were observed and given food and water for 72 hours. The mice that reached the potential mortality endpoint were euthanized immediately. The blood samples of all the mice, which were taken at euthanasia, were tested for TNF-α (40 mice) and IL-6 (33 mice) levels.

2.3.3. Anti-TNF-α Monoclonal Antibody Adjunctive Treatment:

The anti-TNF-α monoclonal antibody was purchased from R & D system company (Minneapolis, U.S.A.). Because we identified a rise in TNF-α peak at 12 hours post-surgery, we wanted to study the role of anti-TNF-α monoclonal antibody on survival of our model. We used antibiotic administration at 12 hours post-surgery (with 100% mortality) as a proof of concept. We also used delayed fluid resuscitation at 12 hours post-surgery (with 100% mortality) for the same reason. Using other time points of treatment strategies (i.e. 25% or 50% mortality) would require many more mice for

statistical analysis and we were limited to a total 300 mice for our research experiment.

2.3.3.1. Anti-TNF-α Treatment for Late Administration (12 hours Post-surgery) of Cefotaxime (group 1):

There were 22 CLP mice and 7 negative control mice in this experiment. 1mL 0.9 % saline fluid was given to each mouse by subcutaneous injection 0 hour post-surgery and then the same amount of saline fluid was continuously given to each of them every 6 hours for up to 72 hours. 0.05mL diluted anti-TNF-α monoclonal antibody was given to each CLP mice 6 hours post-surgery by intraperitoneal injection. Then all the CLP mice were given 0.05mL diluted cefotaxime by intraperitoneal injection 12 hour post-surgery and then the same amount of cefotaxime was continuously given to each of them every 6 hours for up to 72 hours.

2.3.3.2. Anti-TNF-α Treatment for Late Fluid (12 hours Post-surgery) Resuscitation (group 2): Due to the high mortality, there were only 8 CLP mice and 7 negative control mice in this experiment. 0.05mL diluted cefotaxime was given to each CLP mice by intraperitoneal injection 0 hour post-surgery and then the same amount of cefotaxime was continuously given to each of them every 6 hours for up to 72 hours. 0.05mL diluted anti-TNF-α monoclonal antibody was given to each CLP

mice 6 hours post-surgery by intraperitoneal injection. Then all the mice were given 1mL 0.9% saline fluid by subcutaneous injection 12 hours post-surgery and then the same amount of fluid was continuously given to each of them every 6 hours for up to 72 hours.

Mice of both groups were observed and given food and water for 72 hours. The mice reaching potential mortality endpoint were euthanized immediately. The blood samples were tested for TNF-α (group 1: 29 mice, group 2: 15 mice) and IL-6 levels (group 1: 25 mice, group 2: 12 mice).

2.4. Cytokines (TNF-α and IL-6) Kinetics during Late Intervention Strategies:

We observed that some mice were moribund at 48 hours post-surgery and had TNF-α levels as low as the negative control mice. To determine whether the TNF-α level dropped over time from a peak and correlated with high mortality, we repeated cefotaxime 12 hours post-surgery group and 0.9% saline fluid 12 hours post-surgery group, again with timed euthanasia.

2.4.1. Cefotaxime Administration 12 hours Post-surgery Group:

A total of 35 treated CLP mice were euthanized at different time points after the surgery: 2 mice at 0 hour post-surgery, 3 mice at 6 hours post-surgery, 4 mice at 12

hours post-surgery, 11 mice at 18 hours post-surgery, 8 mice at 24 hours post-surgery, 3 mice at 30 hours post-surgery and 4 mice at 48 hours post-surgery. Although we attempted to have equal groups, some of the mice needed to be sacrificed early because they reached the potential mortality endpoint. As well some time point data could be extrapolated from previous measurements.

2.4.2. Fluid Resuscitation 12 hours Post-surgery Group:

Due to high mortality, 24 mice (18 CLP mice and 6 negative control mice) were euthanized at different time points after surgery within 24 hours. 2 CLP mice and 2 negative control mice were euthanized at 0 hour post-surgery, then 4 CLP mice were euthanized every 6 hours within 24 hours. 2 negative control mice were euthanized every 12 hours within 24 hours.

TNF-α and IL-6 levels from both groups were tested from the blood samples and the results were compared between two groups along with the mortalities.

2.5. TNF-α Assay:

The TNF-α Assay Kit was purchased from Assay Design Company (Michigan, U.S.A.). The mouse TNF-α Microtiter Plate in it contains a 96-well plate using break-

apart strips coated with monoclonal antibody specific to mouse TNF-α. The sera of mice were stored at –70℃ until use.

2.5.1. ELISA (Enzyme-linked Immunosorbent Assay) Technique:

Reagent Preparation: Wash buffer (Tris buffered saline containing detergents) was diluted 1: 200 in distilled water. Mouse TNF-α standard solution (20,000 picogram [pg]/mL) was diluted 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 in tube #1 to tube #7 respectively in Assay Buffer 13 (a proprietory recipe containing Tris buffered saline with proteins and detergents). Test sera were diluted 1:8 in Assay Buffer 13.

Assay Procedure: Fifty μL of diluted mouse TNF-α standard solutions (Standards #1 to #7) and Assay Buffer 13 were pipeted into the appropriated wells of the plate. Fifty μL of the test serum was pipeted into each appropriate well. The plate was sealed by plastic sealer and incubated at room temperature on a plate shaker for 2 hours at 500 revolutions per minute (rpm) and then washed with buffer four times. Fifty μL of mouse TNF-α antibody (rabbit polyclonal antibody to mouse TNF-α) was then pipeted into each well. The plate was sealed and incubated at room temperature on a plate shaker for two hours at 500 rpm, washed four times with buffer and then 50μL of cytokine conjugate (a blue solution of donkey anti-rabbit antibody conjugated to horseradish peroxidase) was added into each well. The plate was then sealed and

incubated at room temperature on a plate shaker for 30 minutes at 500 rpm. After four washes in buffer 50μL of Substrate Solution was pipeted into each well, incubated at room temperature for 30 minutes and the optical density was read at 450nm by the plate reader.

2.6. IL-6 Assay:

IL-6 Assay Kit was purchased from R & D system company (Minneapolis, MN, U.S.A.). Mouse sera were stored at –70℃ until use. 96 well polystyrene microplate is coated with a monoclonal antibody specific for mouse IL-6.

2.6.1. ELISA Technique:

Reagent Preparation: Mouse IL-6 Kit Control (buffered protein base with preservatives) was reconstituted with 1.0mL distilled water. Wash buffer was diluted 1: 25 in distilled water. Substrate Solution was mixed by equal volume hydrogen peroxide (Color Reagent A) and chromogen (Color Reagent B). 2.5 nanogram (ng) recombinant mouse IL-6 was reconstituted with 5.0mL of Calibrator Diluent RD5T (buffered protein solution) to produce a stock solution of 500pg/mL. Then the reconstituted standard was diluted 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 in Calibrator Diluent RD5T. Sera were diluted 1:20 in Calibrator Diluent RD5T.

Assay Procedure: Firstly, 50μL of Assay Diluent RD1-14 was added to each well. Then 50μL of diluted Standard solutions, Control solution and sera were added into each well. After that the plate was sealed and incubated at room temperature for 2 hours. The plate was washed four times with buffer 2 hours later and 100μL of mouse IL-6 conjugate (polyclonal antibody against mouse IL-6 conjugated to horseradish peroxidase) was added into each well. The plate was sealed and incubated at room temperature for 2 hours, then washed four times in buffer and 100μL of Substrate Solution was then added into each well. The plate, protected from light by wrapped in the aluminium foil, was incubated at room temperature for 30 minutes and then 100μL of Stop Solution (diluted hydrochloric acid solution) was added into each well. Finally, the optical density of each well was read by microplate reader at 450nm.

2.7. Statistical Analyse:

Fisher's exact test, t-test and analysis of variance (ANOVA) test were used to find out if there were significant differences in mortalities, temperature changes, WBC counts, ALT levels, lactate levels, and cytokine levels between different treatment groups. The Method Center at the Ottawa Hospital provided helpful support with statistical analysis.

2.8. Animal Care:

All experimental procedures and protocols involving animals were reviewed by, and set with the approval of, the University of Ottawa Animal Care Committee, protocol number BMI-71.

3. Results

3.1. Model Development & Parameter Determination:

The mortality of CLP mice with one 18G needle puncture on ceca was low (Fig. 1), thus we used two punctures instead of one. Then we performed preliminary experiments to size the cecal ligation after the puncture and we found that the mortality was 100% (Fig. 1) when more than 1/2 of the cecum was ligated no matter what treatment was given. The ceca were excised at autopsy suggesting vascular compromise.

We then studied lethality in the model based on two different sizes of needles (16 & 18G). Although the mortalities of both needles within the first 30 hours post-surgery were the same (Fig. 1), we selected 18G needle because it enabled abdominal sepsis from fecal contamination within 24 hours post-surgery while 16G needle could not. The 16G needle yielded massive fecal contamination in the mouse's abdominal cavity and caused mice reaching potential mortality endpoint before they showed the signs of sepsis. We settled on this model to enable studies of therapeutic interventions. All the negative control mice in the pilot study survived throughout the observation.

The rectal temperature and weight were measured every three hours after surgery on both CLP mice and negative control mice. The temperature of CLP mice dropped

down gradually (range: 36 to 30℃) after the surgery while the negative control's temperature (range: 36℃ to 38℃) remained normal (Fig. 2). When the CLP mice's rectal temperature dropped down below 32℃ and remained for 1 hour continuously, this was also a signal that the mice were moribund. From the weight measurement of the CLP mice, we observed that a mouse's weight increases when it becomes moribund because the body could not absorb the injected 0.9% saline fluid and the GI tract stops its movement and developed ileus (enlarged intestinal fluid filled). If the mice were hypothermic (<32℃), plus the other behaviors of a moribund condition (gaining weight, anorexia, groggy or laboring breath), they were euthanized immediately (as mandated by Animal Care). Negative control mice did not show significant change in body weight or other body phenomena. Most of CLP mice reached the potential mortality endpoint before 24 hour after surgery; a few reached the potential mortality endpoint around 30 hour after surgery. Untreated CLP mice were used as positive controls in the early intervention strategies to compare with the mice on various treatments.

3.1.1. Pathological Examination:

The pale liver and abdomen that was filled with fluid could be seen during the autopsy of most CLP mice euthanized after reaching potential mortality endpoint. The pathological examination of the five main organs (liver: 34 mice, heart: 9 mice, lung: 9

mice, kidneys: 10mice, spleen: 6 mice) showed that there was necrotic tissue on the surface of the liver (Fig. 3) and there was edema and blood congestion in the lung alveoli (Fig. 4). Bacteria were identified in the peritoneum (one mice), but no bacteria were found within the gut/intestinal wall. The other organs did not show any obvious abnormalities.

3.1.2. Blood Tests:

Because of the small amount of blood from each mouse, only one blood test could be done on each mouse.

3.1.2.1. ALT (Alanine Aminotransferase) Assay:

The ALT assays were done on the blood sample from the CLP mice euthanized at 18 hours (two mice), 24 hours (four mice) and 30 hours (one mouse) post-surgery. Two negative control mice were euthanized at 24 and 30 hours post-surgery respectively as well for comparison. There is no significant difference (Fig. 5) between CLP mice from 18 hours and 24 hours post-surgery groups (t-test, $P>0.05$).

3.1.2.2. Blood Glucose Assay:

Six CLP mice and two negative control mice had blood glucose measured every 6 hours starting 6 hours post-surgery. All six mice's blood glucose levels dropped down

gradually throughout the first 24 hours after surgery (range: 6 mmol/L to <1 mmol/L), while the two negative control mice had stable glucose levels within normal range (range: 8 to 5 mmol/L) in first 24 hours (Fig. 6).

<u>3.1.2.3.</u> Lactate Assay:

Two mice each from the CLP mice were euthanized at 18 hours, 24 hours and 30 hours post-surgery and had blood samples for lactate levels. There is no significant difference (Fig. 7) between different time points due to the limited numbers of samples.

<u>3.1.2.4.</u> TNF-α & IL-6 Assays:

The blood samples of CLP mice and negative control mice euthanized at 18 hours, 24 hours and 30 hours post-surgery were tested for TNF-α and IL-6 levels in the serum. From 18 hour to 30 hour, the TNF-α levels of CLP mice did not keep going up (Fig. 8) significantly (ANOVA test, $P>0.05$).

IL-6 assay of blood sample from the CLP mice and negative control mice at 18 hours, 24 hours and 30 hours post-surgery showed that the IL-6 levels of CLP mice dropped down significantly from 18 hours to 30 hours post-surgery (ANOVA test, $P<0.05$) while the levels of negative control mice were constant (Fig. 9).

3.2. Treatment Strategies:

3.2.1. Timing of Antibiotic Administration:

In the cefotaxime given at 0 hour post-surgery group: all the 8 CLP mice survived for 72 hours. In the cefotaxime given at 3 hours post-surgery group: 6 out of 8 CLP mice survived for 72 hours. In the cefotaxime given at 6 hours post-surgery group, only 1 of 8 mice survived for 72 hours. In the cefotaxime given at 12 hours post-surgery group: all the 10 mice had reached potential mortality endpoint before 72 hours (Fig. 10). The mortalities of cefotaxime 6 & 12 hours post-surgery groups are significantly different from the other 3 groups (Fisher's exact test, $P<0.05$).

Body temperature differed for CLP mice which received cefotaxime, which did not have treatment (positive control mice), and negative control mice (Fig. 11). The average body temperatures of negative control mice at different time points were stable and normal throughout the first 24 hours post-surgery (range: around 38℃). The average body temperatures of CLP mice with cefotaxime 0 hour post-surgery at different time points were unstable with a decline in the first 6 hours (range: 35 to 38℃), then a return to stable baseline (range: 36 to 38℃). The average body temperatures of CLP mice with cefotaxime 3 hours post-surgery at different time points were unstable throughout the first 24 hours post-surgery (range: 35 to 38℃).

The average body temperatures of CLP mice with cefotaxime starting at 6 hours post-surgery and positive control mice at different time points declined during the first 24 hours (range: < 32 to 38℃) until the potential mortality endpoint temperature was reached (Fig. 11).

The autopsy and pathological examination of the CLP mice with treatments, which reached the potential mortality endpoint before 72 hours post-surgery were the same results as described previously (Page 59, Pathological Examination Section). Some CLP mice that survived had pale livers, but they did not have edema and blood congestion in the lung alveoli.

The WBC count of CLP mice with or without cefotaxime all declined after surgery. The WBC count of CLP mice without treatment was significantly different (t-test, $P<0.05$) from that of the same mice before surgery (Fig. 12).

The TNF-α assay results showed that when cefotaxime was given at 0 hour, 3 hours and 6 hours post-surgery groups, average TNF-α level kept going up from 0 hour to 3 hours and from 3 hours to 6 hours post-surgery (ANOVA test, $P<0.05$) and the average level of CLP mice from cefotaxime 6 hours post-surgery was similar (t-test, $P>0.05$) to the average level of positive control mice (Fig. 13). In cefotaxime 12 hours post-surgery, the result were complicated: 3 CLP mice with treatment reached

endpoint around 18 hours post-surgery and their average level of TNF-α was the highest that we measured (Fig. 13). 2 CLP mice reached endpoint around 24 hours post-surgery and 5 CLP mice reached endpoint around 48 hours post-surgery. Their average TNF-α levels kept dropping down (ANOVA test, $P<0.05$) and the average TNF-α level of CLP mice reaching endpoint around 48 hours post-surgery was even significantly lower (t-test, $P<0.05$) than the negative control mice (Fig.13).

IL-6 assay results showed that the average level of IL-6 from the cefotaxime given at 0 hour post-surgery group was similar with the level of negative control mice (Fig. 14). The average level of cefotaxime 3 hours post-surgery group was a little higher than cefotaxime 0 hour post-surgery group but there was no significant difference (t-test, $P>0.05$). The average levels of cefotaxime given at 6 hour and 12 hour post-surgery were similar and much higher than cefotaxime given at 0 hour and 3 hours post-surgery groups (Fig. 14).

We also did bacterial culture from abdominal swabs (taken at surgery & euthanasia) and blood (taken at euthanasia) for both treated (3 mice), untreated CLP mice (3 mice) and negative control mice (one mouse) during surgery and at the time of euthanasia (table 4). In this sample of mice we found heavy growth of Coagulase-negative *Staphylococcus*, *Enterococcus* and *Proteus mirabilis* (fecal flora), from both the abdominal swab and blood taken at euthanasia in a positive control mouse (table 4).

No bacteria were cultured from either abdominal cavity or blood of several negative control mice. From the mice which were treated with cefotaxime, the results showed that *Enterococcus* (table 4) still existed in the blood because this bacterium is not sensitive to the antibiotic cefotaxime.

3.2.2. Fluid resuscitation:

The mortalities of the treatment groups with 0.9% saline fluid starting at 0 hour, 3 hours, 6 hours and 9 hours post-surgery were the same (Fig. 15). Only 1 out of 8 treated CLP mice reached the potential mortality endpoint before 72 hours. In the group with 0.9% saline fluid given at 12 hours post-surgery, 7 out of 8 mice reached the potential mortality endpoint before 72 hours (Fig. 15). The mortality of group with 0.9% saline fluid given at 12 hours post-surgery was significantly higher than all the other four groups (Fisher's exact test, $P<0.05$). All the mice were on the antibiotic cefotaxime from time zero after surgery. Most of the CLP mice reaching the potential mortality endpoint in this study showed characteristics of it within the first 24 hours post-surgery and earlier than the same characteristics seen in the mice in the antibiotic treatment groups above.

The body temperature changes among the first four groups (0 hour, 3 hours, 6 hours and 9 hours post-surgery) were similar and stable (range: 33 to 38℃) within the first 24

hours post-surgery (Fig. 16). The body temperature change of 0.9% saline fluid starting 12 hours post-surgery had a similar pattern with the other groups in the first 18 hours post-surgery (range: 33 to 38℃), while it began to drop down after 18 hours (from 32 to 34℃) in the late fluid resuscitation group (Fig. 16). The autopsy and pathological examination also showed pale liver along with lung edema and blood congestion from mice reaching the potential mortality endpoint in late fluid resuscitation group (Fig. 3).

The TNF-α assay showed that the average levels between CLP mice with fluid resuscitation starting at 0 hour, 3 hours, 6 hours and 9 hours post-surgery were similar, while the level of CLP mice with fluid starting at 12 hours post-surgery was significantly higher (t-test, $P<0.05$) than all of them respectively (Fig. 17). IL-6 assay showed the similar pattern of results with the TNF-assay (Fig. 18).

3.2.3. Anti-TNF-α Adjunctive Treatment:

3.2.3.1. Anti-TNF-α Treatment for Late Administration (12 hours Post-surgery) of Cefotaxime (Group 1):

Treatment with anti-TNF-α antibody was able to neutralize TNF-α and mitigate the inflammatory response after surgery. Thus it decreased mortality by 37.4% throughout 72 hours of observation compared to the mortality when cefotaxime was given at 12

hours post-surgery, which was 100%. The mortalities between two groups (Fig. 19) are significantly different (Fisher's exact test, $P<0.05$).

Temperature change in this group showed that it gradually dropped down to 34℃ from 0 hour to 6 hours post-surgery. Then it went up and stabilized between 36℃ and 38℃ (Fig. 20).

3.2.3.2. Anti-TNF-α Treatment for Late Fluid (12 hours Post-surgery) Resuscitation (Group 2):

In this group, 7 out of 8 mice reached the potential mortality endpoint before 72 hours of observation. The temperature change in this group showed that it kept going down from 0 hour to 6 hours post-surgery and stayed stable between 32 and 33℃ (Fig. 20). The average temperature change between 0 and 24 hours post-surgery was significantly different from group 1 (t-test, $P<0.05$).

The TNF-α assay showed that the CLP mice which reached the potential mortality endpoint before 72 hours of observation had higher TNF-α levels than the mice which survived throughout 72 hours in both group 1 and group 2 (Fig. 21). IL-6 assay showed similar results with TNF-α assay but the average IL-6 level of CLP mice reaching the potential mortality endpoint before 72 hours was nearly one hundred times higher than the mice which survived (Fig. 22). There were no differences (Fig.

21& 22) in TNF-α and IL-6 levels between CLP mice which survived and negative control mice in both groups (t-test, P>0.05). There was a significant difference of IL-6 levels (Fig. 22) in moribund mice between group 1 and group 2 (t-test, P<0.05), while there was no significant difference of TNF-α levels (Fig. 21) between the two groups (t-test, P>0.05).

3.3. Cytokines (TNF-α and IL-6) Kinetics during Late Intervention Strategies:

Since the TNF-α results were not always correlated with mortality, we studied the kinetics of TNF-α within 24 hours post-surgery by applying the cefotaxime 12 hours post-surgery group. TNF-α was measured at time intervals from immediately after surgery to time of euthanasia. The average TNF-α levels of CLP mice at 0 hour, 6 hours and 12 hours post-surgery were low and similar (Fig. 23). The average level of CLP mice at 18 hours post-surgery peaked and then subsequently dropped down after that (Fig. 23). The TNF-α level went down to the similar level with 0 hour post-surgery after 30 hours of observation (Fig. 23). In the 0.9% saline fluid 12 hours post-surgery group, the average TNF-α levels of CLP mice kept increasing as time progressed (Fig. 24). No declining level was observed. All the CLP mice were euthanized before 24 hours post-surgery (Fig. 24).

The IL-6 levels in the cefotaxime 12 hours post-surgery group kept increasing throughout the 48 hours of observation. Although it dropped down after 30 hours of observation, it was still hundreds of times higher than the level of CLP mice that were euthanized at 0 hour post-surgery (Fig. 25). The result of 0.9% saline fluid 12 hours post-surgery group was similar. Although IL-6 levels dropped down after 12 hours of observation, the levels were still higher than the levels of negative control mice and CLP mice that were euthanized at 0 hour post-surgery (Fig. 26).

4. Discussion

4.1. Model Development & Parameter Determination:

Since we wanted to evaluate the availability of the models and establish the most appropriate one for our project, different surgical procedures had been administered on mice. Both one puncture and two punctures on the 1cm ligated ceca had been tried and mice with two punctures had obviously higher mortality rate (100%) within the first 30 hours post-surgery than mice with one puncture (nearly 20%). In the mean time, mice with two punctures were able to show obvious signs of sepsis, such as drowsiness (unresponsive to touching), groggy (unable to walk in a straight line), anorexia, labored breathing, dehydration, GI tract shutdown and hypothermia (<32℃). Along with the number of punctures, the length of ligation was also studied. Ligations longer than 1cm caused the death of mice even before the signs of potential mortality endpoint showed. Therefore, after the preliminary experiment, we concluded that 1cm cecal ligation from the end of the cecum and a through and through puncture with two holes is an optimal and reproducible sepsis model for our further research.

Autopsy results showed a pale liver in most CLP mice, which might indicate hepatic dysfunction as an early signal in this sepsis model. The tissue examinations indicated the presence of necrotic hepatocytes in the tissue, which is consistent with the white

color on the surface of the liver and corroborates the theory of the liver suffering early damage in sepsis (Fig. 3). The lung tissue showed congestion with red blood cells and edema in the alveoli throughout the lungs, which might suggest that the immediate cause of death of CLP mice may be from pulmonary congestion (Fig. 4). The gut was filled with fluid when the abdomen was opened after the euthanasia, but the pathological examination did not find any inflammatory focus within the colon tissue. All the inflammatory response in the abdomen was found outside the gut within the peritoneum and related to the fecal contamination. The fluid accumulation in the intestine is likely due to decreased GI tract motility casued by intraperitoneal sepsis.

Compared to the liver and the lung, we did not find any substantial changes in the characteristics of other organs. This suggests that the current model causes an acute septic picture. The infectious process causes death before the onset of multi-organ dysfunction.

The results of bacterial blood cultures indicate that the CLP mice developed bacteremia with similar bacterial isolates found in the gut (table 4). The bacteremia is likely caused by the fecal contamination of the abdominal cavity and the presence of the blood cultures (table 4) are influenced by antibiotic treatment.

The WBC count indicates that there is a decrease of WBC after the surgery in both CLP mice and negative control mice (Fig. 12). CLP mice have their WBC counts drop significantly (t-test, $P<0.05$) compared to the count of same mice 24 hours pre-surgery (Fig. 12). Although we did not measure leukocyte leak infiltration these findings may support previous research showing that there is a correlation between hepatic leukocyte infiltration and hepatic dysfunction (Molnar et al. 1997). However WBC recruitment to the site of infection (peritoneum) is the most likely explanation for the decreased WBC count.

Because the CLP mice were anorexic, we tested the blood glucose level every 6 hours after surgery and found the CLP mice's blood glucose levels dropped down throughout the observation whereas the negative control kept comparatively constant and above normal level (Fig. 6). We suspect that because of the necrotic hepatic tissue changes found, the gluconeogenetic pathway in liver may be altered or inhibited and thus led to hypoglycemia in the anorexic CLP mice. In some early experiments, a number of mice were euthanized just around the time of reaching the first temperature endpoint (<32℃) before other moribund characteristics were seen. We suspected that hypoglycemia might cause mice hypothermia before other signs of sepsis and made us modify the model as described in previous section (Page 67) to remove this phenomenon. The result of the ALT assay showing elevation may also corroborate the

hypothesis that the CLP mice suffered hepatic dysfunction at an early stage of infection in this model (Fig. 5).

The results of the lactate assay showed an increasing lactate level in CLP mice during sepsis, which indicate they might develop metabolic acidosis (Fig. 7). This probably explained some of the labored breathing along with the lung congestion described. As lactate was elevated in CLP mice without treatment, this supports the theory of microvascular circulatory compromises early in sepsis (Fowler et al. 2002).

From the TNF-α assay (Fig. 8), we tested the blood sample from 18, 24 and 30 hours post-surgery and the results indicated an increasing trend of TNF-α level compared to the level in negative control mice. However these results were not statistically significant. Since the mice were euthanized when they showed potential mortality endpoint, thus TNF-α may stop its releasing at late stage of sepsis and may not be a good marker of ongoing inflammation.

IL-6 assay showed a different phenomenon (Fig. 9). Although the IL-6 level dropped down from 18 hours post-surgery dramatically, the lowest average level was still almost five times higher than the negative control mice at the same time point. This indicates ongoing inflammation and supports the hypothesis that IL-6 may be a better

predictive marker of severe sepsis compared to TNF-α, especially later in the sepsis presentation.

The CLP mice have similar vital signs with human clinical sepsis, such as hypothermia and dehydration. The bacterial culture, blood count and blood biochemical tests indicate that CLP mice might develop bacteremia, metabolic acidosis, hypoglycemia and also hepatic dysfunction, which are consistent with patients suffering from severe sepsis. The moribund mice all showed similar signs seen in human severe sepsis. The immunologic tests further support the evidence of an ongoing inflammatory response in CLP mice. Therefore, our mouse model provided consistent results in sepsis and enabled us to control treatment factors, such as the stage of sepsis and the timing of fluid resuscitation and antibiotics, which are not controllable in human sepsis. Autopsy of model also suggests that hepatic dysfunction is an early marker of sepsis (Gorrasi et al. 2006).

4.2. Treatment Strategies:

4.2.1. Timing of antibiotic Administration:

The mortality (Fig. 10) between four different groups indicated early administration of antibiotics, in this case cefotaxime is very important for the survival rate. The earlier

we started cefotaxime, the more CLP mice survived. This also correlated with the decreased clinical and biochemical results.

Temperature changes (Fig. 11) showed the effect of timing of antibiotics on CLP mice. The average temperature change of CLP mice with cefotaxime started at 0 hour post-surgery was similar with the change of negative control mice. Both groups had stable temperatures during the first 24 hours post-surgery (Fig. 11). In contrast, the average temperature change of CLP mice with cefotaxime started at 3 hours post-surgery is variable but the lowest temperature was never below 34℃ (Fig. 11) and is consistent with the low mortality in this group. When the antibiotic was started at 6 hours post-surgery, the average temperature change of CLP mice was similar to the positive control mice. Both groups had a temperature decline gradually below 32℃ within first 24 hours post-surgery and these were consistent with the high mortality in both groups (Fig. 10 & 11). Since hypothermia is a sign of sepsis severity, the maintenance of stable temperature is very important as this may indicate that the infectious condition and inflammation is diminished. In our model, because hypothermia was an endpoint for potential mortality maintaining temperature was crucial for survival. Therefore, earlier administration of cefotaxime in the experimental model led to a decreased mortality by reducing inflammatory response and hence the temperature remained relatively stable.

The TNF-α assay indicated early administration of cefotaxime inhibits the inflammatory response to sepsis significantly compared to delayed administration (Fig. 13). As cefotaxime was injected directly into the infection focus by IP injection and eliminated the pathogen (such as *Proteus mirabilis* and Coagulase-negative *Staphylococcus*), cefotaxime given at 0 hour post-surgery inhibited pathogen growth before it was able to spread into the blood stream. Hence all the mice were able to survive throughout 72 hours of observation and their average TNF-α and IL-6 levels stayed at a normal range (Fig. 13 & 14) because the inflammatory response had never started. 3 hours post-surgery administration of cefotaxime inhibited some pathogens growth before the inflammatory response was fully activated. This group might have less bacteremia due to less pathogen spread into blood stream. The signs of CLP mice in this group were intermediate and the average TNF-α and IL-6 levels (Fig. 13 & 14) were a little higher than early treatment or negative control groups but there was no significant difference (t-test, $P>0.05$) in results. Administration of cefotaxime 6 hours post-surgery appears less able to prevent pathogens spread into the bloodstream because the infection is more established when the cefotaxime was administrated into abdominal cavity. Inflammatory response had been fully activated during this delaying cefotaxime treatment and organ damage in CLP mice became manifest. This is likely the main reason why CLP mice with cefotaxime 6 hours post-surgery had a higher mortality (nearly 100%) than the two other groups above (Fig. 10).

The original intention of giving CLP mice cefotaxime 12 hours post-surgery was to further confirm the hypothesis that delayed administration of cefotaxime would cause more severe inflammatory response in the mice. However, a very interesting phenomenon emerged in this group. The average IL-6 level in this group was somewhat higher than CLP mice with cefotaxime started at 6 hours post-surgery (Fig. 14) as we thought, but average TNF-α level was a little confusing. There were differences in TNF-α levels in this group depending upon the time they became moribund. Mice which survived longer had a lower TNF-α level (Fig. 13). Did the mice survive due to low TNF-α levels or did the TNF-α levels start to fall in CLP mice despite ongoing inflammation? Data from previous research suggests that TNF-α is an acute phase reactant that falls with ongoing inflammation in sepsis (Cannon et al. 1990; Hesse et al. 1988; Suffredini et al. 1989); therefore the 3 mice that became moribund around 18 hours post-surgery showed a significantly high TNF-α level (Fig. 13) because they might have a higher initial infection compared to the other mice with the same treatment and high TNF-α level seemed to correlate with early mortality although in small numbers. This result also suggests that TNF-α levels cannot predict the mortality rate if measured later in the inflammation response. On the other hand, high IL-6 levels in CLP mice were consistent with the mortality (Fig. 14). IL-6 is a predictor of progress of inflammatory response and death in this model even though the levels are below the peak IL-6 concentration.

The autopsies indicated that earlier administration of cefotaxime not only prevented hypothermia but also prevented the damage of key organs in the mice. The likely reason is that early antibiotic administration eliminated the bacterial pathogens before their invasion into blood along with the decrease in inflammation. This is also consistent with the decrease in hepatic dysfunction and other pathologic changes seen with early antibiotic administration.

The blood cultures from treated CLP mice found *Enterococcus* in the bloodstream (table 4). Interestingly cefotaxime is not effectively against *Enterococcus*. However, this fact might not affect the impact of early administration of cefotaxime on decreasing the mortality of this model. This might be due to the local infection is too small to cause severe infection or the fact that *Enterococcus* is not considered as a significant pathogen in primary peritonitis in humans and does not require antibiotic coverage in intra-abdominal sepsis (Edgeworth et al. 1999).

4.2.2. Fluid Resuscitation:

All the mice had 1mL sterile water injected into the abdominal cavity before closing the incision in order to spread the infection. This may affect the initial time when mice reach potential mortality endpoint in fluid studies (to 9 hours post-surgery) because it

could be considered as part of fluid resuscitation, but it did not affect the observation of the importance of fluid resuscitation in sepsis.

Because all the mice received antibiotics at 0 hour post-surgery, infection was not the primary factor in ongoing sepsis inflammation as demonstrated above. The mortalities (Fig. 15) among the five groups indicated that starting fluid resuscitation within first 12 hours post-surgery or sepsis is crucial for the overall effect of treatment in the CLP model. The temperature changes (Fig.16) also confirmed this because it shows that early fluid resuscitation was necessary to prevent hypothermia in CLP mice.

TNF-α and IL-6 assays (Fig. 17 & 18) shows that delayed fluid resuscitation alone can induce an inflammatory response during sepsis. Average levels of TNF-α and IL-6 in the first four groups (Fig. 17 & 18) indicated there was no acute inflammatory response with fluid provided within 9 hours post-surgery (early fluid resuscitation), while the fifth group (Fig. 17 & 18) had an acute inflammatory response linked to high mortality (Fig. 15) related to the 12-hour delayed fluid resuscitation. The results also indicated that a 9-hour delay in fluid resuscitation in this model does not induce an inflammatory response caused by surgery in CLP mice if it is combined with early administration of cefotaxime (started at 0 hour post-surgery). Most CLP mice with delayed fluid resuscitation reached the potential mortality endpoint hours earlier than CLP mice with delayed administration of cefotaxime. These findings suggest that fluid

resuscitation may be more important on early survival and certainly can aggravate the inflammatory response of an untreated infection.

Autopsy of CLP mice reaching the potential mortality endpoint in this group did not show damaged hepatic tissue. But they all showed blood congestion and edema in lung tissue that may have been the cause of mortality. The reason of the pulmonary damage is probably linked to ARDS caused by sepsis (Costa et al. 2006).

Above all, early fluid resuscitation is able to inhibit the inflammatory response and maintain the circulatory system. When it is combined with early administration of cefotaxime, CLP mice survived throughout 72 hours' study period. Despite early administration of cefotaxime (0 hour post-surgery), the inflammatory response could continue due to the administration of late fluid resuscitation. Therefore early fluid resuscitation plays a key role in the treatment of intraperitoneal infection-caused sepsis.

4.2.3. Anti-TNF-α Adjunctive Treatment:

Anti-TNF-α adjunctive treatment given at 6 hours post-surgery could decrease the mortality (Fig. 19) in the group with cefotaxime started at 12 hours post-surgery (cefotaxime 12 hours post-surgery, Fisher's exact test, $P<0.05$) but it was not effective (Fig. 19) when fluid resuscitation was started at 12 hours after surgery (fluid 12 hours

post-surgery). The reason for this is not clear. Anti-TNF-α is able to decrease the inflammatory response by neutralizing TNF-α when caused by infection but it was ineffective when dehydration caused by delayed fluid resuscitation was the cause of the inflammatory response. This suggests that the mechanisms of inflammation caused by delayed antibiotics administration are different from those caused by delayed fluid resuscitation. It could also explain the negative results of clinical studies of anti-TNF-α in sepsis especially if fluid resuscitation timing and amount was not meticulously controlled in the clinical trial design.

The body temperatures of moribund mice in this treatment are different from those of the previous experiment; they did not show clear hypothermia (Fig. 20). The reason is probably the neutralization of TNF-α in the mice.

There was no significant difference in average TNF-α levels at sacrifice between dying and living CLP mice with anti-TNF-α treatment (Fig. 21), while there was still a significant difference in average IL-6 levels (Fig. 22). TNF-α is an early signal during sepsis; therefore the neutralization of it will block the process of inflammatory response at early stage. When it is combined with fluid resuscitation and administration of cefotaxime, which could eliminate the pathogens and maintain the circulatory system, the treatment can reduce hypothermia and improve survival. This

also suggests that TNF-α is not a predictor of mortality in our model especially after 18 to 24 hours post-surgery.

TNF-α assays from CLP mice when cefotaxime was given 12 hours post-surgery (Fig. 23) indicated that the mice reaching endpoint at 18 hours post-surgery had the highest TNF-α level because they might have a significant high initial infection after surgery compared to the mice showing moribund after 18 hours. It also indicated that TNF-α levels at late stages of sepsis (30-48 hours post-surgery) are similar with that of negative control mice (Fig. 23). The reason is likely because TNF-α had been used up by the immune system during the process of inflammatory response. Although TNF-α level became normal, a huge amount of IL-6 had been released due to the ongoing inflammatory response (Fig. 25). IL-6 can continue stimulating the inflammatory response and may either cause the late stage morbidity and mortality or is at least a predictor of death.

We could not reproduce similar results in TNF-α assay from CLP mice with delayed fluid resuscitation started at 12 hours post-surgery because mice in this group cannot survive for more than 24 hours. We could only show that both TNF-α and IL-6 were at higher levels than those of negative control mice as inflammatory response continued (Fig. 24 & 26). We did show that delayed fluid resuscitation had increased levels of TNF-α and IL-6 during the process of sepsis in CLP mice's body despite effective

early antibiotics treatment. Clearly the mechanism of inflammatory response from delayed fluid resuscitation is different from delayed antibiotics treatment.

According to our results from the TNF-α and IL-6 assays, IL-6 may play a role, which is more important than TNF-α in the mortality of this sepsis model. IL-6 is one of the cytokines that may perpetuate the inflammatory response and aggravate mortality even when TNF-α levels fall.

Now that we have a reliable animal model for sepsis, we will apply the model to study the impact of different fluid resuscitation strategies on mortality and inflammation. We can study other means of modulation of inflammation; observe the impact on mortality and on end organ damages in the liver and lungs.

4.3. Limitation of Mouse Model:

Animal Care Committee limited the numbers of mice for the project to 300 every year, thus the numbers of mice we used were limited. Since death of mouse is unacceptable in animal model, we applied potential mortality endpoint as a surrogate for the euthanasia of mice. Mouse model was unable to totally mimic clinical signs of sepsis in human because there is difference between each other in the immune system. We can only take one mL blood from each mouse, thus the numbers of assays we had done for each mouse was limited.

Table 1 Model Development & Parameter Determination:

Number of Mice	Length of Ligation			Number of Puncture& Size of Needle		
	1/3 Cecum	1/2 Cecum	2/3 Cecum	18G×1	18G×2	16G×2
34	√	X	X	√	X	X
29	√	X	X	X	√	X
6	X	√	X	X	√	X
6	X	X	√	X	√	X
6	√	X	X	X	X	√

"√": Yes. "X": No.

Table 2 ALT Assay Procedures:

Substance	Volume (mL)					
	0	1	2	3	4	5
pyruvic acid	0	0.05	0.10	0.15	0.2	0.25
ALT substrate	0.5	0.45	0.40	0.35	0.30	0.25
0.1mol/L phosphate	0.10	0.10	0.10	0.10	0.10	0.10
Mix, 37℃, 30min						
2,4-M nitrobenzene	0.50	0.50	0.50	0.50	0.50	0.50
Mix, 37℃, 20min						
0.4mol/L sodium hydroxide	5.00	5.00	5.00	5.00	5.00	5.00

10min room temperature, 500nm wave length

Substance	Volume (mL)	
	Control Tube	Test Tube
Serum	0.1	0.1
ALT substrate	-	0.5
Mix, 37℃, 30min		
2,4-M nitrobenzene	0.5	0.5
Mix, 37℃, 20min		
ALT substrate	0.5	-
0.4mol/Lsodium hydroxide	5.0	5.0

10min room temperature, 500nm wave length

Table 3 Treatment Strategies:

Number of Mice	Cefotaxime (0.05mL) Start Time Points (Post-surgery)				0.9% Saline Fluid (1mL) Start Time Points (Post-surgery)					Anti-TNF-α* (0.05mL)
	0h	3h	6h	12h	0h	3h	6h	9h	12h	6h Post-surgery
Timing of Antibiotic										
8 mice	√				√					
8 mice		√			√					
8 mice			√		√					
10 mice				√	√					
Fluid Resuscitation										
8 mice	√				√					
8 mice	√					√				
8 mice	√						√			
8 mice	√							√		
8 mice	√								√	
Anti-TNF-α Adjunctive Treatment										
22 mice				√	√					√
8 mice	√								√	√
Cytokine Kinetics										
35 mice				√	√					
24 mice	√								√	

*Anti-TNF-α was only administrated at 6 hours post-surgery, while the other two treatments were administrated every 6 hours for up to 72 hours since they started.

"√": the starting time of treatment.

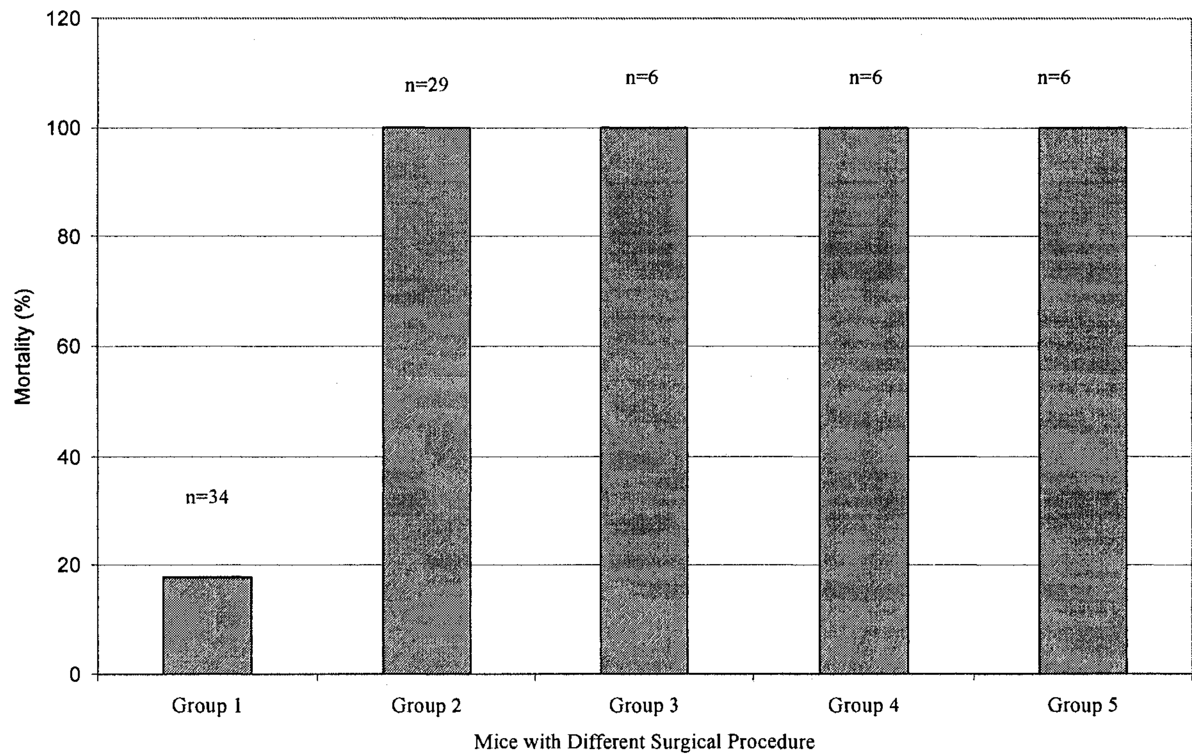
Table 4 Bacterial Cultures of CLP Mice with & without Cefotaxime and Negative Control Mice

(1) Swab	No.129 (endpoint within 24h)	No.131(survive for 72h)
Surgery	No Enterobacteriaceae isolated	No Enterobacteriaceae isolated
Euthanasia	Heavy growth of Coagulase-negative *Staphylococcus* Heavy growth of *Proteus mirabilis* Heavy growth of *Enterococcus gallinarum*	Moderate growth of *Proteus mirabilis* Moderate growth of Coagulase-negative *Staphylococcus* Moderate growth of *Enterococcus gallinarum*

(2) Blood	No.168 (Positive Control)	No.171 (Cefotaxime)	No.184 (Negative Control)
Euthanasia	Heavy growth of Coagulase-negative *Staphylococcus* Heavy growth of *Enterococcus* Heavy growth of *Proteus mirabilis*	Moderate growth of *Enterococcus*	None

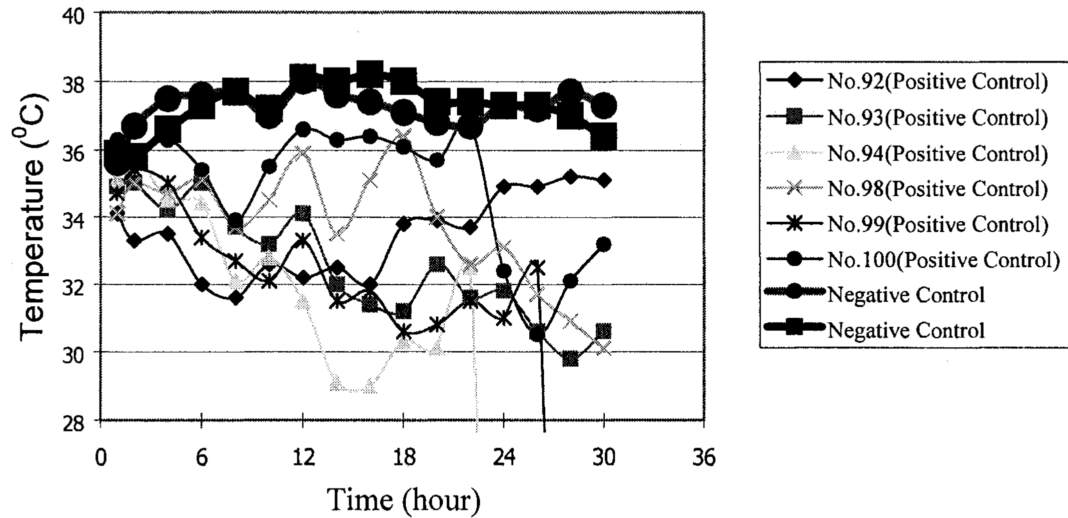
Blood sample was taken by cardiac puncture

Fig. 1 Mortalities of CLP Mice with Different Surgical Procedures within First 30 Hours Post-surgery



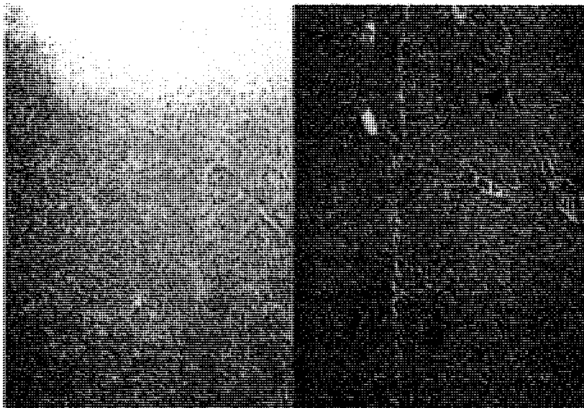
Group 1: CLP mice with 1/3 ceca ligated and one puncture with 18G needle
Group 2: CLP mice with 1/3 ceca ligated and two punctures with 18G needle
Group 3 CLP mice with 2/3 ceca ligated and two punctures with 18G needle
Group 4 CLP mice with whole ceca ligated and two punctures with 18G needle
Group 5 CLP mice with 1/3 ceca ligated and two punctures with16G needle
All the mice in this experiment were given 1mL 0.9% saline fluid every 6 hours for up to 30 hours, started from 0 hour post-surgery.
The mortality of group 1 is significantly lower than all the other 4 groups. (Fisher's exact test, $P<0.05$).

Fig. 2 Body Temperatures of Positive Control Mice & Negative Control Mice



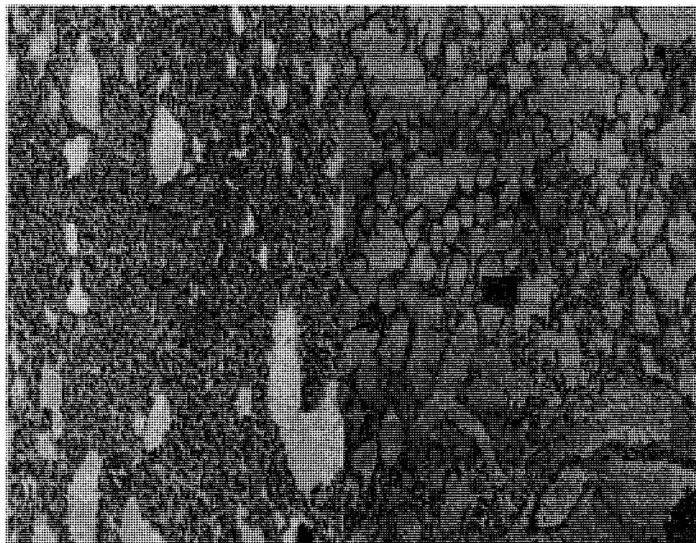
Body Temperature (CLP mice vs. Negative Control). The body temperatures of CLP mice dropped down gradually after surgery, CLP mice No. 94 & 99 dropped down below 32℃ for 1 hour and reached the endpoint. The body temperatures of two negative controls kept constant throughout the observation after surgery.

Fig. 3 Images of Hepatic Tissue



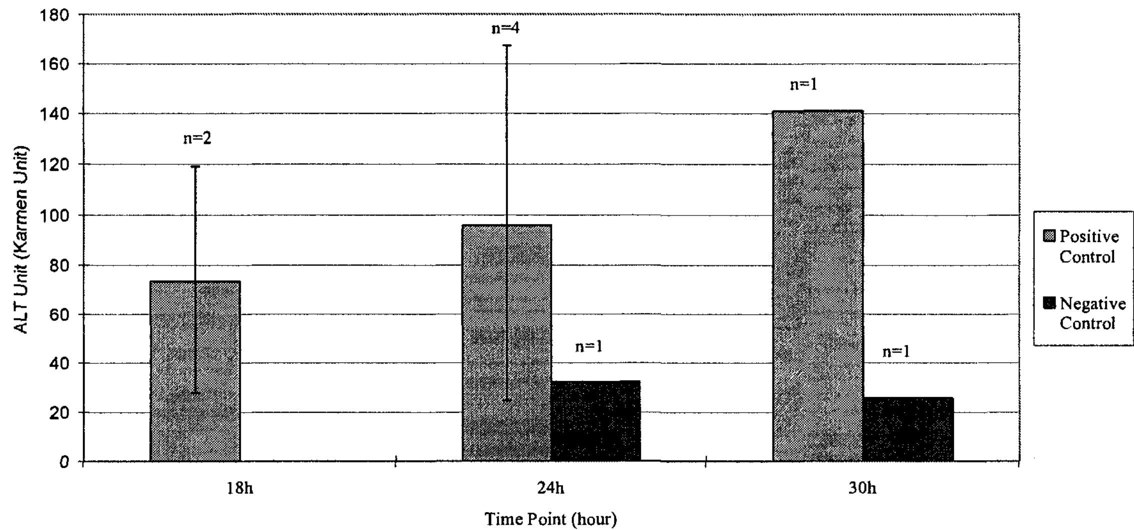
Left image is the necrotic tissue of the liver from a CLP mouse. The left part of the image is necrotic hepatic tissue and the right is the normal hepatic tissue. Right image is the hepatic tissue from a negative control mouse.

Fig. 4 Images of Pulmonary Tissue



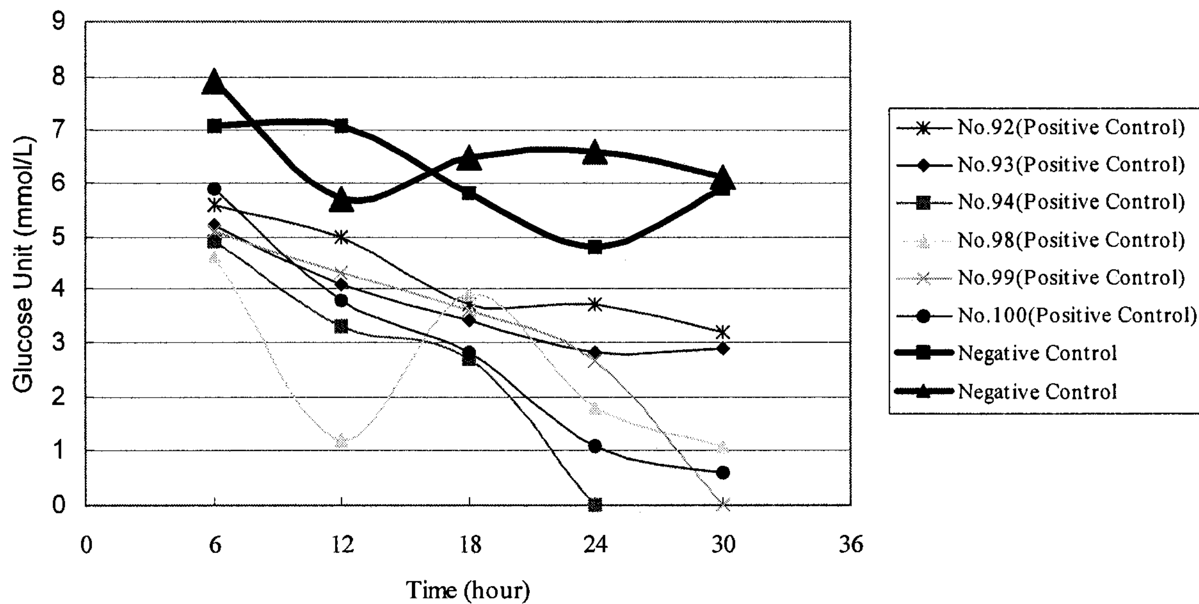
Tissue is from a treated CLP mice with fluid resuscitation delayed for 12 hours post-surgery
Left image is the lung tissue with the blood cell congestion (inside the red circle)
Right image shows the edema in alveolus (Red Arrow)
Both right and left images are from the same CLP mice.

Fig. 5 ALT Assay of Positive Control Mice & Negative Control Mice at Different Time Points Post-surgery



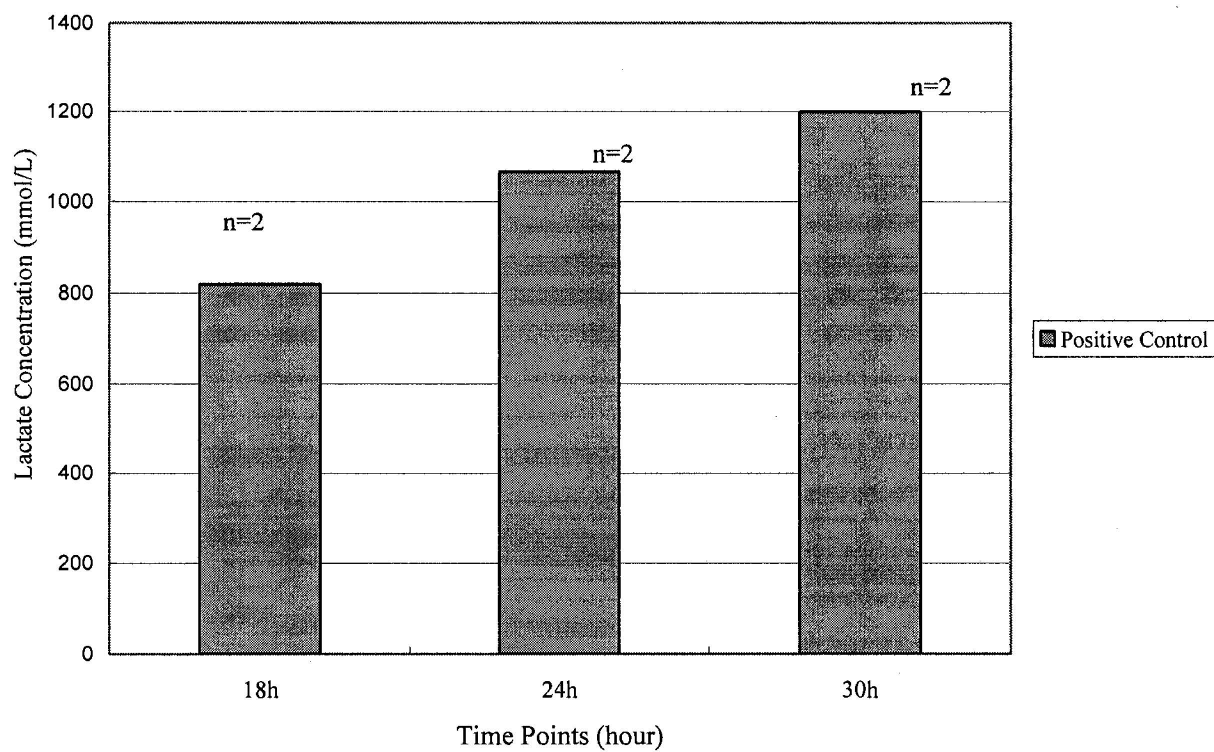
There is no significant difference between 18 hours and 24 hours post-surgery groups (t-test, P>0.05). ALT is an important measure for the test of hepatic function; its increasing level indicates hepatic dysfunction (Error bar is standard deviation).

Fig. 6 Blood Glucose Assay of Positive Control Mice & Negative Control Mice



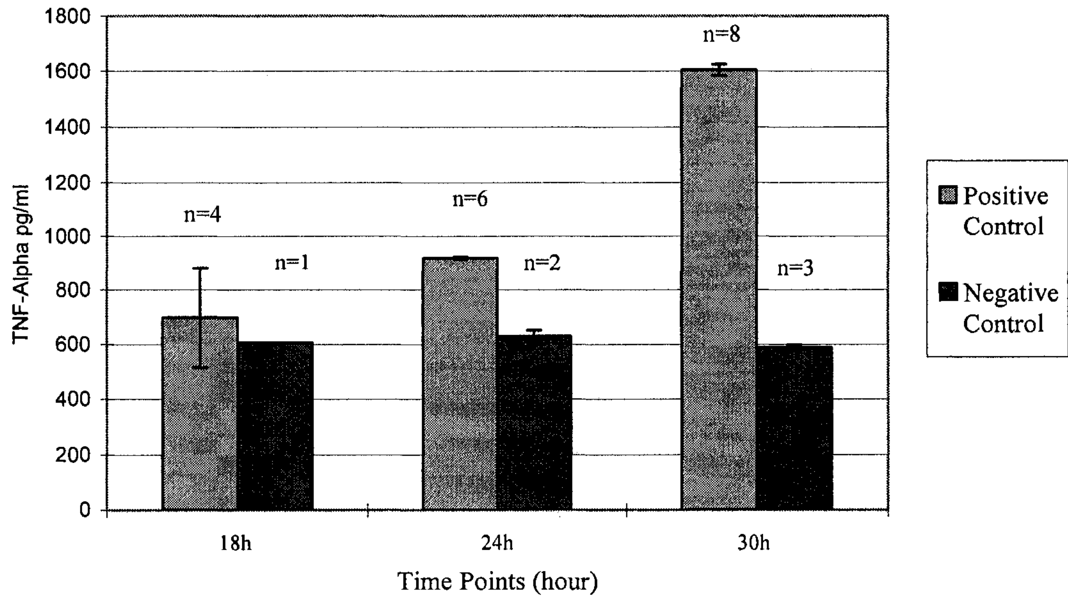
The blood glucose levels of all the CLP mice dropped down gradually from normal to extremely low level, while the two negative control mice had normal levels throughout the observation after surgery.

Fig. 7 Lactate Assay of Positive Control Mice at Different Time Points Post-surgery



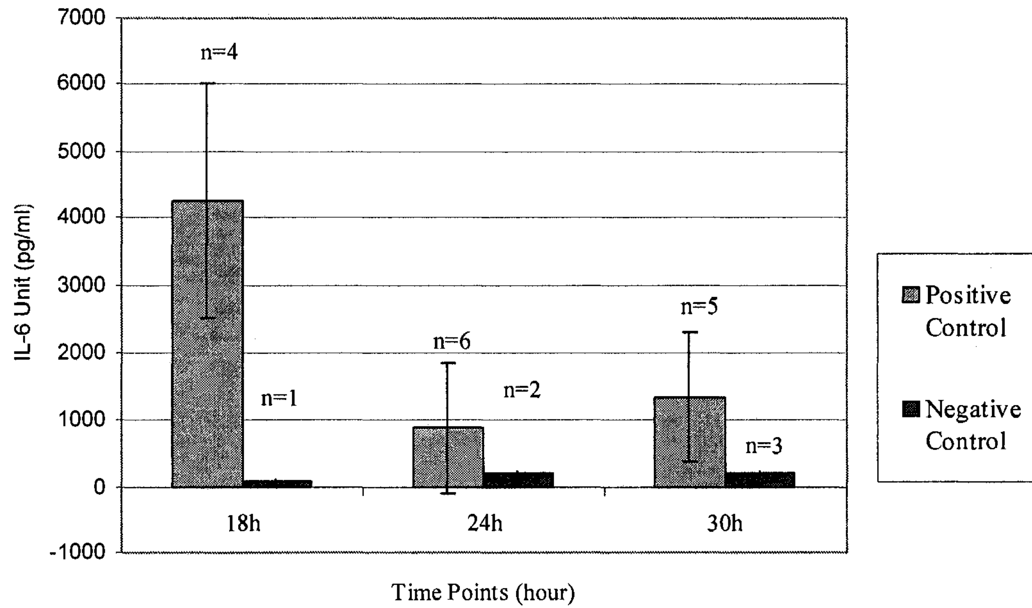
The lactate assay of blood samples from CLP mice shows no significant difference between different time points due to the limited numbers of samples.

Fig. 8 TNF-α Assay of Positive & Negative Control Mice From Different Time Points



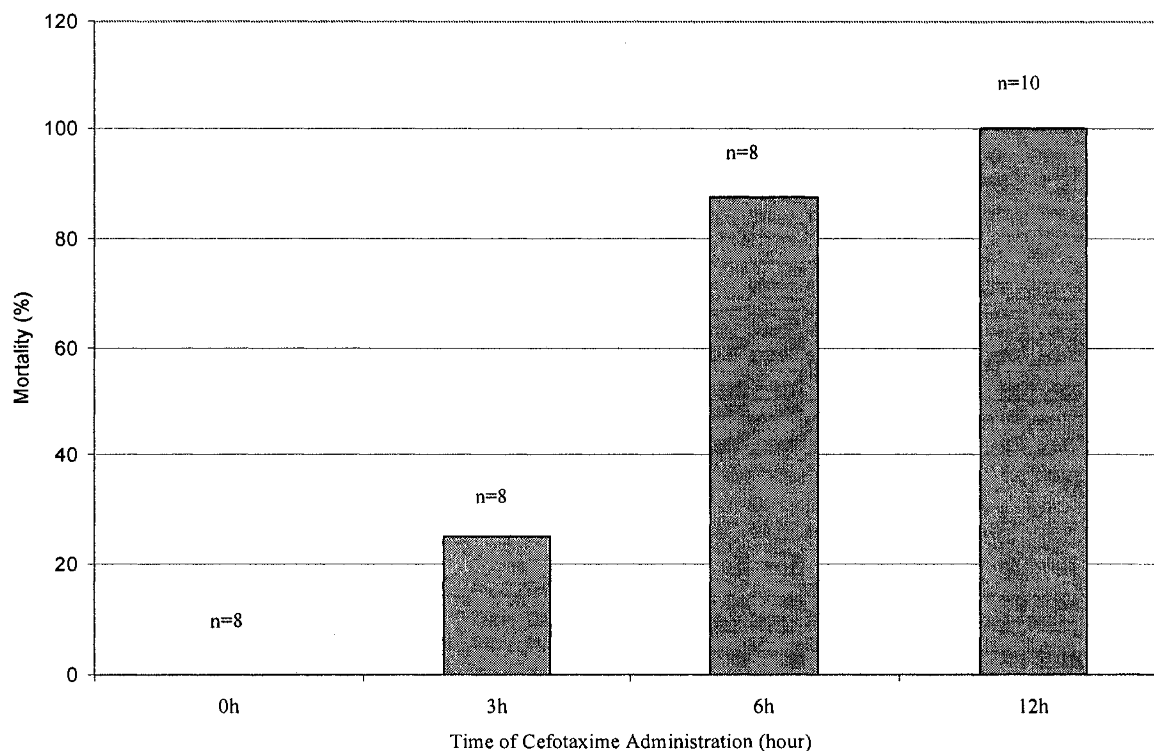
There is no significant change of TNF-α level in positive control groups at different time points (ANOVA test, $P>0.05$). There is also no significant change in negative control groups at different time points due to the limited numbers of samples (Error bar is standard deviation).

Fig. 9 IL-6 Assay of Positive & Negative Mice at Different Time points



IL-6 levels of serum from CLP mice (blue) drop down after 18 hours post-surgery (ANOVA test, $P<0.05$) while levels of negative control mice (red) are low and remain stable (Error bar is standard deviation).

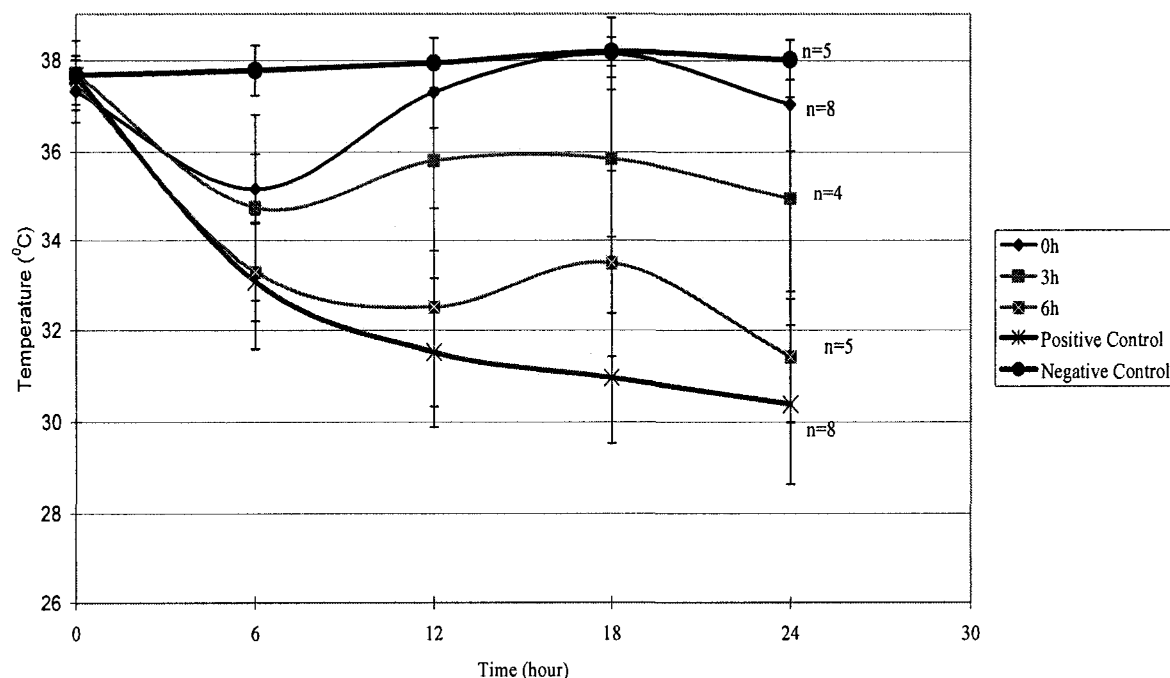
Fig. 10 Mortalities of CLP Mice with Cefotaxime at Different Starting Time Points



As the figure shows, the mortality keeps increasing as the time of cefotaxime administration is delayed. The mortality of group with cefotaxime started at 0 hour post-surgery is significantly different from the mortalities of groups with cefotaxime started at 6 & 12 hours post-surgery (Fisher's exact test, $P<0.05$). The mortality of group with cefotaxime started at 3 hours post-surgery is significantly different from the mortalities of groups with cefotaxime started at 6 & 12 hours post-surgery (Fisher's exact test, $P<0.05$). There are no differences between the two groups with cefotaxime started at 0 hour and 3 hours post-surgery or the two groups with cefotaxime started at 6 & 12 hours post-surgery.

All the mice in this experiment were given 1mL 0.9% saline fluid every 6 hours for up to 72 hours, which started at 0 hour post-surgery. All the mortalities were measured within 72 hours of observation.

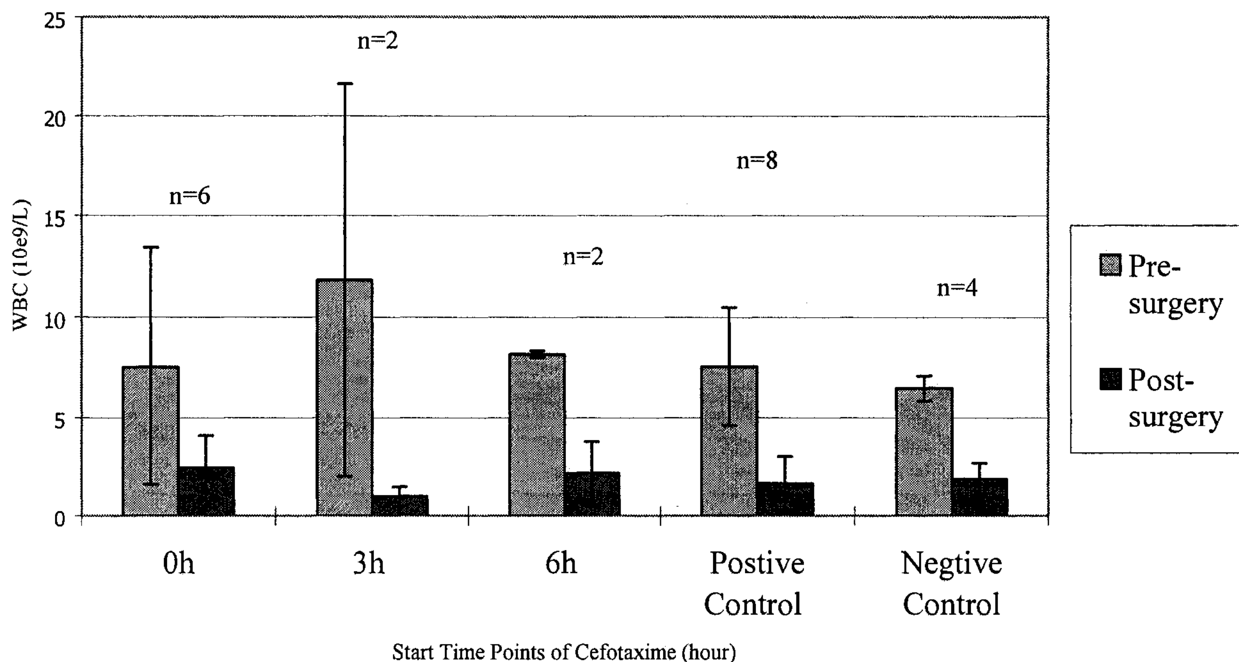
Fig. 11 Body Temperatures of CLP Mice with Cefotaxime at Different Time Points (including Positive & Negative Control)



Body temperature of the negative control group (black) keeps stable above baseline (36 to 38℃). Body temperature of cefotaxime 0 hour post-surgery group (violet) drops down at first 6 hours but goes up and keeps stable (36 to 38℃) at the next 18 hours. Body temperature of cefotaxime 3 hours post-surgery group (pink) is unstable between 34 and 36℃ throughout the first 24 hours post-surgery. Body temperatures of cefotaxime 6 hours post-surgery and positive control (blue and brown) groups keep going down (<32℃). Error bar is standard deviation.

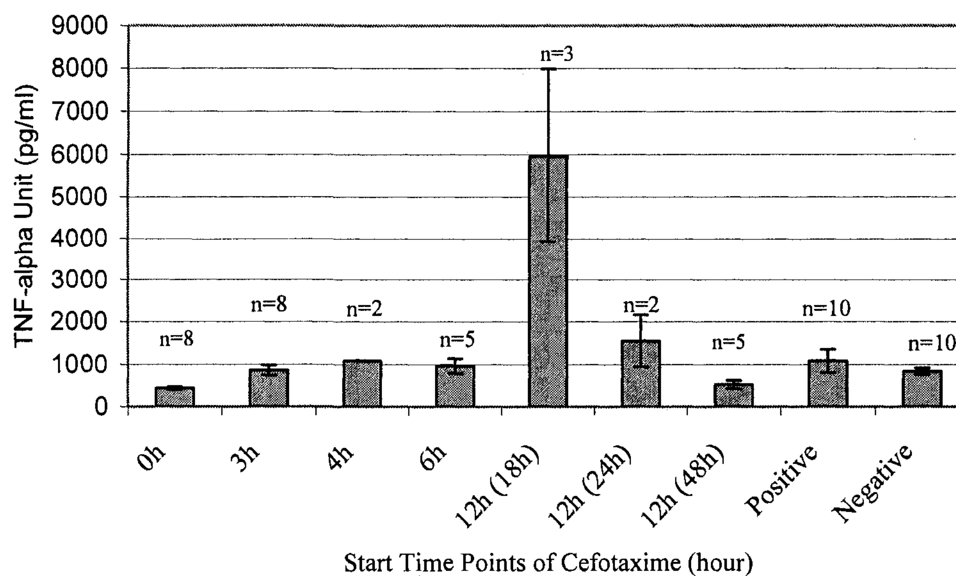
There is a significant difference (ANOVA test, $P<0.05$) of average body temperature changes between 0 and 24 hours post-surgery between five groups.

Fig. 12 WBC Count (Pre-surgery vs. Post-surgery) of CLP Mice with Cefotaxime at Different Time Points (Including Positive & Negative Control)



Blue bars represent WBC counts of the blood taken from mice 24 hours pre-surgery. Red bars represent WBC counts of the blood taken from the same mice when they were euthanized. Thus we can compare the difference of WBC counts between before and after surgery from the same mice. All the mice's WBC counts declined after surgery; the WBC count of positive control post-surgery is significantly different from that of pre-surgery (t-test, $P<0.05$). Error bar is standard deviation.

Fig. 13 TNF-α Assay of CLP Mice with Cefotaxime at Different Starting Time Points at time of Euthanasia



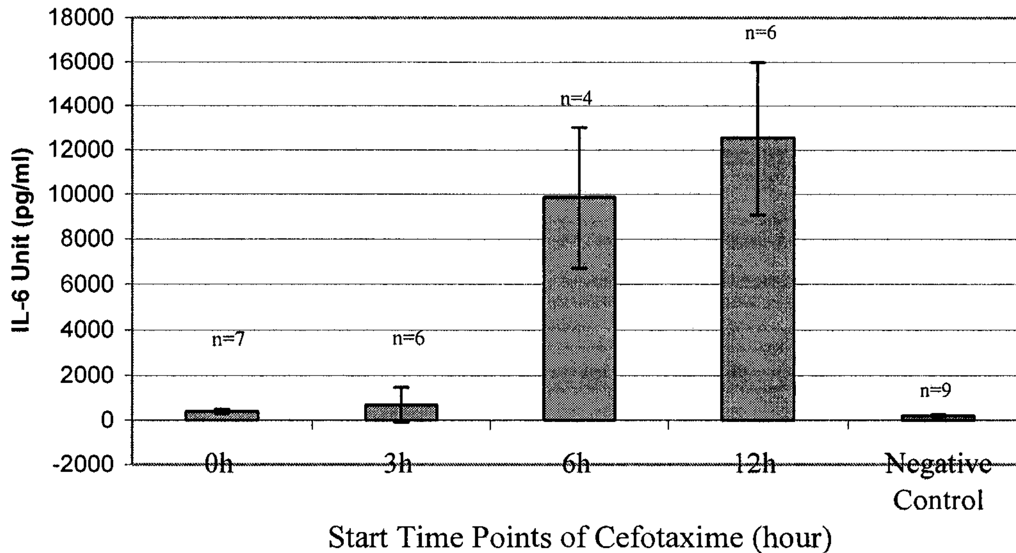
The average level of TNF-α keeps going up from cefotaxime 0 to 3 hours post-surgery and from 3 to 6 hours post-surgery. In cefotaxime 12 hours post-surgery group, the CLP mice reaching endpoint around 18 hours post-surgery have a very high average level of TNF-α, the average TNF-α levels of CLP mice reaching endpoint around 24 and around 48 hours post-surgery keep dropping down and average TNF-α level of CLP mice around 48 hours post-surgery is significantly lower (t-test, $P<0.05$) than the level of negative control mice. The average level of positive control mice is not significantly different (t-test, $P>0.05$) from cefotaxime 6 hours post-surgery group (Error bar is standard deviation).

There is a significant increasing of TNF-α level as the start time points of cefotaxime delayed (ANOVA test, $P<0.05$).

Within cefotaxime 12 hours post-surgery group, the CLP mice showed a significant change (ANOVA test, $P<0.05$) of TNF- α level at different time points of euthanasia.

All the CLP mice in this treatment strategy were kept for 72 hours. The mice reaching the potential mortality endpoint were euthanized immediately. All the negative control mice were kept for 72 hours.

Fig. 14 IL-6 Assay of CLP Mice with Cefotaxime Starting at Different Time Points at time of Euthanasia

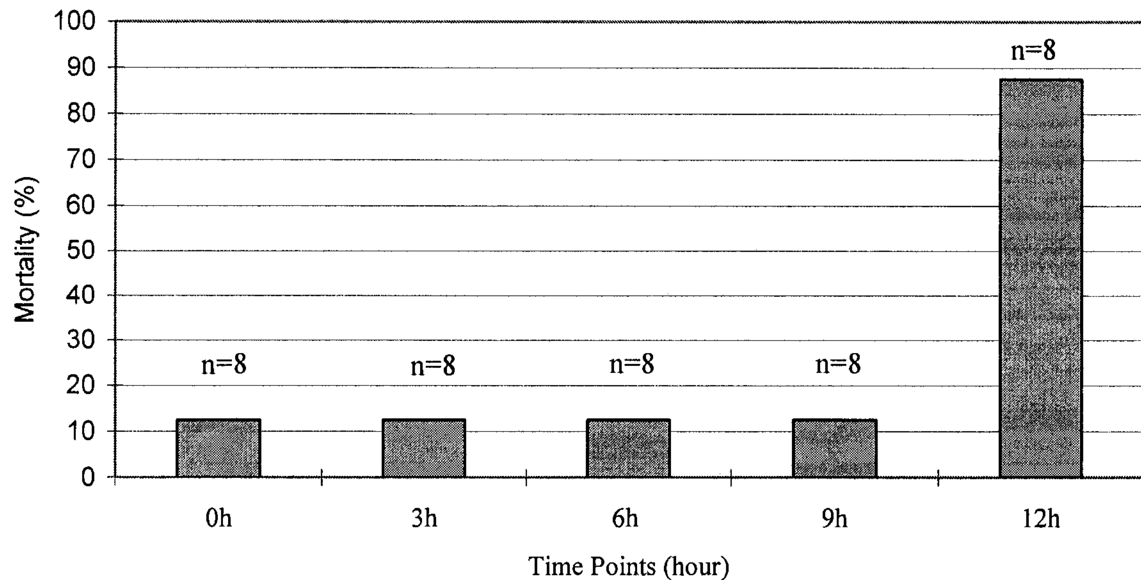


The average IL-6 levels of cefotaxime 0 hour & 3 hours post-surgery groups are not significantly different from negative control mice (t-test, $P>0.05$). The average IL-6 levels of cefotaxime 6 & 12 hours post-surgery groups are not significantly different between each other (t-test, $P>0.05$), but they are both significantly higher than cefotaxime 0 hour & 3 hours post-surgery groups (t-test, $P<0.05$). Error bar is standard deviation.

There is a significant increasing of IL-6 level as the start time points of cefotaxime delayed (ANOVA test, $P<0.05$).

All the CLP mice in this treatment strategy were kept for 72 hours. The mice reaching the potential mortality endpoint were euthanized immediately. All the negative control mice were kept for 72 hours.

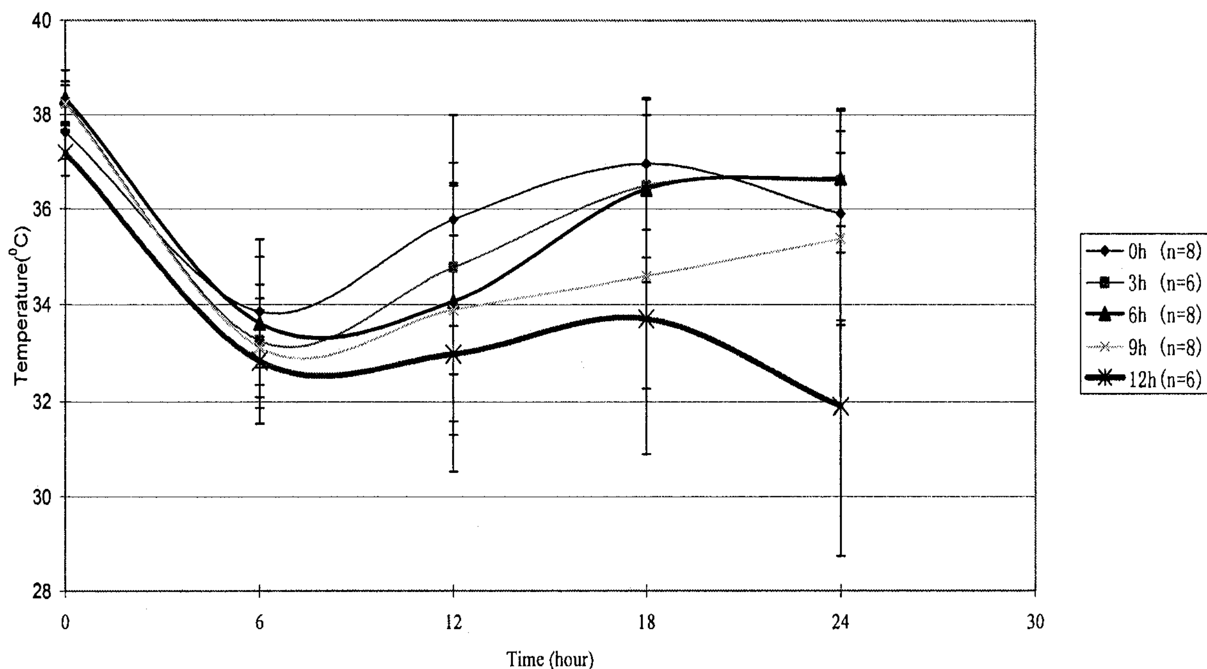
Fig. 15 Mortalities of CLP Mice with Fluid Resuscitation at Different Starting Time Points (0, 3, 6, 9, 12 hours post-surgery)



The mortalities of the first 4 groups with fluid resuscitation started at 0, 3, 6, 9 hours post-surgery are the same, while the mortality of the 5th group with fluid resuscitation started at 12 hours post-surgery is significant higher than the other 4 groups (Fisher's exact test, $P<0.05$). 7 out of 8 mice in the 5th group reached potential mortality endpoint within first 24 hours post-surgery.

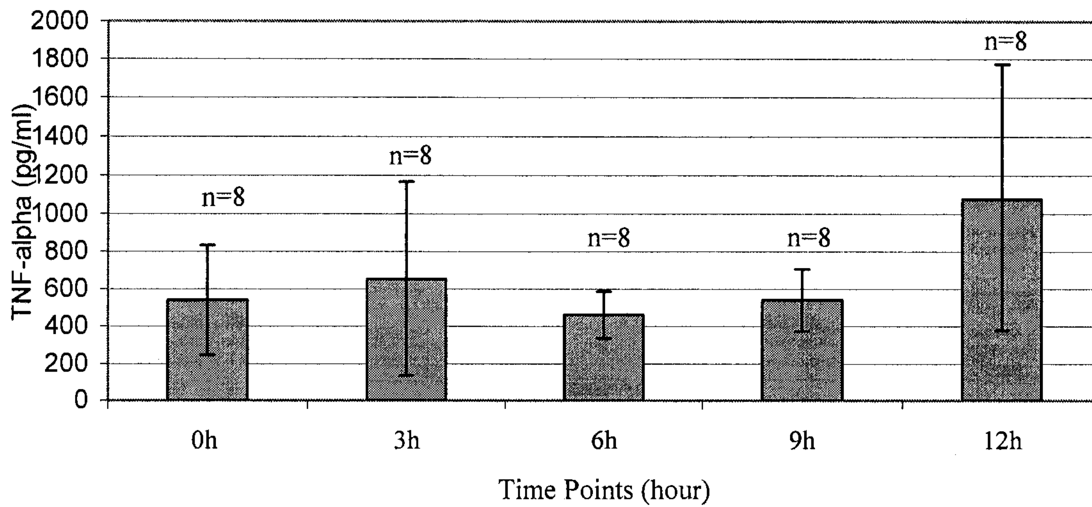
All the mice in this experiment were given 0.05mL diluted cefotaxime every 6 hours for up to 72 hours, which started at 0 hour post-surgery.

Fig. 16 Body Temperatures of CLP Mice with Fluid Resuscitation Starting at Different Time Points



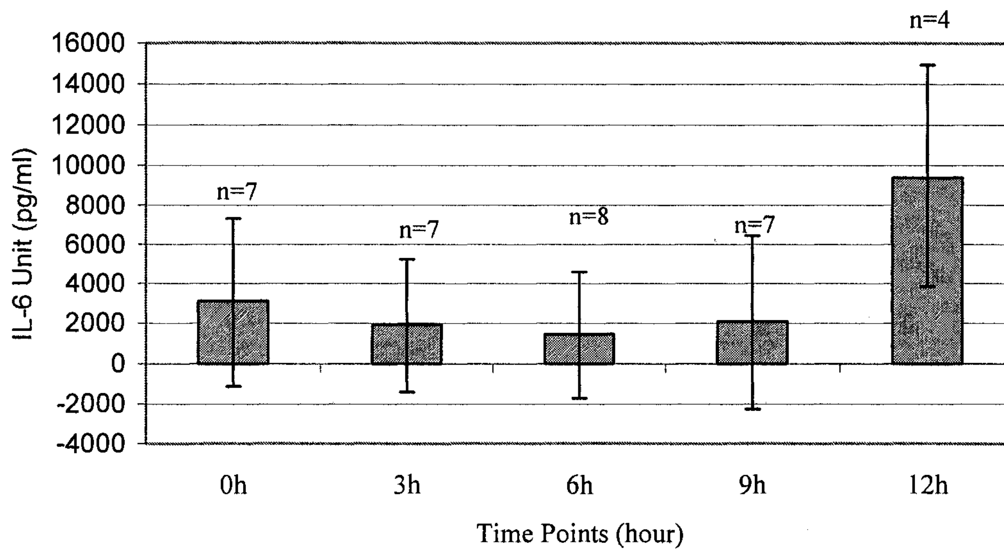
At 1 hour post-surgery, the average temperature between 0.9% saline fluid starting at 0 hour (violet), 3 hours (pink), 6 hours (black), 9 hours (light blue) and 12 hours (purple) post-surgery are similar. The temperature change of 0.9% saline fluid starting at 12 hours post-surgery is going down during the first 24 hours. There is a significant difference of average temperature changes between 0 and 24 hours post-surgery between five groups (ANOVA test $P<0.05$). Error bar is standard deviation.

Fig. 17 TNF-α Assay of CLP Mice with Fluid Resuscitation at Different Starting Time Points



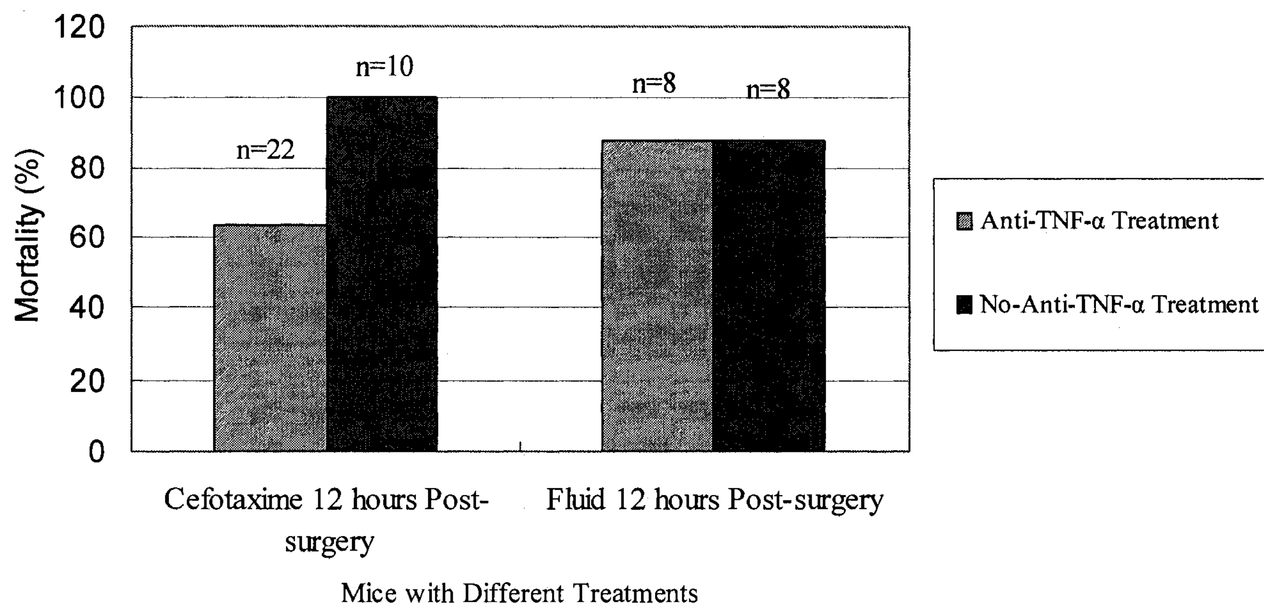
Average levels of TNF-α from the serum of CLP mice in the first 4 groups are similar (0.9% saline fluid starting at 0 hour, 3, 6 & 9 hours post-surgery). The average level of TNF-α in the group with 0.9% saline fluid starting at 12 hours post-surgery is significantly higher than the other 4 groups (ANOVA test, $P<0.05$). Error bar is standard deviation.

Fig. 18 IL-6 Assay of CLP Mice with Fluid Resuscitation at Different Starting Time Points



Average levels of IL-6 from the serum of CLP mice in the first 4 groups are similar (0.9% saline fluid starting at 0 hour, 3, 6 & 9 hours post-surgery). The average level of IL-6 in the group with 0.9% saline fluid starting at 12 hours post-surgery is significantly higher than the other 4 groups (ANOVA test, $P<0.05$). Error bar is standard deviation.

Fig. 19 Mortalities of CLP Mice with and without Anti-TNF-α Adjunctive Treatment in Different Groups with Late Administration of Cefotaxime and Fluid



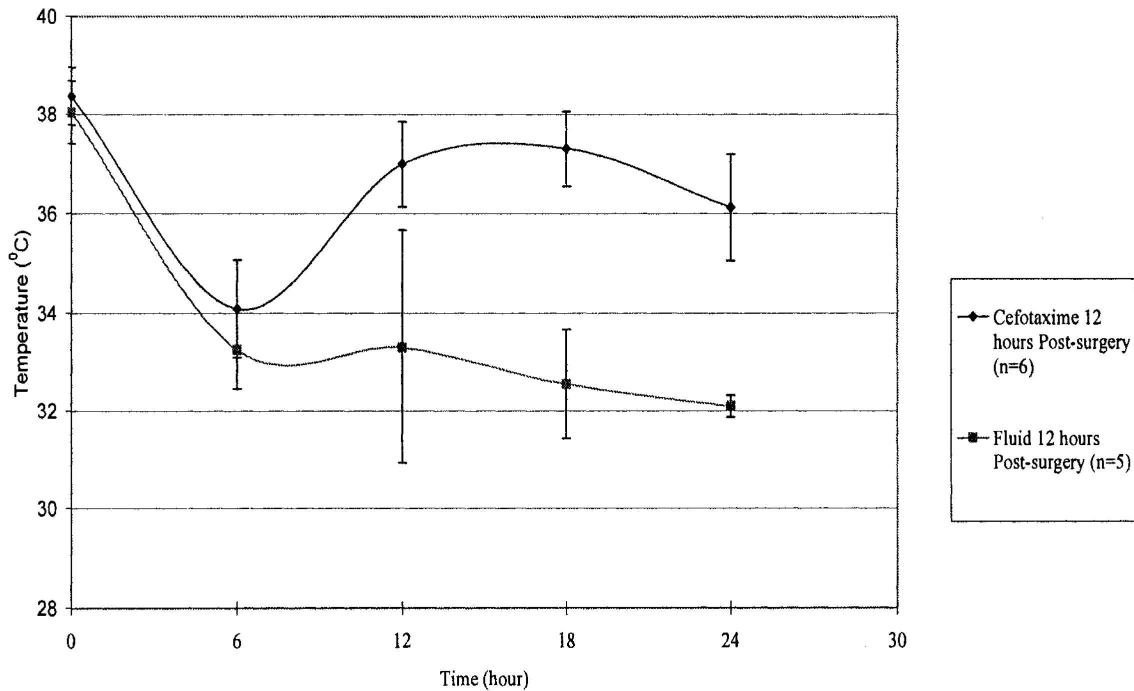
Cefotaxime 12 hours post-surgery: All the mice were given 0.05mL cefotaxime every 6 hours for up to 72 hours starting at 12 hours post-surgery and 1mL 0.9% saline fluid every 6 hours for up to 72 hours starting at 0 hour post-surgery.

Fluid 12 hours post-surgery: All the mice were given 0.05mL cefotaxime every 6 hours for up to 72 hours starting at 0 hour post-surgery and 1mL 0.9% saline fluid every 6 hours for up to 72 hours starting at 12 hours post-surgery.

In cefotaxime 12 hours post-surgery group, the mortality of mice without anti-TNF-α adjunctive treatment is significantly higher than that of mice with anti-TNF-α adjunctive treatment (Fisher's exact test, $P<0.05$).

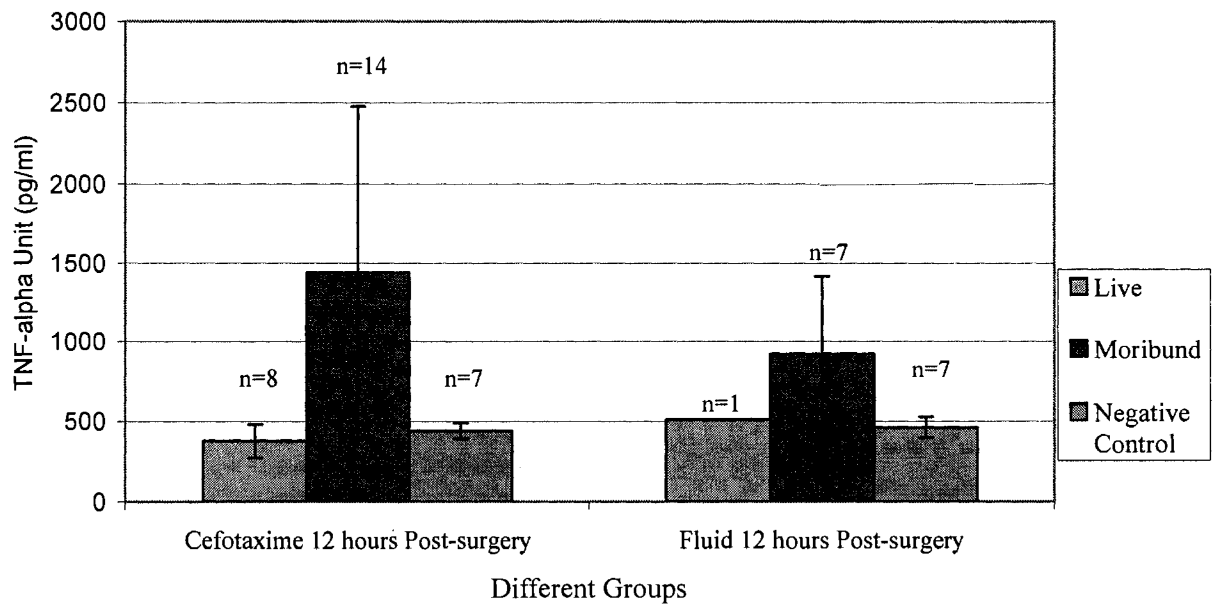
In fluid 12 hours post-surgery group, the mortality of mice without anti-TNF-α adjunctive treatment is the same with that of mice with anti-TNF-α adjunctive treatment.

Fig. 20 Body Temperatures of CLP Mice with and without Anti-TNF-α Adjunctive Treatment in Different Groups with Late Administration of Cefotaxime and Fluid



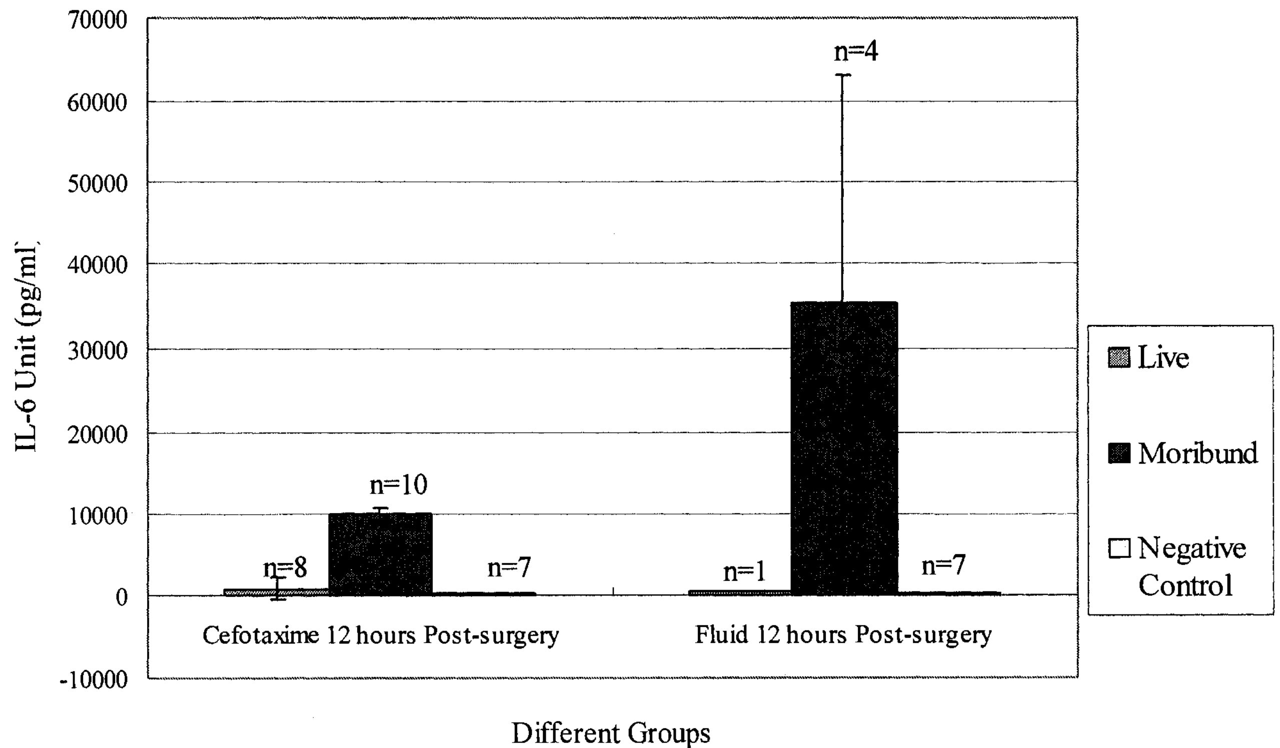
At 0 hour post-surgery, the average temperature of cefotaxime 12 hours post-surgery group (violet) is similar with that of fluid 12 hours post-surgery group (pink). At 24 hours post-surgery, the average temperature of cefotaxime 12 hours post-surgery group is different from that of fluid 12 hours post-surgery group. The average temperature changes of both groups between 0 and 24 hours post-surgery is significantly different (t-test, $P<0.05$). Error bar is standard deviation.

Fig. 21 TNF-α Assay of CLP Mice with and without Anti-TNF-α Adjunctive Treatment in Different Groups with Late Administration of Cefotaxime and Fluid



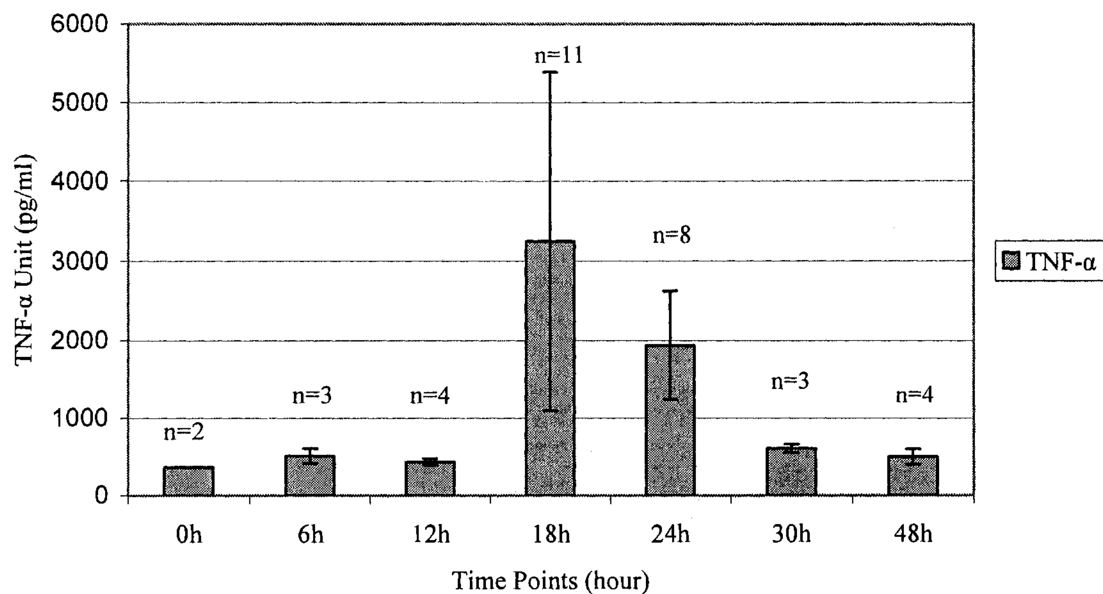
In cefotaxime 12 hours post-surgery group (Left), 14 CLP mice reaching endpoint before 72 hours has a significantly higher average level of TNF-α than both survived CLP mice and negative control mice respectively (t-test, $P<0.05$). Fluid 12 hours post-surgery group (Right) has the similar result (Error bar is standard deviation).

Fig. 22 IL-6 Assay of CLP Mice with and without Anti-TNF-α Adjunctive Treatment in Different Groups with Late Administration of Cefotaxime and Fluid



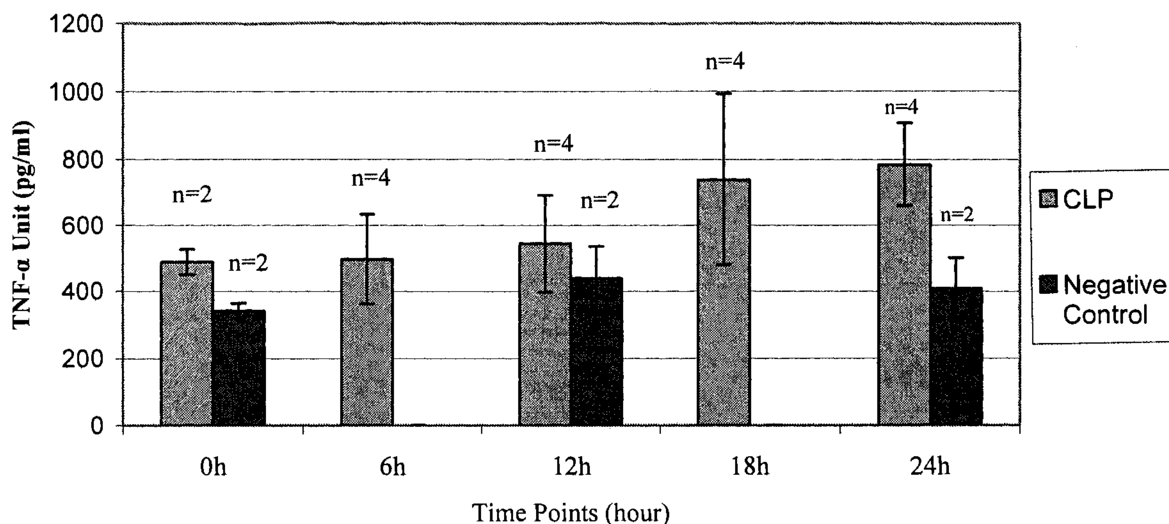
In cefotaxime 12 hours post-surgery group (Left), 10 CLP mice reached endpoint before 72 hours and had a significantly (t-test, $P<0.05$) higher average (*100 times) level of IL-6 than both survived CLP mice and negative control mice. Fluid 12 hours post-surgery group (Right) has the similar result (Error bar is standard deviation).

Fig. 23 TNF-α Assay of CLP Mice with Cefotaxime 12h post-op at Different Time Points



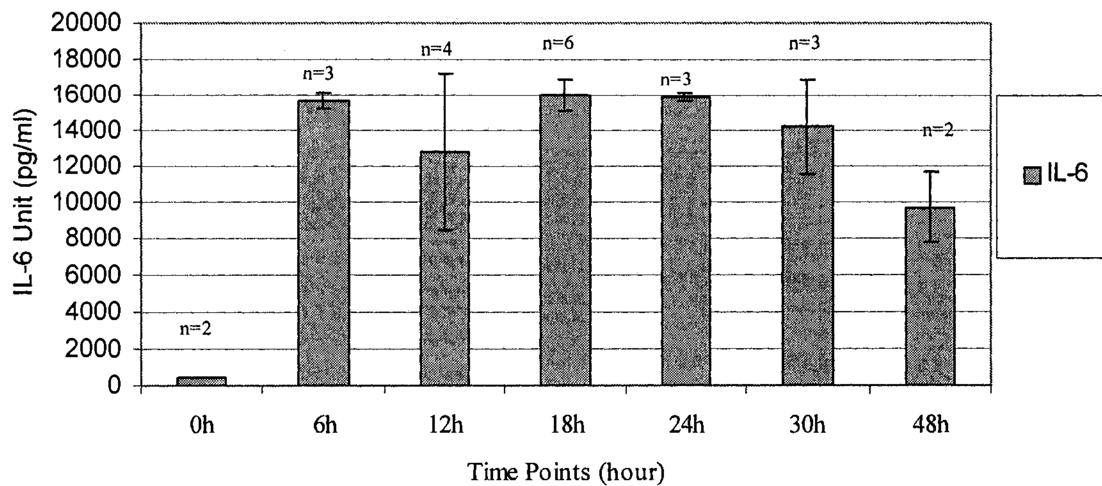
In cefotaxime 12 hours post-surgery group, the TNF-α levels of CLP mice are similar at 0, 6 and 12 hours post-surgery. It peaks at 18 hours post-surgery and drops down. Thereafter the level goes back to pre-peak levels (Error bar is standard deviation). There is a significant change of TNF-α levels in CLP mice as time progresses (ANOVA test, $P<0.05$).

Fig. 24 TNF-α Assay of CLP Mice with Fluid Resuscitation 12 hours Post-surgery at Different Time Points



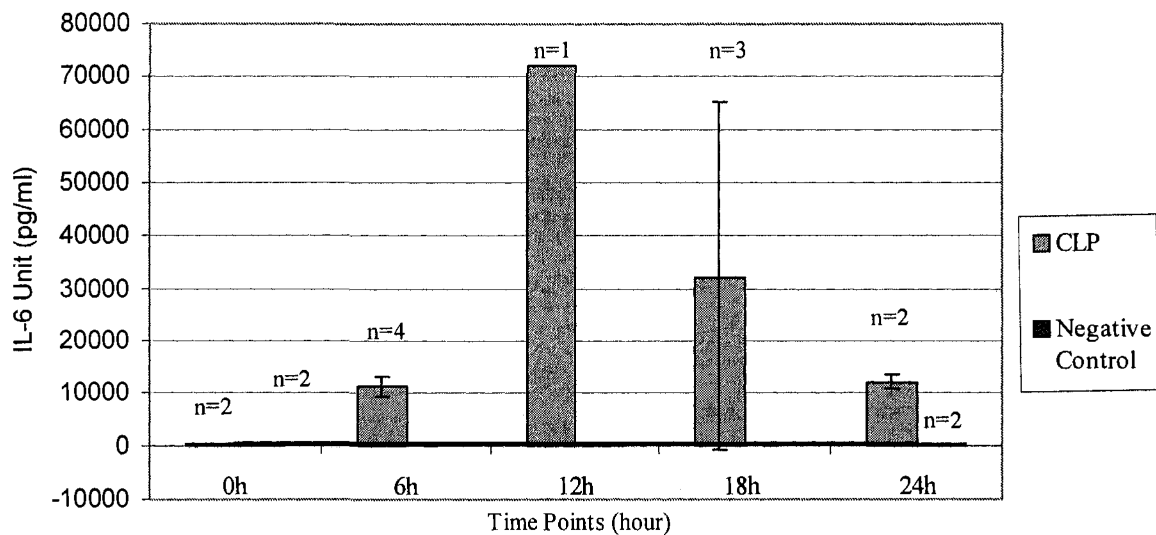
In 0.9% saline fluid 12 hours post-surgery group, the TNF-α levels of CLP mice (blue) keep going up from 0 hour to 24 hours post-surgery. The levels of negative control mice (red) stay constant over time (Error bar is standard deviation). There is no significant change of TNF-α levels in CLP mice as time progresses (ANOVA test, $P>0.05$).

Fig. 25 IL-6 Assay of CLP Mice with Cefotaxime 12 hours Post-surgery at Different Time Points



In cefotaxime 12 hours post-surgery group, IL-6 level of CLP mice increases dramatically right at 6 hours post-surgery and stays high throughout 48 hours post-surgery. Although it drops down after 24 hours' observation, but it is still much higher than the level of 0 hour post-surgery (Error bar is standard deviation). There is a significant change of IL-6 levels as time progresses (ANOVA test, $P<0.05$).

Fig. 26 IL-6 Assay of CLP Mice with Fluid Resuscitation 12 hours Post-surgery at Different Time Points



In 0.9% saline fluid 12 hours post-surgery group, IL-6 level of CLP mice (blue) increases dramatically at 6 hours post-surgery. Although the level drops down after 12 hours post-surgery, it is still hundreds of times higher than the level of negative control mice (red). The IL-6 level of negative control mice is constant throughout the observation (Error bar is standard deviation). There is a significant change of IL-6 levels in CLP mice as time progresses (ANOVA test, $P<0.05$).

List of References

Abraham, E. P. 1980. Fleming's discovery. Reviews of Infectious Diseases . 2: 140-141

Aderka Le J, Vilcek J. 1989. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 Cells and Mice. The Journal of Immunology. 143: 3517-3523.

Akca S, Haji-Michael P, deMedonca A, Suter PM, Levi M, Vincent JL. 2002.The time course of platelet counts in critically ill patients. Critical Care Medicine. 30: 753–756.

Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. 2001. Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. Critical Care Medicine. 29: 1303-1310.

Annane D, Sébille V, Charpentier C, Bollaert PE, François B, Korach JM, Capellier G, Cohen Y, Azoulay E, Troché G, Chaumet-Riffaut P, Bellissant E. 2002. Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. The Journal of the American Medical Association. 288: 862-871.

Astiz ME, DeGent GE, Lin RY, Rackow EC. 1995. Microvascular function and rheologic changes in hyperdynamic sepsis. Critical Care Medicine 23: 265–271.

Badr KF. 1994. Novel mediators of sepsis-associated renal failure. Semianrs in Nephrology. 14: 3-7.

Bagby GJ, Plessala KJ, Wilson LA, Thompson JJ, Nelson S. 1991. Divergent efficacy of antibody to tumor necrosis factor-alpha in intravascular and peritonitis models of sepsis. The Journal of Infectious Diseases163: 83.

Baker CC, Chaudry IH, Gaines HO, Baue AE. 1983. Evaluation of factors affecting mortality rate after sepsis in a murine cecal ligation and puncture model. Surgery. 94: 331-335

Barza M. 1978. A critique of animal models in antibiotic research. Scandinavian Journal of Infectious Diseases. 14: 109-117

Baskurt OK, Gelmont D, Meiselman HJ. 1998. Red blood cell deformability in sepsis. American Journal of Respiratory and Critical Care Medicine. 157:421–427.

Baughman RP, Lower EE, Flessa HC, Tollerud DJ. 1993. Thrombocytopenia in the intensive care unit. Chest. 104: 1243–1247.

Bauss F, Dröge W, Männel DN. 1987. Tumor necrosis factor mediates endotoxic effects in mice. Infection and Immunity. 55:1622-1625.

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA.1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proceedings of the National Academy of Sciences. 87: 1620-1624

Bedell SE, Bush BT.1985. Erythrocyte sedimentation rate. The American journal of medicine.1985; 78:1001-1009.

Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, Steingrub JS, Garber GE, Helterbrand JD, Ely EW, Fisher CJ Jr. 2001. Recombinant human protein C worldwide evaluation in severe sepsis (PROWESS) study group. Efficacy and safety of recombinant human activated protein C for severe sepsis. The New England Journal of Medicine. 344: 699-709.

Beutler B, Milsark IW, Cerami AC.1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science. 229: 869.

Bickell WH, Wall MJ Jr, Pepe PE, Martin RR, Ginger VF, Allen MK, Mattox KL. 1994. Immediate versus delayed fluid resuscitation for hypotensive patients with penetrating torso injuries. The New England Journal of Medicine. 331: 1105-1109.

Bisno AL, Freeman JC. 1970. The syndrome of asplenia, pneumococcal Sepsis, and disseminated intravascular coagulation. Annals of Internal Medicine. 72: 389-393.

Bochud PY, Calandra T. 2003. Pathogenesis of sepsis: new concepts and implications for future treatment. British Medical Journal. 326: 262-266.

Bogart D, Biggar WD, Good RA. 1972. Impaired intravascular clearance of pneumococcus type-3 following splenectomy. Journal of the Reticuloendothelial

Society. 11: 77-87.

Bone RC, Fisher CJ Jr, Clemmer TP, Slotman GJ, Metz CA, Balk RA. 1989. Sepsis syndrome: a valid clinical entity. Critical Care Medicine. 17: 389-393.

Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. 1992. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest. 101: 1644-1655.

Bone RC. 1996. Sir Isaac Newton, sepsis, SIRS, and CARS. Critical Care Medicine. 24: 1125-1128.

Borden EC, Chin P. 1994. Interleukin-6: a cytokine with potential diagnostic and therapeutic roles. Journal of Laboratory and Clinical Medicine. 123: 824-829.

Brown EJ, Hosea SW, Frank MM. 1981. The role of the spleen in experimental pneumococcal Bacteremia. Journal of Clinical Investigation 67: 975-982.

Buttrum SM, Drost EM, MacNee W, Goffin E, Lockwood CM, Hatton R, Nash GB. 1994. Rheological response of neutrophils to different types of stimulation. Journal of Applied Physiology. 77:1801–1810.

Cannon JG, Tompkins RG, Gefland JA, Michie HR, Standford GG, van der Meer JWM, Endres S, Lonnemann G, Corsetti J, Chernow B, Wilmore DW, Wolf SM, Burke JF, Dinarello CA. 1990. Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. Journal of Infectioius Diseases. 160: 79-84.

Casey LC, Balk RA, Bone RC. 1993. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. Annuals of Internal Medicine. 119: 771-778.

Chadda K, Annane D. 2002. The use of corticosteroids in severe sepsis and acute respiratory distress syndrome. Annals of Medicine. 34:582-589.

Champman PB, Lester TJ, Casper ES, Gabrilove JL, Wong GY, Kempin SJ, Gold PJ, Welt S, Warren RS, Starnes HF, Sherwin SA, Old LJ, Oettgen HF. 1987. Clinical pharmacology of recombinant tumor necrosis factor in patients with advanced cancer. Journal of Clinical Oncology.5: 1942-1951.

Chen J, Li T, Li J, et al. 1986. Biochemical Experiment. Science Press. Experiment 87.

Costa EL, Schettino IA, Schettino GP. 2006. The lung in sepsis: Guilty or Innocent? Endocrine, metabolic & immune disorders-Drug targets. 6: 213-216.

Cunningham MA, Romas P, Hutchinson P, Holdsworth SR, Tipping PG. 1999. Tissue factor and factor VIIa receptor/ligand interactions induce proinflammatory effects in macrophages. Blood. 94: 3413-3420.

Damas P, Ledoux D, Nys M, Vrindts Y, De Groote D, Franchimont P, Lamy M. 1992. Cytokine serum level during severe sepsis in human. IL-6 as a marker of severity. Annals of Surgery. 215: 356-362.

Dear JW, Yasuda H, Hu X, Hieny S, Yuen PS, Hewitt SM, Sher A, Star RA. 2006. Sepsis-induced organ failure is mediated by different pathways in the kidney and liver: Acute renal failure is dependent on MyD88 but not renal cell apoptosis. Kidney International. 69: 832–836.

Debets JM, Kampmeijer R, van der Linden MP, Buurman WA, van der Linden CJ. 1989. Plasma tumor necrosis factor and mortality in critically ill septic patients. Critical Care Medicine. 17: 489.

Dellinger RP, Carlet JM, Masur H, Gerlach H, Calandra T, Cohen J, Gea-Banacloche J, Keh D, Marshall JC, Parker MM, Ramsay G, Zimmerman JL, Vincent JL, Levy MM. 2004. Surviving sepsis campaign management guidelines committee. Surviving sepsis campaign guidelines for management of severe sepsis and septic shock. Critical Care Medicine 32: 858-873.

Deitch EA. 2004. Role of the Gut in Multiple Organ Dysfunction Syndrome UMDNJ Research. The University of Medicine & Dentistry of New Jersey. 4-6.

Dhainaut JF, Marin N, Mignon A, Vinsonneau C. 2001. Hepatic response to sepsis: Interaction between coagulation and inflammatory processes. Critical Care Medicine. 29 Supplement 1:S42-S47.

Dinarello CA, Thompson RC. 1991. Blocking IL-1: interleukin-1 receptor antagonist *in vivo* and *in vitro*. Immunology Today. 77: 1627-1652.

Domagk G. 1986. Classics in infectious diseases. A contribution to the chemotherapy

of bacterial infections. Reviews of Infectious Diseases. 8 (1): 163-166.

Drost EM, Fisher TC, Kassabian G, Gelmont D, and Meiselman HJ. 1995. Neutrophil deformability in sepsis syndrome and following TNF-exposure in vitro (abstract). Am. J. Respir. Critical Care Medicine. 151: A322.

Echtenacher B, Falk W, Mannel DN, Krammer PH. 1990. Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. The Journal of Immunology. 145: 3762.

Edgeworth JD, Treacher DF, Eykyn SJ. 1999. A 25-year study of nosocomial bacteremia in an adult intensive care unit. Critical Care Medicine. 27:1421-1428.

Emerson TE, Lindsey DC, Jesmok GJ, Duerr ML, Fournel MA. 1992. Efficacy of monoclonal antibody against tumor necrosis factor alpha in an endotoxemic baboon model. Circulation Shock. 38: 75-84.

Endo S, Inada K, Inoue Y, Kuwata Y, Suzuki M, Yamashita H, Hoshi S, Yoshida M. 1992. Two types of septic shock classified by the plasma level of cytokines and endotoxin. Circulatory Shock. 38: 264-274.

Eppihimer MJ, Lipowsky HH. 1994. Leukocyte sequestration in the microvasculature in normal and low flow states. The American Journal of Physiology. 67: H1122–H1134.

Eraklis AJ, Kevy SV, Diamond LK, Gross RE. 1967. Hazard of overwhelming infection after splenectomy in childhood. The New England Journal of Medicine. 276: 1225-1229.

Eskandari MK, Bolgos G, Miller C, Nguyen DT, DeForge LE, Remick DG. 1992. Anti-tumor necrosis factor antibody therapy fails to prevent lethality after cecal ligation and puncture or endotoxemia. The Journal of Immunology 148: 2724.

Esmon CT, Fukudome K, Mather T, Bode W, Regan LM, Stearns- Kurosawa DJ, Kurosawa S. 1999. Inflammation, sepsis, and coagulation. Haematologica. 84: 254–259.

Esmon CT. 2000. Introduction: are natural anticoagulants candidates for modulating the inflammatory response to endotoxin? Blood. 95:1113-1116.

Esmon CT. 2002. New Mechanism for Vascular Control of inflammation mediated by natural anticoagulant Proteins The Journal of Experimental Medicine. 196: 561-564.

Facklam RR, Collins MD. 1989. Identification of Enterococcus species isolated from human infections by a conventional test scheme. Journal of Clinical Microbiology. 27: 731-734.

Febbraio MA, Pedersen BK. Jul. 2005 Contraction-induced myokine production and release: is skeletal muscle an endocrine organ? Exercise and Sport Sciences Reviews. 33:114-119.

Ferguson KL, Brown L. 1996. Bacteremia and sepsis. Emergency Medicine Clinics of North America. 14:185-195.

Fischer E, Marano MA, Van Zee KJ, Rock CS, Hawes AS, Thompson WA, DeForge L, Kenney JS, Remick DG, Bloedow DC, Thompson RC, Lowry SF, Moldawer LL. 1992. Interleukin-1 receptor blockade improves survival and hemodynamic performance in *Escherichia coli* septic shock, but fails to alter host responses to Sublethal Endotoxemia. Journal of Clinical Investigation. 89: 1551-1557.

Fong Y, Tracey KJ, Moldawer LL, Hesse DG, Manogue KB, Kenney JS, Lee AT, Kuo C, Allison AC, Lowry SF, Cerami A. 1989. Antibodies to Cachexin/Tumor Necrosis Factor reduces interleukin-1β and interleukin-6 appearance during lethal Bacteremia. The Journal of Experimental Medicine. 170: 1627-1633.

Fowler DE, Wang P. 2002. The cardiovascular response in sepsis: proposed mechanisms of the beneficial effect of Andrenomedullin and its binding protein. International Journal of Molecular Medicine. 9: 443-449.

Franco RF, de Jonge E, Dekkers PE, Timmerman JJ, Spek CA, van Deventer SJ, van Deursen P, van Kerkhoff L, van Gemen B, ten Cate H, van der Poll T, Reitsma PH. 2000. The in vivo kinetics of tissue factor messenger RNA expression during human endotoxemia: relationship with activation of coagulation. Blood. 96: 554-559.

Goldie AS, Fearon KC, Ross JA, Barlay GR, Jackson RE, Grant IS, Ramsay G, Blyth AS, Howie JC. 1995. Natural cytokine antagonist and antiendotoxin core antibodies in sepsis syndrome. The sepsis intervention group. The Journal of American Medical Association. 274: 172-177.

Gorrasi J, Takala J, Jakob SM. 2006. The Vascular Bed during Critical Illness: Evaluation in Animal Models. Yearbook of Intensive Care and Emergency Medicine. 119-128.

Gutiérrez-Peña EJ, Knab J, Büttner DW. 1996. Neutrophil granule proteins: evidence for the participation in the host reaction to skin microfilariae of onchocerca Volvulus after diethylcarbamazine administration. Parasitology. 113: 403-414.

Hack CE, De Groot ER, Felt-Bersma RJ, Nuijens JH, Strack Van Schijndel RJ, Eerenberg-Belmer AJ, Thijs LG, Aarden LA. 1989. Increased plasma levels of interleukin-6 in sepsis. Blood. 74: 1704-1710.

Havell EA. 1989. Evidence that tumor necrosis factor has an importance role in antibacterial resistance. The Journal of Immunology. 143: 2894.

Hesse DG, Tracey KJ, Fong Y, Manogue KR, Palladino MA Jr, Cerami A, Shires GT, Lowry SF. 1988. Cytokines apperance in human endotoxemia and primate bacteremia. Surgery, Gynecology and Obstetrics. 166: 147-153.

Hinshaw LB, Emerson TE, Taylor FB, Chang ACK, Duerr M, Peer GT, Flournoy DJ, White GL, Kosanke SD, Murray CK, Xu R, Passey RB, Fournel MA. 1992. Lethal staphylococcus aureus-induced shock in primates prevention of death with anti-TNF antibody. Journal of Trauma. 33: 568-573.

Hinshaw LB. 1996. Sepsis/septic shock: Participation of the microcirculation. an abbreviated review. Critical Care Medicine 24: 1072-1078.

Homels CL, Russell JA, Walley KR. 2003. Genetic polymorphisms in sepsis and septic shock: role in prognosis and potential for therapy. Chest. 124: 1103-1115.

Houck PM, Bratzler DW, Nsa W, Ma A, Bartlett JG. 2004. Timing of antibiotic administration and outcomes for medicare patients hospitalized with community – acquired pneumonia. Archives of Internal Medicine. 164: 637-644.

Houck PM, Bratzler DW. 2005. Administration of First Hospital antibiotics For community-acquired pneumonia: Does timeliness affect outcomes? Current Opinion in Infectious Diseases. 18: 151-156.

Hughes JE, Stewart J, Barclay GR, Govan JR. 1997. Priming of neutrophil respiratory burst activity by lipopolysaccharide from burkholderia cepacia. Infection and Immunity. 65: 4281-4287.

Janeway CA Jr, Medzhitov R. 2002. Innate immune recognition. Annual Review of Immunology 20: 197-216.

Jones A, Geczy CL. 1990. Thrombin and factor Xa enhance the production of interleukin-1. Immunology. 71: 236-241

Jones A, Geczy CL. 1990. Thrombin and factor Xa enhance the production of interleukin-1. Immunology. 71: 236-241

Julian D, Saunders WB. 1996. Acute myocardial infarction: pre-hospital and in-hospital management: The task force on the management of acute myocardial infarction of the European Society of Cardiology. European Heart Journal. 17: 43-63.

Keel M, Ungethüm U, Steckholzer U, Niederer E, Hartung T, Trentz O, Ertel W. 1997. Interleukin-10 counterregulates roinflammatory cytokine-Induced inhibition of neutrophil apoptosis during severe sepsis. Blood. 90: 3356-3363.

Kishimoto T, Akira S, Narazaki M, Taga T. 1995. Interleukin-6 family of cytokines and gp130. Blood. 86: 1243-1254.

Koch T. 1998. Origin and mediators of sepsis and SIRS Kidney International. 53: S66-S69.

Kollef MH, Sherman G, Ward S, Fraser VJ. 1999. Inadequate antimicrobial treatment of infections: a risk factor for hospital mortality among critically ill patients. Chest. 115: 462-474.

Koppensteiner R. 1996. Blood rheology in emergency medicine. Seminars in Thrombosis and Hemostasis.22: 89-91.

Kriegler M, Perez C, DeFay K, Albert I, Lu SD. 1988. A novel form of TNF/cachectin is a cell surface cytotoxic Transmembrane protein: ramifications for the complex physiology of TNF. Cell. 53: 45-53.

Kuhns DB, Alvord WG, Gallin JI. 1995. Increased circulating cytokines, cytokine

antagonist, and E-selectin after intravenous administration of endotoxin in humans. The Journal of Infectious Diseases. 171: 145-152.

Lam C, Tyml K, Martin C, Sibbald W. 1994. Microvascular perfusion is impaired in a rat model of normotensive sepsis. The Journal of Clinical Investigation. 94: 2077-2083.

Lamberts SW, Bruining HA, de Jong FH. 1997. Corticosteroid therapy in severe illness. The New England Journal of Medicine. 337: 1285-1292.

Letarte J, Longo CJ, Pelletier J, Nabonne B, Fisher HN. 2002. Patient characteristics and costs of severe sepsis and septic shock in Quebec. Journal of Critical Care. 17: 39-49.

Leung LS, Szal GJ, Drachman RH. 1972. Increased susceptibility of splenectomized rats to infection with Diplococcus pneumoniae. The Journal of Infectious Diseases. 126:507-513.

Levi M, ten Cate H, Bauer KA, van der Poll T, Edgington TS, Büller HR, van Deventer SJ, Hack CE, ten Cate JW, Rosenberg RD. 1993. Inhibition of endotoxin-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. The Journal of Clinical Investigation. 91: 114-120.

Levi M, van der Poll T, ten Cate H, van Deventer SJ. 1997.The Cytokine-mediated imbalance between cagulant and anticoagulant mechanisms in sepsis and endotoxaemia. European Journal of Clinical Investigation. 27: 3-9.

Levi M, Dörffler-Melly J, Reitsma P, Buller H, Florquin S, van der Poll T, Carmeliet P. 2003. Aggravation of endotoxin-induced disseminated intravascular coagulation and cytokine activation in heterozygous protein C deficient mice. Blood. 101: 4823-4827.

Levi M, van der Poll T, Büller HR. 2004. Bidirectional relation between inflammation and coagulation. Circulation. 109: 2698–2704.

Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G. 2003. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Critical Care Medicine. 31: 1250-1256.

Lichanska AM, Hume DA. 2000. Hume origins and functions of phagocytes in the

embryo Experimental Hematology. 28: 601–611.

Liolios A. 2003. Sepsis Review. St Luc Hospital, Montreal, Canada. http://www.md.ucl.ac.be/virtanes/Sepsis%20for%20Laterre.doc

Lowe GDO. 1988. Rheology of Disease. In: Lowe GDO, ed. Clinical Blood Rheology. Florida: CRC Press. 89-111.

Machiedo GW, Powell RJ, Rush BF Jr, Swislocki NI, Dikdan G. 1989. The incidence of decreased red blood cell deformability in sepsis and the association with oxygen free radical damage and multiple-system organ failure. Archives of Surgery. 124:1386–1389.

Matthay MA. 2001. Severe Sepsis- A new treatment with both anticoagulant and antiinflammatory properties. The New England Journal of Medicine. 344: 699-709.

Mavrommatis AC, Theodoridis T, Orfanidou A, Roussos C, Christopoulou- Kokkinou V, Zakynthinos S. 2000. Coagulation system and platelets are fully activated in uncomplicated sepsis. Critical Care Medicine. 28: 451–457.

McCallum RE, Becker KP, Urbaschek R, Urbasche B. 1986. Effect of endotoxin pretreatment on the microbial kinetics of acute peritonitis and sepsis. Circulation of Shock 18: 337.

McLeay AM. 2004. Drotrecogin Alfa: A role in emergency department treatment of severe sepsis? Emergency Medicine Australasia. 16: 324-335.

Meehan TP, Fine MJ, Krumholz HM, Scinto JD, Galusha DH, Mockalis JT, Weber GF, Petrillo MK, Houck PM, Fine JM. 1997. Quality of care, process, and outcomes in elderly patients with pneumonia. The Journal of the American Medical Association. 278: 2080-2084.

Mira JP, Cariou A, Grall F, Delclaux C, Losser MR, Heshmati F, Cheval C, Monchi M, Teboul JL, Riché F, Leleu G, Arbibe L, Mignon A, Delpech M, Dhainaut JF. 1999. Association of TNF2, a TNF-alpha promoter polymorphism, with septic shock susceptibility and mortality: a multicenter study. The Journal of The American Medical Association. 282:561-568.

Molnar RG, Wang P, Ayala A, Ganey PE, Roth RA, Chaudry IH. 1997. The role of

neutrophils in producing hepatocellular dysfunction during the hyperdynamic stage of sepsis in rats The Journal of Surgical Research. 73: 117-122.

1991. Serum levels of tumor necrosis factor determine the fatal or non-fatal course of endotoxic shock. Immunology Letters. 27: 157-162.

Munford RS, Hall CL, Grimm L. 1984. Detection of free endotoxin in cerebrospinal fluid by the limulus lysate test. Infection and Immunity. 45:531-533.

Murakami K, Okajima K, Uchiba M, Johno M, Nakagaki T, Okabe H, Takatsuki K. 1996. Activated protein C attenuates endotoxin-induced pulmonary vascular injury by inhibiting activated leukocytes in rats. Blood. 87: 642-647.

Natanson C, Esposito CJ, Banks SM. 1998. The sirens' songs of confirmatory sepsis trials: Selection bias and sampling error. Critical Care Medicine. 26: 1927-1931.

Negussie Y, Remick DG, DeForge LE, Kunkel SL, Eynon A, Griffin GE. 1992. Detection of plasma tumor necrosis factor, and interleukins 6 and 8 during the Jarisch-Herxheimer reaction of relapsing fever. The Journal of Experimental Medicine. 175:1207-1212.

Newcomb D, Bolgos G, Green L, Remick DG. 1998. Antibiotic treatment influences outcome in murine sepsis: mediators of increased morbidity. Shock. 10: 110-117.

Noris M. 1998. NO as a mediator of hemodynamic disturbances in ARF associated with sepsisin Claudio Ronco & Rinaldo Bellmo (eds). Critical Care Nephrology Kluwar Academic Publishers. 1: 575-89.

Ohlsson K, Björk P, Bergenfeldt M, Hageman R, Thompson RC. 1990. Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. Nature. 348: 550.

Okajima K. 2001. Reguation of inflammatory response by natural anticoagulants. Immunological Reviews. 184: 258-274.

Pascual JL, Khwaja KA, Ferri LE, Giannias B, Evans DC, Razek T, Michel RP, Christou NV. 2003. Hypertonic Saline resuscitation attenuates neutrophil lung sequestration and transmigration by diminishing leukocyte-endothelial interactions in a two-hit model of hemorrhagic shock and infection. Journal of Trauma-Injury Infection & Critical Care 54: 121-132.

Philip AG. 1982. Detection of neonatal sepsis of late onset. The Journal of The American Medical Association. 247: 489-492.

Pinksy MR, Vincent JL, Deviere J, Alegre M, Kahn RJ, Dupont E. 1993. Serum cytokine levels in Human septic shock. Relation to multiple-system organ failure and Mortality. Chest. 103: 565-575.

Pixley RA, De La Cadena R, Page JD, Kaufman N, Wyshock EG, Chang A, Taylor FB Jr, Colman RW. 1993. The Contact System Contributes to Hypotension but no Disseminated Intravascular Coagulation in Lethal Bacteremia: *In vivo* use of a monoclonal anti-factor XII antibody to block contact activation in baboons. The Journal of Clinical Investigation. 91: 61-68.

Porembka DT. 1993. Cardiovascular abnormalities in sepsis. New Horizon. 1: 324-341.

Póvoa P. 2002. C-reactive protein: a valuable marker of sepsis. Intensive Care Medicine. 28: 235-243.

Powell RJ, Machiedo GW, Rush BF Jr, Dikdan G. 1991. Oxygen free radicals: effect on red cell deformability in sepsis. Critical Care Medicine.19:732–735.

Redl H, Schlag G, Ceska M, Davies J, Buurman WA. 1993. Interleukin-8 release in baboon septicemia is partially dependent On tumor necrosis factor. The Journal of Infectious Diseases. 167: 1464-1466.

Reinhart K, Karzai W. 2001. Anti-tumor necrosis factor therapy in sepsis: Update on clinical trials and lessons learned. Critical Care Medicine. 29 Supplement 1: S121-S125.

Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. 2002. Six at six: Interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. Shock 17: 463-467.

Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M; Early Goal-Directed Therapy Collaborative Group. 2001. Early goal-directed therapy in the treatment of severe sepsis and septic shock. The New England Journal of Medicine. 345:1368-1377.

Rothwell PM, Udwadia ZF, Lawler PG. 1991. Cortisol response to corticotropin and survival in septic shock. Lancet. 337: 582-583.

Sampson AP. 2000. The role of eosinophils and neutrophils in inflammation. Clinical & Experimental Allergy. 30: 22-27.

Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA. 1990. Correlation and interactions in the production of interleukin-6 (IL-6), IL-1 and tumor necrosis factor (TNF) in human blood mononuclear Cells: IL-6 Suppresses IL-1 and TNF. Blood. 75: 40-47.

Schulak DJ, Rayhack JM, Lippert FG 3rd, Convery FR. 1982. The Erythrocyte sedimentation rate in orthopaedic patients. Clinical Orthopaedics and Related Research. 167: 197-202.

Selby P, Hobbs S, Viner C, Jackson E, Jones A, Newell D, Calvert AH, McElwain T, Fearon K, Humphreys J, Shiga T. 1987. Tumor necrosis factor in man: clinical and biological observations. British Journal of Cancer. 56: 803-808.

Shalaby MR, Waage A, Aarden L, Espevik T. 1989. Endotoxin, Tumor Necrosis Factor-α and Interleukin-1 induce interleukin-6 production *in vivo.* Clinical Immunology and Immunology Pathology. 53: 488-498.

Shebuski RJ, Kilgore KS. 2002. Role of inflammatory mediators in thrombogenesis. J. Pharmacology and Experimental Therapeutics. 300: 729-735.

Shenep JL, Mogan KA. 1984. Kinetics of endotoxin release during antibiotic therapy for experimental gram-negative bacterial sepsis. The Journal of Infectious Diseases. 150: 380-388.

Sherman ML, Spriggs DR, Arthur KA, Imamura K, Frei E 3rd, Kufe DW. 1988. Recombinant human tumor necrosis factor administered as a five-day continuous infusion in cancer patients: phase I toxicity and effects on lipid metabolism. Journal of Clinical Oncology. 6: 344-350.

Shinefield HR, Steinberg CR, Kaye D. 1966. Effect of splenectomy on the susceptibility of mice inoculated with diplococcus pneumoniae. The Journal of Experimental Medicine. 123: 777-794.

Singer DB. 1973. Postsplenectomy Sepsis. Perspect. Pediatric Pathology / Affiliated With The International Paediatric Pathology Association. 1: 285-311.

Smith J, Urba W, Steis R, Janik J, Fenton B, Sharfman W, Conlon K, Sznol M, Creekmore S, Wells N, Elwood L, Keller J, Hestdal K, Ewel C, Rossio J, Kopp W, Shimuzut M, Oppenheim J, Longo D. 1990. Interleukin-1 alpha: results of a phase I toxicity and immunomodulatory Trial. American Society of Clinical Oncology. 9:717.

Soni A, Pepper GM, Wyrwinski PM, Ramirez NE, Simon R, Pina T, Gruenspan H, Vaca CE. 1995. Adrenal insufficiency occuring during septic Shock: Incidence, outcome, and relation to peripheral cytokin levels. The American Journal of Medicine. 98: 266-271.

Stark ME, Szurszewski JH. 1992. Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology. 103: 1928-1949.

Strauss R, Wehler M, Mehler K, Kreutzer D, Koebnick C, Hahn EG. 2002. Thrombocytopenia in patients in the medical intensive care unit: bleeding prevalence, transfusion requirements, and outcome. Critical Care Medicine. 30:1765–1771.

Suffredini AF, Fromm RE, Parker MM, Brenner M, Kovacs JA, Wesley RA, Parrillo JE. 1989. The Cardiovascular response of normal humans to the administration of endotoxin. The New England Journal of Medicine. 321: 280-287.

Taylor FB Jr, Chang A, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE. 1987. Protein C prevents the coagulopathic and lethal effects of Escherichia coli infusion in the baboon. The Journal of Clinical Investigation. 79: 918-925.

Thijs A, Thijs LG. 1998. Pathogenesis of renal failure in sepsis. Kidney International. 53: S34-S37.

Tiwari SC, Vikrant S. 2004. Sepsis and the kindney. Journal of Indian Academy of Clinical Medicine. 5: 45-54.

Todd JC 3rd, Mollitt DL. 1995. Effect of sepsis on erythrocyte intracellular calcium homeostasis. Critical Care Medicine. 23:459-465.

Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. 1987. Anti-cachetin/TNF monoclonal antibodies prevent septic shock during lethal

bacteremia. Nature. 330: 662-664

Tugrul S, Esen F, Celebi S, Ozcan PE, Akinci O, Cakar N, Telci L. 2002. Reliability of procalcitonin as a severity marker in critically ill patients with inflammatory response. Anaesthesia and Intensive Care. 30: 747-754.

Ueda M, Iwasaki Y, Harada H, Kubota Y, Yokomura I, Hashimoto S, Mizobuchi K, Nakagawa M. 2001. Role of neutrophils in acute lung injury induced by Candida sepsis. Nihon Kokyuki Gakkai Zasshi. 39: 726-731.

Urban CF, Reichard U, Brinkmann V, Zychlinsky A. 2006. Neutrophil extracellular traps capture and kill *Candida albicans* Yeast and Hyphal forms. Cellular Microbiology 8: 668.

Urbaschek B, Ditter B, Becker KP, Urbaschek R. 1984. Protective effects and role of endotoxin in experimental septicemia. Circulation of Shock.14: 209-222.

Urbaschek R, Urbaschek B. 1987. Tumor necrosis factor and interleukin-1 as mediators of endotoxin-induced beneficial effects. Reviews of Infectious Diseases. 9: 607-615

van den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, Vlasselaers D, Ferdinande P, Lauwers P, Bouillon R. 2001. Intensive insulin therapy in the critically ill patients. The New England Journal of Medicine. 345: 1359-1367

van der Poll T, Coyle SM, Levi M, Jansen PM, Dentener M, Barbosa K, Buurman WA, Hack CE, ten Cate JW, Agosti JM, Lowry SF. 1990. Activation of coagulation after administration to TNF to Normal subjects. The New England Journal of Medicine. 322: 1622-1627.

van der Poll T, Levi M, Hack CE, ten Vate H, van Deventer SJ, Eerenberg AJ, de Groot ER, Jansen J, Gallati H, Buller HR, ten Cate JW, Aarden LA. 1994. Elimination of interleukin-6 attenuate coagulation activation in experimental endotoxemia in chimpanzees. The Journal of Experimental Medicine. 179: 1253-1259.

van der Poll T, Keogh CV, Guirao X, Buurman WA, Kopf M, Lowry SF. 1997. Interleukin-6 Gene-deficient mice show impaired defense against pneumococcal pneumonia. The Journal of Infectious Diseases. 176: 439-444.

van der Poll T, de Jonge E, Levi M. 2001. Regulatory role of cytokines in disseminated intravascular coagulation .Seminars in Thrombosis and Hemostasis. 27: 639-651.

Vanderschueren S, De Weerdt A, Malbrain M, Vankersschaever D, Frans E,Wilmer A, Bobbaers H. 2000. Thrombocytopenia and prognosis in intensive care. Critical Care Medicine. 28: 1871–1876.

Van Zee KJ, DeForge LE, Fischer E, Marano MA, Kenney JS, Remick DG, Lowry SF, Moldawer LL. 1991. IL-8 in septic shock, endotoxemia, and after IL-1 administration. The Journal of Immunology. 146: 3478-3482.

Vincent JL, Sun Q, Dubois MJ. 2002. Clinical trials of immunomodulatory therapies in severe sepsis and septic shock. Clinical Infectious Disease. 34: 1084-1093.

Waage A, Halstensen A, Espevik T. 1987. Association between tumor necrosis Factor in serum and fatal outcome in patients with meningococcal disease. Lancer. 1: 355-359.

Waage A, Brandtzaeg P, Halstensen A, Kierulf P, Espevik T. 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. The Journal of Experimental Medicine. 169: 333-338.

Wang P. 2001. Andrenomedullin and cardiovascular responses in sepsis. Centre National De La Recherche Scientifique. 22: 1835-1840.

Whitaker AN. 1968. The effect of previous splenectomy on the course of pneumococcal bacteremia in mice. The Journal of Pathology and Bacteriology. 95: 357-376.

Wichterman KA, Baue AE, Chaudry IH. 1980. Sepsis and septic shock, a review of laboratory models and a proposal. Journal of Surgical Research. 29: 189-201.

Williams DL, Ha T, Li C, Kalbfleisch JH, Schweitzer J, Vogt W, Browder IW. 2003. Modulation of tissue Toll-like receptor 2 and 4 during the early phases of polymicrobial sepsis correlates with mortality. Critical Care Medicine. 31: 1808-1818.

Wilson MA, Chou MC, Spain DA, Downard PJ, Qian Q, Cheadle WG, Garrison RN. 1996. Fluid resuscitation attenuates early cytokine mRNA expression after peritonitis.

Journal of Trauma-Injury Infection & Critical Care. 41: 622-627.

Windsor AC, Mullen PG, Walsh CJ, Fisher BJ, Blocher CR, Jesmok G, Fowler AA 3rd, Sugerman HJ. 1994. Delayed tumor necrosis factor alpha blockade attenuates pulmonary dysfunction and metabolic acidosis associated with experimental gram-negaive sepsis. Archive of Surgery 129: 80.

Yaguchi A, Lobo FL, Vincent JL, Pradier O. 2004. Platelet function in sepsis. Journal of Thrombosis and Haemostasis. 2: 2096–2102.

Zak O. 1980. Scope and limitations of experimental chemotherapy. Experientia. 36(4):479-83.

Appendix I

Calculation:
Ref: http://ocw.tufts.edu/courses/5/content/216005

a. Model spp=human; wt= 70kg; cefotaxime dose= 30mg/kg q8h
b. Subject spp=mouse; wt=0.025kg

1. Convert mg/kg to mg/kcal dose:
   a. MEC for 70kg human= 70 $(70)^{0.75}$ =1694 kcal/day
   b. Dose per treatment= 70kg*30mg/kg=2100mg per treatment
   c. MEC dose=2100/1694=1.24mg/kcal per day
2. Periodicity of dosing
   a. MEC for mouse= 70 $(0.025)^{0.75}$ =4.2 kcal/day
   b. Mass specific metabolic rates:
      A. Human=1694 kcal per day/ 70kg=24.2 kcal/day
      B. Mouse= 4.2 kcal per day/ 0.025kg=168 kcal/day
   c. Treatment interval; for humans=q8h; for a mouse it equals: 8hrs(24.2/168) = 1.2hrs
3. Dose required to treat mouse= MEC mouse * MEC dose human
   = 4.2 kcal per day* 1.24mg/kcal per day
   = 5.2 mg per day
   = 5.2 mg/0.025kg
   = 208mg/kg

Appendix II

The average level of serum TNF-α level in CLP mice is 800pg/mL (0.8ng/mL) in our experiment and a 25 gram Balb/c mouse has about 1.4mL blood. The dose was calculated by the 50% neutralization dose (ND_{50}) of the antibody.

ND_{50} of anti-TNF-α monoclonal antibody from R&D System company is 0.05μg-0.2μg Anti-TNF-α : 0.25ng TNF- α. It means 0.05 to 0.2μg can neutralize 50% of 0.25ng TNF-α. We take the maximum proportion (0.2μg: 0.25ng TNF- α) and the maximum dose needed to neutralize 100% TNF-α in a 25 gram Balb/c mouse is:

0.8ng/mL*1.4mL/0.25ng*0.2μg*2≈2.2μg

Therefore the dose used was 2.2 μ g/mice which was calculated to be enough to neutralize almost all the TNF cytokine in a CLP mouse. According to the calculation, we diluted the antibody from the original 200 μ g/mL to 44 μ g/mL by PBS and administered 0.05mL diluted anti-TNF-α antibody to each CLP m mouse.