SINGLE, ULTRA-HIGH DOSE AMINOGLYCOSIDE THERAPY IN A RAT MODEL

OF *E. COLI* INDUCED SEPTIC SHOCK

By

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A Thesis Submitted to the Faculty of Graduate Studies of The University of Manitoba

In Partial Fulfillment of the Requirements of the Degree of

Doctor of Philosophy

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ABSTRACT

Bacterial infections are a major cause of morbidity and mortality in both the community and nosocomial settings, particularly among the elderly and chronically ill. Sepsis is the body's response to antigens and toxins released by the invasive pathogenic organisms that cause infection. When infection is not effectively controlled, sepsis may develop and progress to severe sepsis and septic shock. Early diagnosis and treatment is pivotal for survival in severe sepsis and particularly, septic shock. Our research focuses on developing a novel treatment strategy for septic shock by using single, ultra-high doses of aminoglycosides. In this project, the effect of a single, ultra-high dose of gentamicin in clearing bacteria from the blood and reducing the bacterial burden in vital organs was evaluated in a rat model of *E. coli* (Bort strain) induced peritonitis with severe sepsis/septic shock. Serum cytokine levels and serum lactate levels were serially measured. Further, the potential adverse effects of ultra-high dosing of aminoglycoside antibiotics in a short-term (9 h) invasive study and long-term (180 days) non-invasive study were assessed. Neuromuscular paralyses due to ultra-high doses of aminoglycosides were assessed. In addition, renal injury markers such as serum creatinine and urinary Neutrophil Gelatinase Associated Lipocalin (NGAL) were assayed. The auditory and vestibular function was also assessed after ultra-high dosing of aminoglycoside in the long-term study.

We conclude that animals can tolerate ultra-high doses of aminoglycosides with appropriate support. Animals were under neuromuscular paralysis for 28 – 50 minutes and were on ventilator support after single ultra-high doses (80 and 160 mg/kg) of aminoglycoside antibiotics (gentamicin and tobramycin). There was no significant acute or delayed renal or ototoxicity associated with the single, ultra-high dose aminoglycoside therapy. Histology

studies of the kidneys and the cochlea of single, ultra-high aminoglycoside dosed animals and untreated control animals were performed after 180 days (6 months). Results indicated that there were no morphological differences between the treated and untreated control animals. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of kidney tissue indicated that there was no apoptosis of endothelial cells in the tubular and glomerular regions with single, ultra- high dose of aminoglycosides consistent with an absence of ultra-high dose induced nephrotoxicity. In the septic shock model, the *E. coli* Bort was below the limit of detection from the blood of the animals within minutes after single, ultra-high dose aminoglycoside administration. After necropsy, bacterial load was determined from all the vital organs and peritoneal fluid (site of infection). The bacterial levels were below the detection limit from the kidneys and there was a significant reduction in bacterial counts from all the remaining organs compared to the infected control animals. A decrease in serum cytokine and serum lactate levels compared to baseline was observed after ultra-high doses of aminoglycosides in the septic shock animals.

Our studies have indicated that the ultra-high dose gentamicin is well tolerated by rats. It is highly effective in clearing *E. coli* Bort from the blood and reducing the bacterial burden in the organs in an experimental model of bacterial peritonitis/septic shock. Further studies in larger animals such as rabbits, sheep, pigs or dogs are required to confirm these results. If these findings are replicated in larger animals, this therapy may be developed further from 'lab to bedside' to treat septic shock patients in intensive care units (ICUs).

ACKNOWLEDGEMENTS

In the lab, I had a chance to work with motivated and cheerful colleagues like Khalil and Dr. Ehsan, who extended their warm support during my research work. I am grateful to Dr. Oliver, our experienced technician for all his help and commitment and for his fun-loving nature that has made our work easier in the lab. I thank all my friends Kallesh, Sai Pydi, Manoj, Anurag and all other friends for their support during my studies at University of Manitoba.

I thank my parents and my family, my wife Radha and my children, Dharaneesh and Aditya for their unconditional love, patience and endurance during my study. I thank one and all who have directly or indirectly helped me during my research work

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9.3. LIST OF ABBREVIATIONS

ABR study	Auditory Brain Stem Response study
AG	Arabinogalactan
AIF	Apoptosis induction factor
AKI	Acute Kidney Injury
ANOVA	Analysis Of Variance
APC	Activated Protein C
ARDS	Acute Respiratory Distress Syndrome
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under the Concentration Curve
Brpm	Breaths per minute
CaMHB	Cation adjusted Mueller-Hinton Broth
CaSR	Calcium Sensing Receptor
CFU	Colony Forming Units
CLP	Cecal ligation and puncture
CLSI	Clinical and Laboratory Standards Institute

C_{max}	Concentration maximum
CNF-1	Cytotoxic Necrotizing Factor-1
CTLs	Cytolytic T-cells
DIC	Disseminated Intravascular Coagulation
EDP	End Diastolic Pressure
EDP	Energy-dependent Process
ELISA	Enzyme-linked Immunosorbent Assay
EPCR	Endothelial Protein C Receptor
ER	Endoplasmic Reticulum
ESBLS	Extended spectrum beta-lactamase
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GEE	Generalized estimating equations
HA1A	Human monoclonal antibody-1
H & E staining	Haematoxylin and Eosin staining
HIV	Human Immunodeficiency Virus
Hly	Hemolysin
HMGB1	High Mobility Group Protein B1
HPA	Heart Performance Analyzer

ICU	Intensive Care Unit
IL	Interleukin
IV	Intravenous
LD	Loading dose
LPS	Lipopolysaccharide
LAM	Lipoarabinomannon
MA	Mycolic acid
MHA	Mueller-Hinton Agar
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphococcus aureus*
MyD88	Myeloid Differentiation Factor 88
NDM-1	New Delhi metal-beta-lactamase-1
NFkβ	Nuclear Factor Kappa beta
NGAL	Neutrophil Gelatinase Associated Lipocalin
NLRs	Nucleotide-Oligomerization Domain Leucine Rich Repeat Proteins
NMDA receptor	N-Methyl - D-Aspartate receptor

NO	Nitric Oxide
OD	Optical Density
ODN-CpG	Oligodeoxynucleotides (ODN) containing CpG motifs
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor - 1
PAMPs	Pathogen Associated Molecular Patterns
pDCs	Plasmacytoid Dendritic Cells
PIM	Phosphatidylinositol Mannose
pK	Pharmacokinetics
PRRs	Pattern Recognition Receptors
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SC route	Sub Cutaneous route
SD rats	Sprague Dawley rats
SIRS	Systemic Inflammatory Response Syndrome
$T_{1/2}$	Half Life
TGF- β	Transforming Growth factor – β

TF   Tissue Factor

TPI-1   Tissue Factor Pathway Inhibitor – 1

TLR   Toll-like Receptor

TIR domain   Toll-Interleukin-1Receptor domain

TDM   Trehalose Dimycolate

TSA   Trypticase Soy Agar

TSST-1   Toxic Shock Syndrome Toxin -1

TUNEL   Terminal Deoxy-Nucleotidyl transferase dUTP Nick End Labeling

UPR   Unfolded Protein Response

USA   United States of America

UTC   Untreated Control

Vd   Volume of distribution

CHAPTER 1

1. Introduction

Bacterial infections resulting in septic states are a major cause of morbidity and mortality in both the community and nosocomial settings, particularly among the elderly and those with chronic illnesses. Severe sepsis and septic shock in particular, are life-threatening conditions and are associated with high mortality [1, 2]. Although these conditions can affect anyone, debilitated persons are especially at high risk. For that reason, sepsis is one of the most common causes of death in hospitalized patients and is the leading cause of death in non-coronary intensive care units (ICU) [3]. Health Canada has reported that 10.9% of all deaths that occurred in hospitals throughout Canada (excluding Quebec) during 2008 – 2009 were related to sepsis [4].

Sepsis describes the systemic response to severe infection. About 45% of hospitalized patients with sepsis progress to severe sepsis and septic shock with an eventual requirement of organ support therapies [5]. For severe sepsis, the length of stay (LOS) is twice that of sepsis. Patients admitted to the ICU with sepsis have a median ICU and hospital LOS of 7 and 16 days respectively, compared to 3 days in non-sepsis cases [4]. Angus et al reported an average LOS of 19 days in the severe sepsis patients [6]. Martin et al conducted a prospective observational study involving 1238 confirmed cases of severe sepsis in 12 different ICUs from different provinces of Canada and reported median ICU length of stay of 10.3 days [7]. Health Canada, during 2008 – 2009, reported a median length of stay for each patient suffering from sepsis as 6.4 days in the ICU and 29.1 days on the ward [4]. All these factors have a major effect on the cost of patient care and overall expenses in hospitals.

1.1. Sepsis and impact on society

1.1.1. Epidemiology

The incidence of sepsis throughout the world has increased rapidly in the past three decades [6, 8-10]. The incidence of sepsis in Canada is estimated to be between 27,000 and 30,000 cases per year with an overall mortality rate of 30.5% (45.2% in patients with severe sepsis and 20.9% in those with sepsis which does not progress to severe sepsis) [4]. The economic burden of treating sepsis patients in ICUs and onwards using activated protein-c was approximately $20,528 and $12,422 respectively (a total cost of $32,950 for each patient) [11].

The treatment cost depends on the number of organ failures and supportive care required in that condition and is proportional to the age of the patient and frequency of other co-morbidities [6]. The total cost of treating septic and septic shock patients is $ 16.7 billion dollars per year in the United States of America (USA) alone [12].

1.1.2. Definitions

1.1.2.1. Sepsis

Sepsis is the body's response to antigens and toxins released by pathogenic microorganisms (typically bacteria or fungi) at the site of infection or in the blood stream. Systemic inflammatory response syndrome (SIRS) [13] is the body's systemic response to a variety of insults and is characterized by at least 2 of the following criteria;

- increased heart rate/ tachycardia (> 90 beats/minute),

- increased breath rate/ tachypnea (> 20 breaths/minute),

- fever > 38^0C or hypothermia < 36^0C

- elevated leukocyte count > 12,000/mm^3 or leukopenia (< 4,000 / mm^3) or generation of immature forms of leukocytes

Sepsis can be defined as SIRS in association with suspected or confirmed infection [13].

1.1.2.2. Severe sepsis: Defined as sepsis along with acute organ dysfunction, hypotension and perfusion abnormality.

1.1.2.3. Septic shock: Defined as severe sepsis with hypotension, even after adequate fluid resuscitation. Clinical manifestations of sepsis are presented in **table 1.** The conceptual relationship between sepsis, severe sepsis, and septic shock are illustrated in **figure 1**.

1.2. Factors influencing the incidence of sepsis

Sepsis is a serious systemic syndrome that can progress to septic shock (which is immediately life-threatening) if the underlying infection is not diagnosed and treated in a timely manner. The main reason for the increase in number of septic patients is an aging population with a high frequency of co-morbid secondary illnesses (e.g. diabetes, hepatic failure, renal failure) and frequent use of immunosuppressive chemotherapeutic drugs (cancer, transplant patients) [14], as well as increased use of invasive procedures (e.g. hemodialysis) [15] and the increase of antibacterial resistance in pathogenic bacteria [16].

A large fraction of patients with septic shock are elderly [17, 18]. Most of these individuals also have severe co-morbidities. Schroder et al has previously reported men to be more prone to sepsis compared to women [18, 19]. The number of patients admitted to hospitals with septic shock is higher during winter as compared to other seasons [20]. This is

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probably due to increased viral pulmonary infections during winters that lead to super-infections with the pathogens of bacterial pneumonia.

1.3. Etiology of sepsis

Sepsis occurs in response to severe infections caused by different pathogenic organisms. Bacteria including Gram positive and Gram negative are responsible for approximately 90% of cases of sepsis and septic shock in North American ICUs [21, 22]. The dominant organisms associated with sepsis evolve. In the early era of antibiotics, Gram positive bacteria were the most frequent organisms that caused sepsis. By the 1960's, Gram negative bacteria became dominant in both community and hospital settings [23]. More recently, Gram positives are again increasing in frequency. *Staphylococcus aureus*, *Streptococcus pneumoniae, Klebsiella* spp., *Escherichia coli*, and *Pseudomonas aeruginosa* are among the most frequently isolated organisms in sepsis [2, 22, 24].

Among bacteria, *E. coli*, *Listeria* spp, and group B streptococci particularly cause infections and sepsis in neonates whereas *S. aureus*, pneumococci and meningococci are common agents in children and young adults [2, 9, 22, 25-27]. In elderly patients, septic shock is often caused by Gram negative bacterial isolates from the urinary tract, gastrointestinal tract or the respiratory tract. While *M. tuberculosis* does rarely cause septic shock [28], direct shock from viral infections is uncommon in North America. When it does occur (dengue and hemorrhagic fever viruses), the pathophysiology is quite distinct and toxin driven vasodilation does not generally occur [29, 30]. Respiratory tract infections are responsible for the highest proportion of sepsis cases [2, 9]. One reason is the occurrence of secondary bacterial respiratory infections following influenza virus pneumonia [31]. An

increase in the number of sepsis cases due to fungal infections, particularly *Candida* species, has been reported in recent years, particularly in the USA [9, 32].

The outcome of patients with sepsis and septic shock is highly dependent on the type of organism causing infection and whether or not the organism is resistant to the antimicrobial used for empirical therapy [33]. Many studies have shown that timely, appropriate antibiotic use can save the life of the patient with severe sepsis or septic shock due to bacterial infection. Kumar et al have reported that initiation of microbially inappropriate initial empiric antimicrobials (i.e. absent activity for the likely pathogens) results in fivefold reduction of survival in septic shock patients [22].

1.4. Pathogenesis of sepsis, severe sepsis, and septic shock

The current accepted immunologic paradigm of sepsis as defined by Bone et al. emphasizes that sepsis is characterized by SIRS [34]. Modern thought has held that the process of sepsis begins with toxins associated with microorganisms leading to release of endogenous mediators which drive the pathophysiology of the illness [35].

Sepsis and septic shock usually begin with a nidus of infection at a specific site [36] such as urinary tract, lungs, peritoneum, wound, post-operative surgical wounds etc. The pathogen causing infection replicates at these sites. Once the organism load increases sufficiently, they disseminate or release antigens systemically. This triggers SIRS in part to help eliminate the microbial pathogen [37, 38]. Many virulence mechanisms of bacteria are responsible for the propagation of infection. Some of these mechanisms are described below.

1.4.1. Bacterial toxins

Severe bacterial infections can lead to the release of antigens and toxins in the blood and tissues of the individual. These toxins are classified as exotoxins (antigens released by living bacteria into their surrounding environment) and structural toxins like endotoxins released after the death/lysis of the bacteria. Bacterial nucleic acids (CpG oligonucleotides) have also significant antigenic properties in mammals and can reproduce almost all of the same responses as classic bacterial toxins [39, 40].

1.4.1.1. Exotoxins

Exotoxins are low molecular weight proteins. There are three major types of exotoxins associated with sepsis that are usually released by Gram positive and Gram negative bacteria along with various enzymes that cause tissue injury [41]. The three exotoxins are Type I, type II and type III toxins. Superantigens belong to the type I toxins and are regarded as the most important of the three exotoxins.

1.4.1.1.1. Superantigens

Notable Gram positive exotoxins associated with sepsis and septic shock are superantigens associated with *S. aureus* (Toxic Shock syndrome Toxin-1(TSST-1)) [42] and streptococcal species (pyrogenic toxin)[43]. Superantigens are a type of Type-1 toxin that cause injury to the host without entering the cellular cytoplasm. These toxins bind to their receptors on the surface of the cells and induce intracellular signals. Exotoxin can trigger aggressive cellular immune response, leading to toxic shock [44]. Superantigen mediated toxic shock syndrome is usually produced by either *Staphylococcus aureus* or *Streptococcus*

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pyogenes. These antigens are processed by leukocytes, bind to the outer region of the major histocompatibility complex II (MHC II) and are presented to T-cells resulting in a robust immune response [45, 46]. In normal infections, antigens trigger 0.01% of T-cells, but superantigens can trigger >20% of T-cells in the body. Recent reports suggest that superantigens also bind to cluster of differentiation 28 (CD28 receptors), co-stimulatory molecules on T-cells [47]. Binding of superantigens to both MHC II and T-cell receptors induces a massive production of Th1 pro-inflammatory response. This leads to the expression of tumor necrosis factor-α (TNF-α), interferon-γ (IFN- γ) and interleukin-2 (IL-2) in high concentrations, leading to extensive tissue damage and shock [48]. Various other exotoxins (such as enterotoxins and cytolysins) produced by bacteria can result in other pathogenic phenomenon as noted in **table 2**.

1.4.1.2. Structural toxins

Structural toxins such as endotoxin/ lipopolysaccharide (LPS) from the cell wall of Gram negative bacteria, teichoic acid of Gram positive bacteria, peptidoglycan, nucleic acid etc. are released after the death of bacteria. Gram positive and Gram negative bacterial pathogen associated molecular patterns (PAMPs) (peptidoglycan, LPS, CpG-ODN etc.) are recognized by pattern recognition receptors (PRRs) and trigger the transcription of inflammatory cytokines [49] **(figure 2)**.

1.4.1.2.1. Endotoxin

Endotoxin (also referred to as LPS) is the most studied and best understood of these structural toxins. LPS has 3 parts; the outer hydrophilic O-side chain, a center core

oligosaccharide and the inner hydrophobic lipid A which is the toxicity mediator [50]. Pico-molar concentration of LPS can trigger pro-inflammatory responses, expression of tissue factor and release of TNF-α and IL-1β by macrophages [51]. LPS also causes endotoxic shock with pro-inflammatory cytokine expression in experimental animals [52]. Notably, lipid A alone reproduces all the toxic manifestations of LPS *in vitro* and *in vivo* [53-55]. LPS is recognized by Toll-like receptor - 4 (TLR- 4) on immune cells [56].

In circulation, LPS is solubilized by LPS-binding proteins in serum and are bound to cluster of differentiation 14 (CD14) receptors on macrophages [57]. CD14 is also present in a soluble form and can independently bind LPS. On macrophages, CD14 transfers LPS to MD2 (a novel molecule) and this in turn binds to TLR4 forming a TLR4-MD2 receptor complex [58]. This complex dimerizes and interacts with toll-interleukin-1 receptor domain (TIR domains). Activation of TLR4 by LPS triggers myeloid differentiation factor 88 (MyD88) that in turn activates nuclear factor kappa beta (NFκβ) migration in the cytoplasm of the cells. NFκβ moves to the nucleus and activates the genes responsible for the release of pro-inflammatory cytokines [59]. Hoshino et al reported that TLR4 knockout mice are hypo-responsive to LPS challenge indicating the role of TLR4 in recognizing LPS from Gram negative bacteria [60].

1.4.1.2.2. Other structural toxins

Apart from LPS, teichoic acid, lipoteichoic acid, peptidoglycans, flagellum, bacterial CpG oligo-deoxynucleotides of Gram positive and Gram negative bacteria [61-65], trehalose dimycolate (TDM), mycolic acid (MA), lipoarabinomannon (LAM), arabinogalactan (AG), phosphatidylinositol mannose (PIM) of *Mycobacterium* spp. [66, 67] and β-glucans, chitin

and mannoproteins from the fungal cell wall are PAMPs recognized by TLR2, TLR4 and CD14 receptors expressed on the surface of the immune cells [49, 68]. These PAMPs are recognized by corresponding TLRs and cause downstream signaling through MyD88 and activation of NFκβ. Consequently, inflammatory cytokines are produced by immune cells. TLRs and their ligands/ PAMPs from different pathogenic organisms are illustrated in **figure 3** and listed in the **table 3** [69]. This convergence in the signaling mechanism underlies the similarity in the clinical sepsis presentation with severe infection due to different pathogens.

1.4.1.2.2.1. cterial nucleic acids

Bacterial DNA itself can reproduce septic shock in experimental animals by triggering immune cells to produce pro-inflammatory mediators [64, 65]. The nucleic acid of bacteria contains unmethylated CpG motifs that are not found in mammalian DNA. The oligo-deoxynucleotides containing CpG motifs (ODN-CpG) are capable of activation of host defense mechanisms leading to innate and acquired immune responses [70, 71]. The ODN-CpG are short single-stranded DNA molecules that contain a cytosine triphosphate deoxynucleotide (C) linked by a phosphodiester bond (P) with a guanine triphosphate deoxynucleotide (G). The ODN-CpG motifs are reported to be recognized by the endosomally expressed pathogen recognition receptor (PRR), TLR9 [72]. Once the TLR9 is triggered, downstream signaling and the production of pro-inflammatory cytokines, interferons and chemokines is initiated.

1.5. Responses of the host

A number of factors are involved in the pathogenesis of sepsis. Untreated or inadequately treated severe sepsis leads to septic shock. During the early stages of severe sepsis (i.e. sepsis with organ failure), the patient may experience confusion or decreased level of consciousness due to encephalopathy, respiratory failure and oliguria [73]. Coagulation abnormalities and liver dysfunction are observed in the later stages of severe sepsis [74]. Multi-organ dysfunction is driven in part by cardiovascular collapse and by excessive inflammatory stimulation. Organ dysfunction includes renal failure, acute respiratory distress syndrome (ARDS), hepatic failure, and disseminated intravascular coagulation (DIC) each of which can contribute to the death of the patient [75, 76]. With further progression, overt septic shock with cardiovascular collapse characterized by severe hypotension and metabolic acidosis may manifest. In septic shock, the severity and number of underlying co-morbidities and the total number of organs failed has high impact on morbidity and mortality of the patient [77].

Sepsis and septic shock manifest as a consequence of uncontrolled infection and an ultimately maladaptive response to the infection by the body's immune system. In normal infections, the body's pro-inflammatory and anti-inflammatory responses work together to support the clearance of invasive pathogens while limiting excessive tissue damage. The syndrome of sepsis in an individual represents a complex dynamic interaction between the host immune system and microbial virulence factors which can trigger maladaptive pro-inflammatory and anti-inflammatory responses in combination with a network of signaling. This may result in induction of apoptosis, activation of coagulation and stimulation of neuro-endocrine pathways [78, 79]. Inflammatory responses during sepsis are presented in **figure 4**.

The major inflammatory mediators in the pathogenesis of sepsis and septic shock are presented in **table 4**. IL-1β and TNF-α cytokines are both considered particularly important. IL-1β and TNF-α are found to induce shock when administered in experimental animals [80, 81]. These cytokines induce the production of adhesion receptors on endothelial cells, thereby facilitating the adhesion and migration of activated macrophages, neutrophils and platelets [82, 83]. These activated cells release prostaglandins and oxidants causing injury to the endothelial cells, resulting in vasodilation and plasma extravasations. Sepsis also causes increased production of inducible nitric oxide synthase (iNOS) in leukocytes, vascular endothelial and smooth muscles [84]. In leukocytes, phagocytic killing of pathogens is augmented. In the vasculature, iNOS-mediated release of nitric oxide (NO) in excessive amounts causing vasodilation [85]. Further, NO-mediated alterations in the intracellular endothelial junction integrity lead to organ injury and generalized edema which may contribute to organ failure.

1.5.1. Immune responses to infection

The innate immune system is the first line of defense against invading pathogens, but also has a central role in the genesis of the septic response. The innate immune system comprises immune elements that are normally present and available to neutralize pathogens. Elements include intact mucosal/epithelial barriers, phagocytes including neutrophils, macrophages, dendritic cells, mast cells and eosinophils, natural killer cells and certain plasma proteins including opsonins, bacteriolysin, hemolysin and the complement system [86]. The adaptive immune system involves elements that are normally inactive but become active under appropriate stimulation when the innate immune response is inadequate. There

are two aspects of the adaptive immune system: humoral immunity involving antibody production by B lymphocytes and cell-mediated immunity involving T lymphocytes. Cell-mediated immunity involves various lymphocyte subgroups, including, in particular, cytotoxic and helper T-cells among other subgroups. T lymphocytes have a central role in the pathophysiology of sepsis. Given space limitations, the discussion that follows will focus aspects of the immune system that bear particular relevance to sepsis.

1.5.1.1. Innate immune system

Many immune cells, including macrophages and dendritic cells have specific receptors called the PRRs on their surfaces with which they recognize the invading pathogens [87]. PRRs are germ-line encoded, and each receptor has broad specificities for microorganisms. As mentioned earlier, PAMPs are often components of the microbial cell wall, such as lipopolysaccharide, peptidoglycan, lipoteichoic acids, β-glucan and lipoproteins. Bacterial nucleic acids can also be recognized by these receptors [49, 88-90].

Detection of these structures by PRRs of the innate immune system can signal the presence of microorganisms. There are three important indicators of PAMP's key role in microbial recognition by PRRs; first, they are invariant among different genera of microbes. Second, they are products that are unique to microorganisms, allowing discrimination between self and non-self molecules. Third, they have essential roles in microbial physiology.

As mentioned earlier, toll-like receptors (TLRs) and CD14 are an important type of PRR and play a major role in defense against bacteria [49]. These receptors are present on the surface of various cells that play a vital role in the innate immune system. There are different

types of TLRs and each of them has a specific function. Of the TLRs, TLR 2 and TLR 4 are of major importance as they bind to different bacterial surface structures such as LPS, peptidoglycan, lipopeptides and lipotechoic acid etc. [68, 91]. TLRs also bind to bacterial nucleic acid and bacterial DNA itself can induce a severe immune response in the host [64, 65]. The nucleotide-binding oligomerization domain leucine-rich repeat proteins (NLRs) are a type of PRR found in cytoplasm. [90, 92].

Attachment of the PRRs to their specific ligands triggers the downstream signaling mechanisms through intracellular proteins that in turn trigger the production of pro-inflammatory endogenous mediators such as interleukin-1 (IL-1), IL-2, IL-6, IL-8, tumor necrosis factor-α (TNF-α), platelet activating factor (PAF), eicosanoids, nitric oxide (NO), oxygen free radicals, high mobility group protein B1 (HMGB1) and others [92, 93]. These molecules are known to have profound effects on the cardiovascular system, kidneys, lungs, liver, central nervous system, and the coagulation cascade and play a central role in the pathophysiology of sepsis and septic shock.

1.5.1.2. Complement system

The complement system is one of the arms of the innate immune system. It plays a vital role in the elimination of bacteria, fungi, parasites and other pathogens [94]. Many infection-causing organisms that enter the body are first attacked by the complement system. Complement factors are released as a part of the inflammatory process in response to infection. In sepsis, elevated levels of anaphylatoxins, C3a and C5a are detected in the blood [95-97].

There are three different pathways through which the complement is activated; classical, lectin and the alternate pathway. All these pathways converge at the central step of complement activation. When any of the three pathways interact with the surface of microbial pathogens in the body, an enzyme called C3 convertase is generated. This leads to cleavage of C3 covalently bound to the microbial surface into C3a and C3b. C3b is bound to the microbial surface, but C3a is released and is responsible for the inflammatory responses. C3b is an opsonin and is helpful in clearance of the microbial pathogen by the phagocytes. C3b also combines with the C3 convertase forming a multi-subunit enzyme, a C5 convertase. The C5 convertase cleaves C5 to form C5a and C5b. C5a is highly active peptide with inflammatory properties and act as anaphylatoxin. C5b reacts with other components of complement pathway and forms a membrane attack complex on the surface of the microbe which creates pores on the microbial cell surface thereby causing bacterial cell lysis [97].

C3a and C5a that are generated after activation of the complement pathways induce chemotaxis in other immune cells as well as priming of the cell's responses to a second stimulus [97-99]. The primary effect of activation of complement pathways is lysis of the microbial pathogen causing an infection. But during sepsis, C5a is produced in excessive amounts that trigger a series of events leading to septic shock, respiratory distress syndrome, and multiorgan failure. The excessively produced C5a is taken up by C5a receptors of neutrophils, macrophages/ monocytes. Loss of responsiveness in these cells to both C5a and other stimuli is observed [100]. Solomkin et al reported that neutrophils from blood of sepsis patients lose their ability to respond chemotactically *in vitro* to C5a during the early phases of sepsis and to other chemotactic peptides as sepsis progresses [101]. Neutrophils exhibit substantial loss of H_2O_2 production that is necessary for effective bactericidal activity. The

loss of responsiveness of neutrophils after contact with C5a is known as 'desensitization' or 'deactivation'[102].

An *in vitro* study on C5a receptors expressed by human PMNs suggests that these receptors are essential for modulation of the inflammatory response [103, 104]. C5a complement plays an important role in enhancing septic shock; anti-C5a receptor blockers may be a potential approach to manage the inflammatory response of septic shock. *In vivo* studies on the use of anti-C5a antibody were promising in sepsis models with i) attenuation of septic shock and pulmonary edema; ii) decreased IL6 activity in response to anti-C5a mAb; iii) attenuated coagulopathy, reduction in lymphoid cell apoptosis and improved survival rate with application/administration of neutralizing antibodies [104-107]. Further large multicenter clinical trials are required to ascertain the findings and develop appropriate treatment strategy.

1.5.1.3. Coagulation

In normal individuals, the coagulation cascade is highly controlled. In inflammatory conditions such as sepsis, both pro-coagulant and anti-coagulant mechanisms are activated. Disseminated intravascular coagulation (DIC) is observed in about 25 – 50% septic shock patients [108]. Normally, three different anti-coagulant proteins play a major role in controlling the coagulation process. They are anti-thrombin, tissue factor pathway inhibitor-1 (TFPI-1) and activated protein C (APC). Anti-thrombin inhibits formation of thrombin. TFPI-1 is the main inhibitor of TF-factor VIIIa complex. In vascular endothelial cells, APC is formed from protein C due to binding of thrombin to thrombomodulin. APC formation in the vascular endothelium is amplified by the presence of the endothelial protein C receptor (EPCR) [108]. The main function of APC is to inactivate factor Va and VIIIa of the

coagulation cascade as well as decrease the synthesis of plasminogen activator inhibitor-1 (PAI-1) and prevent fibrin clot formation.

In sepsis, deposition of characteristic fibrin clots occurs through three main mechanisms; tissue factor (TF) mediated thrombin generation, dysfunctional physiological anticoagulant mechanisms, and impaired fibrin removal due to depression of the fibrinolytic system. TF is exposed on the surface of the endothelial cells as well as the circulating monocytes when stimulated by bacteria, LPS, and TNF-α. Exposed TF then binds to different coagulation factors (Va and VIIIa) and activates the conversion of prothrombin to thrombin [109]. This further converts fibrinogen to fibrin. Fibrin binds to platelets and forms fibrin clots on the endothelial surface, causing microvascular obstruction leading to tissue hypoxia (e.g. digital ischemia) (**figure 5**).

Many preclinical studies and multiple sepsis models have supported the anti-coagulant potency and anti-inflammatory properties of activated protein C [110]. The PROWESS phase III clinical trial reported that, recombinant human APC reduced 28 day severe sepsis mortality, but in subsequent PROWESS – SHOCK study, APC did not exhibit any benefit in septic shock patients [110, 111]. Subsequently, APC was withdrawn from the market. Despite this, the use of APC in treating septic shock patients is under debate and further investigation into new anticoagulant molecules is needed.

1.5.1.4. Adaptive immune system

Adaptive immunity has two components; humoral and cell-mediated. The primary effector of humoral and cell-mediated immunity are respectively, B-cells and T-cells. B-cells

are responsible for the production of antibodies against specific antigens. T-cells are involved in a broad range of cell-mediated responses including cytokine expression.

Cell-mediated responses involving T-cells are central to the pathogenesis of sepsis. Two key types of T-cells are present in the body; CD4 T-cells (Th1 cells, Th2 cells, and Th17 cells) and CD8 T-cells [112, 113]. During infection, macrophage-line cells in the affected tissues (primarily dendritic cells) capture pathogen antigens and migrate to lymph nodes. These antigen presenting cells deliver the antigen in conjunction with MHC class II complex to naïve T-cells. CD4 T-helper cells (Th cells) recognize antigens presented and are stimulated to differentiate to Th1, Th2, Th17 and Regulatory T-cell (Treg) phenotypes.

Th1 cell activation leads to cell-mediated immunity and generation of pro-inflammatory cytokines like IFN-γ, TNF-α, IL-2, IL-6, and IL-1β. These cytokines enter the systemic circulation causing activation of neutrophils and macrophages and other immune cells. This further drives systemic inflammatory responses (i.e. SIRS) in the body. Th2 cells produce IL-4, IL-10, and IL-13, and also support antibody-mediated immune responses. IL-17, IL-21, IL-22, IL-26 are produced by Th17 cells. These cytokines contribute to a neutrophil recruitment and activation and thereby target the extracellular bacteria. Along with interleukins, Th17 response results in the secretion of antimicrobial peptides that contribute to mucosal host defense [114]. CD8+ T-cells produce IFN-γ and TNF-α that destroy target cells by cytolysis and are the major effector cells against intracellular bacteria. These CD8+ T-cells are activated by the antigens expressed on the infected cells and differentiate into cytolytic T-cells (CTLs) [115]. These cells secrete proteins that form pores on the infected cell membranes, leading to apoptosis of the infected cells.

In normal individuals, anti-inflammatory cytokines that impair the pro-inflammatory responses are produced to avoid collateral damage [116]. Usually, anti-inflammatory cytokine levels go down after normalization of pro-inflammatory cytokines. In sepsis, however, anti-inflammatory cytokine levels remain elevated. Immune cells become exhausted because of high levels of anti-inflammatory cytokines and the continuous presence of antigen. This leads to apoptosis of immune cells, such as CD4 T-cells, CD8 T-cells, B-cells, macrophages and neutrophils [112]. An increase in regulatory T-cells (Treg) cells along with altered macrophage activation and reduced production of pro-inflammatory cytokines are common [117, 118]. A shift from a Th1 cell response to a Th2 cell response is also observed. Surviving CD4 and CD8 T-cells either shift from the pro-inflammatory Th1 cell type to an anti-inflammatory Th2 cell phenotype or form an exhausted phenotype with reduced cytokine expression and increased expression of PD1 receptors [118-120]. Immunological irresponsiveness or 'anergy' is observed [118, 121, 122].

As a result, loss of antibody production, decreased antigen presentation and decreased macrophage activation are observed [122]. Because of 'anergy' the primary antigen is not cleared completely and secondary infections occur [123, 124]. Many patients with septic shock die of these late secondary infections [125]. The progress of immune dysfunction in sepsis is shown in **figure 6**.

1.5.2. Cardiovascular dysfunction:

Kumar and Parrillo defined shock as a 'state in which profound and widespread reduction of effective tissue perfusion leads first to reversible, and then if prolonged, to irreversible cellular injury' [126]. Septic shock patients are characterized by substantial

hemodynamic abnormalities typically in combination with multiple organ dysfunction. Hemodynamic alterations include hypovolemia due to vasodilation and interstitial edema, myocardial dysfunction and a decreased vascular tone with marked hypotension [109, 127]. Clinical studies have suggested that microcirculatory alterations are frequent in sepsis and septic shock patients [120, 128].

Microcirculatory dysfunction and microvascular shunting down are mainly due to endothelial dysfunction with increased NO production due to the generation of an inducible NO synthase (iNOs) in endothelial and vascular smooth muscle cells [129]. Microvascular clotting may also occur. Due to microcirculatory dysfunction, delivery of oxygen to some tissues can be impaired even while other areas receive unnecessary perfusion [130]. The presence of areas of tissue hypoxia, which are accompanied by areas of excessive and inappropriate perfusion, results in increased lactate generation (lactic acidosis), in spite of increased global systemic oxygen delivery with increased venous oxygen saturation. This clinical condition is defined as 'cytopathic tissue hypoxia'. This is an oxygen delivery independent state which is associated with a high level of organ failure and mortality in patients [131].

1.6. Conceptualizing sepsis and septic shock: Implications for antimicrobial therapy

In septic shock patients, the cardiovascular system collapses, causing a life-threatening drop in blood pressure. The patients become vasopressor dependant despite adequate volume resuscitation. Even after the administration of vasopressors and restoration of normal macro-hemodynamic parameters, tissue perfusion is not normal. Treatment options are limited. If

the condition persists, death is inevitable and the mortality rate is high in these patients (>

50%) [132]. Treating such patients in a timely manner is challenging for the intensive care

physicians.

1.6.1. Immunologic model

The currently accepted immunologic model of the sepsis pathogenesis suggests that

this disorder is present when SIRS caused by infection exists [13, 133]. This paradigm holds

that the disorder is caused by an infection, which initiates an immunologic (inflammatory

cytokine and eicosanoid) / coagulation cascade that propagates independently of the

underlying infectious trigger [116]. This view of sepsis is shown in **figure 7** [13]. The

assumption from this model is that the causative pathogen is rapidly eliminated by

antimicrobials, so that it is no longer central to the progression of sepsis. Figure 7 indicates

that the progression of the inflammatory phase of sepsis (and a counter-inflammatory,

"immunoparalytic" phase of illness) will occur as a consequence of cellular signaling despite

rapid pathogen elimination [116, 118]. In this model, septic states from sepsis to severe

sepsis to septic shock are related disorders of increasing severity. Septic shock, in particular,

is considered an epiphenomenon to the underlying cellular injury induced by these mediators

rather than a discrete clinical entity with a distinct pathogenesis and pathophysiology.

Overwhelming meningococcemia with septic shock, a condition in which an exquisitely

antimicrobial-sensitive organism can be quickly eliminated, but where massive tissue damage

may still occur is the archetypal infectious syndrome that best fits this paradigm [134].

1.6.2. Microbiologic paradigm

A key deficiency of this immunologic model of sepsis is that most pathogens cannot be eliminated quickly despite current optimal therapy [135-139]. Autopsy studies of septic deaths show persistent infectious foci in as many as 90% of cases [137]. An older model of septic shock emphasizes the role of micro-organisms and active infection in the pathogenesis of septic shock. In this model, the septic process begins with a nidus of infection (peritonitis, pneumonia, etc.)[138]. Within that focus, the organism replicates and, if untreated, the microbial infectious load increases over time [139]. Pathogens release a variety of exo- and endotoxins (toxic burden) that stimulate the production of endogenous mediators including inflammatory cytokines and eicosanoids (inflammatory response). The result is cellular dysfunction, which can be manifested as tissue injury and, ultimately organ dysfunction, including septic shock (cellular dysfunction/tissue injury) **(figure 8)**. The core element of this view of sepsis is that the microbial infectious load drives downstream responses that result in septic shock. This paradigm suggests that elimination of the underlying infection should terminate the downstream inflammatory/coagulant basis for tissue injury and organ dysfunction.

1.6.3. Integrating shock

The microbiologic paradigm of septic shock has its own conceptual deficiency. It overlooks the concept of 'irreversible shock' as developed by Carl J. Wiggers [140]. This concept holds that shock of any etiology, once established, becomes irreversible with an inevitable progression to death if the shock is not reversed in a limited period of time. Clinical and experimental studies have suggested that it is not sufficient to reverse the clinical

manifestations of shock (i.e. reverse hypotension with fluids and vasopressor agents, treat acidosis with bicarbonate infusion, support respiratory failure with mechanical ventilation) for survival. Instead, the underlying source of shock must be addressed. For example, in the context of hemorrhagic shock, bleeding must be stopped as quickly as possible [141]. In the case of a massive myocardial infarction causing cardiogenic shock, the closest correlate of survival is not the initiation of vasopressor support or provision of mechanical assist devices, but rather the speed with which revascularization is implemented [142].

Septic shock can be conceptualized using a similar framework. In this view, the presence of shock drives organ failure and the underlying source of shock is the total infection-associated microbial load [138, 143, 144]. The speed that the pathogenic microbes are eliminated (or at least suppressed to a level below the threshold that causes shock) becomes the primary determinant of survival [138, 145]. Using this paradigm, an alternate model of septic shock that incorporates elements of the current immunologic model and previous microbiologic model with Wigger's irreversible shock concept can be created (**figure 9**). This model is similar to the microbiologic model with two major revisions. First, it suggests that sufficient endogenous inflammatory stimulation through a greater microbial burden and a higher subsequent toxin load will lead patients past a threshold that results in septic shock (above the hatched line in figure 9). Second, sustained shock thereafter will at some point lead to irreversible organ injury and inevitable death [138]. This threshold may be highly variable between individuals based on their age, cardiovascular reserve, and genetic factors. However, all septic patients proceed on a similar trajectory.

This model suggests that the speed that the infectious load is reduced or eliminated, (so that the duration of time for the remaining live pathogens to cause shock is minimized)

irrespective of whether vasopressors are able to maintain blood pressure, is the key determinant of survival. Based on the model, this approach should limit the risk that the indeterminate pathophysiologic point at which recovery is no longer possible (if septic shock persists) is passed [138].

Note that this principle will apply to any infectious condition where there is a time-dependent risk of irreversible and irreplaceable organ failure. In the case of septic shock, persistence of shock causes failure of multiple organs simultaneously. While a single or even two organ failures can be addressed medically or surgically, failure of multiple organs simultaneously is not generally a survivable condition. Bacterial meningitis, where delay-dependent brain injury will be both irreversible and irreplaceable, is another condition that is similarly dependent on rapid antimicrobial therapy for survival [146].

Based on this conceptualization of septic shock, any therapy that accelerates pathogen clearance should improve outcome in septic shock. Fortunately, many studies of antimicrobial therapies in septic shock have been performed. Those that accelerate pathogen clearance have shown to be successful [138, 147, 148]. In contrast, it can be predicted that strategies (particularly non-resuscitative, immune modulator strategies) that do not involve accelerating pathogen clearance may be less useful.

1.7. General treatment options for septic shock

The universally accepted treatments for sepsis are fluid resuscitation, vasopressor use, initiation of broad-spectrum antimicrobials, and source control as recommended by the International Surviving Sepsis Campaign Guidelines Committee 2012 [149].

1.7.1. Resuscitative strategies

There is some data suggesting that basic resuscitative strategies with early aggressive fluid resuscitation may be useful. The River's study of goal-directed fluid resuscitation with transfusion and dobutamine support to maintain a central venous oxygen saturation of >70% was considered a landmark when it was published in 2001 [150]. Patients in the experimental arm (who had a central venous oxygen monitoring catheter) received about 1.5 L more fluid resuscitation in the first 6 hours after presentation to the emergency room with septic shock than did those with a similar catheter not equipped to measure central venous oxygen saturation. However, several more recent, much larger randomized trials have failed to reproduce those results [151, 152].

1.7.2. Antitoxins and immune modulation

Based on the immunological model of sepsis and septic shock, many clinical trials have been performed to test novel immunomodulatory therapies. In the early 1980's, it was thought that the neutralization of the primary Gram-negative toxin (i.e. endotoxin/ lipopolysaccharide) which is common to all such organisms may help in improving the mortality rate in sepsis and septic shock. Initial clinical trials using E5 and human monoclonal antibodies (HA1A) against lipopolysaccharides exhibited improvement in mortality in the group of patients with proven Gram negative sepsis [153, 154]. These studies were performed in a small cohort of patients, but a major study involving 530 patients by Bone et al indicated that there was no survival advantage with monoclonal antibody treatment [155]. In another large clinical trial by Mc Closkey et al [156], HA1A that binds to lipid A of endotoxin was studied in a randomized double-blinded, placebo controlled study. HA1A

antibodies were not effective in reducing the mortality in Gram negative septic shock. Other studies of severe sepsis have been performed using signal inhibitors of toll-like receptor 4 (TLR 4) present on the surface of immune cells [157]. As noted previously, TLR4 binding of LPS from Gram negative bacteria is central to inflammatory responses to Gram negative infections. Inhibition of signaling can limit pro-inflammatory cytokine release and related responses. This study also indicated that there was no difference in mortality in treated and placebo control groups in severe sepsis.

In 1990's, steroids were thought to be effective in the treatment of sepsis as they inhibit the pro-inflammatory cytokines such as TNF-α, IL-1, IL-2, IL-6 and INF-γ, chemokines, eicosanoids and bradykinins. Steroids also elevate anti-inflammatory responses and induce expression of adrenergic receptors in the vascular smooth muscle cells, which are otherwise down-regulated in septic shock patients [158-160]. Clinical trials using high doses of steroids in sepsis and septic shock have demonstrated no benefit compared to controls [161, 162]. However, harmful secondary infections of the patients with increased mortality were reported in these patients [163]. The surviving sepsis guidelines recommend using steroids in patients with vasopressor refractory shock only [149].

The only novel therapy licensed for sepsis was drotrecogin-alfa (activated). This product, a form of activated protein C, is thought to have both anticoagulant and immune-modulatory activity. Following initial regulatory approval in the last decade, further studies after the initial pivotal successful trial failed to demonstrate efficacy and the drug was removed from the market [164].

Many other experimental therapies including inhibitors of the coagulation cascade, antibodies against TNF-α, anti-endotoxin antibodies, statins, immune-stimulants and others

have been assessed for severe sepsis and/or septic shock and have shown no significant benefit in survival [165, 166]. To date, the knowledge gained from animal research and clinical trials has been useful in defining the pathophysiology of sepsis and septic shock conditions, but has not achieved the goal of developing a novel universal treatment option for severe sepsis and septic shock. For the most part, only antimicrobial therapy has consistently been shown to improve the outcome in septic shock [166-168].

1.7.3. Antimicrobial therapy of sepsis and septic shock

According to our composite model of septic shock pathogenesis, accelerated clearance of pathogens should have a significant favorable impact on survival [138]. However, the same model also suggests that such a favorable impact may be substantially less apparent or absent in sepsis without shock because of the lack of a delay-dependent risk of irreversible and irreplaceable organ injury. There are several strategies that can be used to accelerate pathogen clearance (**table 5**). The fundamental impact of all these antimicrobial optimization strategies is to maximize bactericidal activity.

1.7.3.1. Appropriate antimicrobial therapy

If the appropriate empiric antimicrobials are used, accelerated elimination of bacteria is facilitated, favorably influencing survival in septic shock patients. Once bacteria are eliminated, adverse cellular responses, such as maladaptive cytokine and coagulation cascades should eventually decline preventing the patient from entering into irreversible shock (**figure 10**).

As substantially increased mortality rates are associated with inappropriate antimicrobial therapy, it is important for the clinicians to understand the patient's clinical presentation and comorbidities as these will help define the range of probable etiologic pathogens (**figure 11**). The patient's anatomic site of infection, immune status, other risk factors, probable etiologic organism causing infection and resistance pattern of those organisms must be considered. Usually, septic shock patients have significant co-morbidities and are at increased risk of infection [77, 169]. Superior empirical therapy can be achieved by considering local and unit specific antibiograms or consulting the infectious disease specialists in the unit [170, 171]. Some studies suggest improved outcome with such an approach [172]. All these details will assist in choosing appropriate empiric antibacterial therapy.

1.7.3.2.   Early antimicrobial therapy

Early administration of empiric antimicrobial agents that cover 100% of the probable infection causing pathogens can save the life of the septic shock patient [2]. In the emergency department, early initiation of antimicrobials along with other treatments of a 'sepsis bundle' was associated with improved outcome in patients with community-acquired septic shock [173]. Many other studies have also described the importance of early antimicrobial therapy on the improvement of outcome in sepsis and septic shock [174, 175]**.**

1.7.3.2.1.   Delay in antibiotic therapy

The time delay in administering effective antibacterial therapy after the onset of hypotension is a surrogate for the increasing bacterial burden in the body and can have a

significant impact on the outcome. This statement is supported by our murine septic shock model where the time of occurrence of septic shock is dependent on the bacterial load in the animal's blood rather than the amount of bacteria used for the infection [145]. Many studies have reported the importance of time to antimicrobial therapy in relation to mortality risk in septic shock [2, 167, 176]. Accelerated clearance of the causative pathogen can be achieved by rapid administration of broad-spectrum, highly bactericidal antimicrobials. Knudsen and colleagues demonstrated in their animal studies that the timing of antibacterial therapy is very important for the survival of animals with severe pneumonia [177]. Mortality was dependent on the time of penicillin dose after inoculation of the bacteria. Kumar and colleagues have demonstrated in a murine septic shock model using an *E. coli* strain that administering antibiotics at or before 12 hours after peritoneal implantation of organism yielded <20% mortality in animals [145]. When the antibiotic treatment was administered at or after 15 hours of infection, mortality increased to 85-100%.

Kumar and colleagues also retrospectively analyzed the data from 2731 septic shock patients and found that the delay to initial appropriate antimicrobial as the single strongest predictor of survival [167]. They demonstrated that the initiation of appropriate/effective antimicrobial therapy within the first hour after onset of hypotension due to septic shock was associated with 79.9% survival. With every hour of delay to appropriate/effective antimicrobial initiation within the first 6 hours after onset of hypotension, there was a 7.6% drop in survival (**figure 12**).

Many retrospective studies of sepsis, septic shock, surgical infections, infections after organ transplant, community-acquired and hospital-acquired pneumonia have reported delayed antimicrobial therapy as an important factor for mortality of these patients [178, 179].

Therefore, most studies have suggested that utilizing appropriate, but delayed initial antimicrobial therapy for life-threatening infections leads to undesirable outcomes.

1.7.3.3. Loading doses of antimicrobials

The determination of the loading dose (LD) of antimicrobials in septic shock patients is very important. The loading dose is determined by the volume of distribution (Vd) and required maximum/ peak concentration (C_{max}) using the formula below;

$$LD \ (mg/kg) = Vd \ (liter/kg) \ x \ C_{max} \ (mg/L)$$

Vd and C_{max} are known to be altered in critically ill/ septic shock patients [180]. Changes such as increased permeability of microvascular endothelium leading to increased extracellular water in septic shock patients can alter the volume of distribution of hydrophilic antimicrobials (ex. aminoglycosides). In these circumstances, larger amounts of hydrophilic antimicrobials will be required as a loading dose. In contrast, a lipophilic antibiotic (ex. ciprofloxacin) has a greater affinity for adipose tissue. An obese patient may require a higher than predicted dose of lipophilic antibiotic as a loading dose [181]. Since the total content of body lipids does not change quickly, loading dose alteration with acute critical illness is rarely required.

The other crucial factor in determining the loading dose is the maximum concentration (C_{max}) to be achieved in the serum. This depends on the activity of the antimicrobial and the (minimum inhibitory concentration) MIC of the pathogen causing sepsis. The C_{max} is predicted using pharmacokinetic principles, but renal function plays no role in calculation C_{max} or LD [182].

During empiric therapy, the pathogen causing sepsis and its susceptibility may not be known, but the ICUs will have the data regarding common local pathogens and their resistance patterns from their previous sepsis cases. For concentration-dependent antimicrobials, high loading doses are required for a maximum bactericidal effect. Moore et al have reported a lower mortality rate with a high loading dose of aminoglycoside antibiotics [183]. With time-dependent antimicrobials, the LD may not be important for achieving adequate bactericidal effect. However, a larger LD may be administered to ensure good tissue penetration [182]. The use of loading doses is common for some antimicrobials used in life-threatening infections [184]. However, the risk of toxic side effects should be taken into consideration while treating patients who are already unwell with critical illness.

1.8. Antimicrobial Potency:

1.8.1.   Bactericidal and bacteriostatic antimicrobials

Accelerated clearance of pathogens is the key to the positive outcome in severe infections as well as septic shock. Antibiotics and antifungals are classified as cidal and static basing upon their ability to kill and/ or inhibit the growth of the pathogen with time. By definition, bactericidal antibiotics eliminate the bacteria substantially more quickly than bacteriostatic antibiotics. Lepper et al have described the importance of using bactericidal antibiotics in treating meningitis [185]. Meningitis is a condition that is similar to septic shock where the speed of clearance of bacteria is associated with survival of patients. In this study, using a bacteriostatic agent such as tetracycline with a β-lactam in treating pneumococcal meningitis resulted in a lowered survival compared to β-lactams alone. This inferior outcome was thought to be due to antagonism of β-lactam driven bacterial clearance

by tetracycline. Lower clinical response rates in serious infections have been reported with bacteriostatic vs bactericidal agents presumably because of inferior bacterial clearance. For example, a meta-analysis of tigecycline non-inferiority trials of serious infections has shown higher mortality in the treatment arm indicating the failure of tigecycline to clear pathogens [186, 187]. With bacterial osteomyelitis and endocarditis, using highly bactericidal antibiotics are reported to be associated with greater cure rates [188]. There is also evidence to suggest that bactericidal therapies yield better outcomes than static in treatment of invasive candidiasis [189]. Overall, the available evidence suggests that cidal therapy is superior compared to the static therapy specifically in life-threatening bacterial and fungal infections (**figure 13**).

1.8.2   Combination therapy

Monotherapy with beta-lactam antibiotics is often sufficient to eradicate the infection causing bacteria in serious infections. However, combination therapy using two different classes of antibiotics can also generate a synergistic or an additive effect leading to faster elimination of pathogens and a decreased mortality in septic shock [190, 191]. A synergistic effect is reported in β-lactams and aminoglycoside antibiotic combination therapy [192, 193]. Recently, a similar effect was reported in β-lactam and fluoroquinolones for Gram negative bacteria [194]. Studies have reported improvement in survival after combination therapy in severe, life-threatening infections, particularly with severe sepsis [195, 196].

Empiric combination therapy should not be administered for more than 3 to 5 days [149]. Once the causative pathogen is identified or clinical stability improves, the empirical therapy and / or combination therapy can be narrowed down to a single antibiotic to which

organism is susceptible [138]. Such de-escalation is associated with improved survival and also limits development of resistance in the pathogens [197, 198].

In addition, with the emergence of resistant bacterial strains such as carbapenemase-producing Enterobacteriaceae and other multi-drug resistant strains of *Pseudomonas* spp and *Acinetobacter* spp., where, beta-lactam monotherapy cannot be used, definitive combination therapy may be necessary, as alternative antibiotics alone are often insufficient for treatment of severe infections. At present, resistance to carbapenems and other beta-lactams is increasing [199, 200]. New antibiotics that are active against these resistant strains are not available. Exploration of the potential of combination therapy may be advantageous in treating infections due to resistant strains.

1.9. Pharmacokinetics and pharmacodynamics of antibiotics

1.9.1. Pharmacokinetics

The study of absorption, distribution, metabolism and elimination of a drug in the body of an individual describes the pK of an antibiotic or any other drug [201]. Beyond the issues already discussed, an alternate approach to augmentation of pathogen clearance is to utilize dosing strategies that optimize bacterial killing, i.e. optimizing drug pharmacokinetics. There is a gap in the knowledge of pharmacokinetics in patients with severe sepsis and septic shock.

1.9.2. Pharmacodynamic parameters

Pharmacodynamics describes the relationship of antimicrobial concentration

to pharmacologic effect, i.e. bacteriostatic or bactericidal effect on the microbial pathogens. Antimicrobial drugs can be classed as being dominantly time-dependent or concentration-dependent in their effect. The three main pharmacodynamic parameters that drive the efficacy of antimicrobials are 1) peak concentration maximum (C_{max}) of free drug to MIC ratio ($f C_{max}$ /MIC), 2) the 24 h free drug area under the concentration curve (fAUC) to MIC ratio (fAUC$_{24}$/MIC) and 3) the time the free drug concentration remains above the MIC (%fT>MIC) [201]. $f C_{max}$ / MIC parameter is used to describe concentration-dependent antimicrobial efficacy, whereas %T>MIC describes the antimicrobial efficacy of time-dependent drugs. fAUC/MIC is relevant to both concentration-dependent and time-dependent antibiotics. Antimicrobial free drug concentration is the primary parameter associated with drug efficacy.

1.9.2.1. Time-dependent antimicrobial efficacy

Time-dependent antimicrobials exhibit an optimal bactericidal effect when drug concentrations are maintained above the MIC for the entire dosing interval (i.e. 100% T>MIC). Typically, antibiotic concentrations maintained above the MIC for > 40 – 50% of the dosing interval are sufficient to clear moderately severe bacterial infections in immunocompetent animals and patients [202, 203]. With these antimicrobials, higher concentrations do not result in a greater kill of organisms. Antibiotics such as beta-lactams, carbapenems, oxazolidinones, and tetracyclines exhibit %T> MIC as the pharmacodynamic parameter that describes the antibacterial activity [202-205].

Several comparative studies have shown superior clinical cure rates in serious infections with higher T>MIC [206-208]. In one randomized clinical trial with meropenem in

critically ill patients, continuous infusion of meropenem (which yields higher T>MIC) was superior in achieving microbiologic cure compared to standard intermittent doses [209].

1.9.2.2. Concentration-dependent elimination of bacteria

In a condition like septic shock, faster bacterial clearance plays an important role in the survival of the patient. Broad spectrum antimicrobials are recommended within 1 h of recognition of septic shock [149]. Even though time-dependent antibiotics are effective, concentration-dependent antibiotics may also be very useful in patients with difficult-to-treat multidrug-resistant bacterial infections, such as *Pseudomonas* spp and *Acinetobacter* spp, as well as for patients with severe infections associated with respiratory failure and septic shock. Extended spectrum beta-lactams and either a fluoroquinolones or an aminoglycoside are often used as a combination therapy, in these patients [210, 211].

Concentration-dependent antibiotics exhibit increasing bactericidal activity with increasing drug concentration. The efficacy of these antibiotics is determined by their C_{max} and AUC in relation to MIC. Fluoroquinolones and aminoglycosides are concentration-dependent in their antimicrobial efficacy [183, 202, 203, 212]. Fluoroquinolones and aminoglycosides exhibit AUC_{24}/MIC and C_{max} /MIC as the primary parameters associated with efficacy.

1.9.2.2.1. Fluoroquinolones

One study has predicted a cure rate $\geq$ 90% against Pseudomonas bacteremia by using aminoglycosides and/or ciprofloxacin at C_{max} (peak) /MIC value of at least 8 and an AUC_{24}/MIC value of > 123 (total drug) [213]. Aggressive dosing with targeted peak/MIC for

aminoglycosides and ciprofloxacin were strongly associated with the clinical outcome. In

another study, higher AUC24/MIC ciprofloxacin values were associated with better clinical

outcome in Enterobacteriaceae bloodstream infections [214]. In this study, bacterial

eradication was faster when the AUC_{24}/MIC value was > 250 compared to AUC_{24}/MIC $\geq$

125. However, no clinical data is available with fluoroquinolones regarding the relation

between their pharmacokinetic (PK) indices and mortality in sepsis or septic shock.

Unfortunately, an inadequate plasma concentration of fluoroquinolones after dosing in

critically ill and septic shock patients is not uncommon [215].

Toxicity and antimicrobial efficacy of fluoroquinolones are both associated with peak

(Cmax) concentrations. This is a major issue with respect to optimization of antimicrobial

efficacy. The available literature suggests that fluoroquinolone toxicity is associated with

specific chemical moieties present [216]. The most common fluoroquinolones related adverse

effects are mild nausea and diarrhea, headache and dizziness. Some of the severe toxic effects

are arthropathy, QTc interval prolongation, seizures, dysglycemia and phototoxicity, hepatitis

and hemolytic anemia [217-219]. Severe toxic manifestations can be fatal.

1.9.2.2.2. Aminoglycosides

Aminoglycosides exert concentration-dependent killing over a wide range of

aerobic bacterial strains [183, 220]. Aminoglycosides have a high degree of activity for both

Gram negative and Gram positive bacteria [221]. However, these antibiotics have a narrow

therapeutic window and usage is limited due to sub-acute/chronic nephro- and oto-vestibular

toxicity [222-225]. With the increased availability of other classes of antimicrobial agents

(fluoroquinolones, beta-lactams), aminoglycosides have not been considered as primary

therapy in the past except for serious systemic infections where other antibiotics were ineffective or in situations where synergistic combinations were required [226]. In recent years, with the increase in the prevalence of resistance to carbapenems, fluoroquinolones, beta-lactams and beta-lactamase inhibitor combinations, clinicians have started to use aminoglycosides with greater frequency.

1.9.2.2.2.1. Aminoglycoside pharmacology

Aminoglycosides are antibacterial compounds that comprise of an aminocyclitol ring combined with amino-modified sugars [227, 228]. Aminoglycosides have a well-defined pharmacokinetic profile. They are not absorbed in the intestinal tract; hence they must be administered by intravenous (IV) route. They can also be administered by the intramuscular route (IM) if necessary, but this approach is relatively uncommon. A large fraction of administered aminoglycosides are excreted unchanged through urine. The first developed aminoglycoside molecule, streptomycin, was discovered in 1943 from *Streptomyces griseus* by Albert Schatz, a graduate student working with Dr. Selman Waksman at Rutgers University [229]. Streptomycin was found to be highly active against various bacterial strains including *Mycobacterium* spp. After the discovery of streptomycin, a new generation of antimicrobials evolved and many other aminoglycoside antibiotics, including neomycin, kanamycin, paromomycin, gentamicin, and tobramycin were developed from different Streptomyces species [228].

1.9.2.2.2.2. Mode of Action

Aminoglycosides bind to the negatively charged lipopolysaccharides on the cell wall of bacteria. From there, they enter the periplasmic space through the outer membrane porins by passive diffusion. Uptake of aminoglycosides from the periplasmic space is an energy-dependent process (EDP) (O_2 dependent, electron transport system). Once the aminoglycoside antibiotics enter the cytoplasm, they bind to the 30S ribosomal subunit by another EDP [228, 230].

Aminoglycosides bind irreversibly to 16S rRNA (ribonucleic acid) of the 30S ribosomal subunit and prevent translation of proteins or induce false proof readings leading to the formation of aberrant proteins [231, 232]. Aberrant proteins may be inserted into the cell membrane, leading to altered permeability of the bacterial cell. This causes accumulation of aminoglycosides at higher concentrations in the cell cytoplasm and further reduction in protein synthesis.

Inhibition of protein synthesis alone does not confer bactericidal activity. After entering the bacterial cells, aminoglycosides also competitively displace magnesium ions from lipopolysaccharide bridges on the cell wall. These ions are important for bacterial cells to maintain cell wall integrity [233]. Magnesium ion displacement results in weakened cell wall integrity and leaking of cytoplasmic content into the exterior resulting in bacterial death. Aminoglycosides rely on oxygen dependent mechanisms to exhibit bactericidal activity [234-236]. These energy dependent processes are absent in the anaerobic bacteria. Hence, aminoglycosides have no effect on anaerobic bacteria or facultative anaerobic bacteria under anaerobic conditions [237].

1.9.2.2.2.3. rapeutic use of aminoglycosides

Aminoglycosides are useful primarily in infections involving Gram-negative bacteria, such as *Pseudomonas* spp., *E. coli, Acinetobacter* spp., *Enterobacter* spp and some *Mycobacteria* spp. [237]. The most frequent use of aminoglycosides is empiric therapy for serious Gram negative infections such as bacteremia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections. They also have secondary utility for some aerobic Gram positive organisms. Infections caused by Gram positive bacteria can also be treated with aminoglycosides, but usually in conjunction with beta-lactams or vancomycin. Presumably, inhibitors of cell wall synthesis like β-lactams or vancomycin lead to increased bacterial permeability and enhanced uptake of the aminoglycosides. Arbekacin is comparatively new aminoglycoside antibiotic that has anti-MRSA activity along with its activity against Gram negative bacterial strains [238]. Hwang et al has recently reported the usefulness of arbekacin as an alternative to vancomycin in MRSA treatment [239]. The same group conducted a retrospective observational study and reported arbekacin as a safe alternative to vancomycin in the treatment of MRSA skin and soft tissue infections [240]. The small number of subjects was a limitation in this study. Common clinical uses of aminoglycoside antibiotics are tabulated in **table 6**.

Amikacin has the broadest and most potent antibacterial activity of available aminoglycosides. The structure of amikacin is different from the rest of the drug class. This difference helps amikacin evade the inactivating enzymes in otherwise aminoglycoside resistant strains. Amikacin is preferred in treating serious infections due to resistant Gram negative bacterial strains [241, 242]. Effective use of gentamicin and other commonly available aminoglycosides like netilmicin for treatment is completely dependent on the

sensitivity pattern of the bacterial strains in that specific hospital setting [243]. Tobramycin with enhanced activity for *Pseudomonas* spp is often used for treatment of those infections [244, 245].

1.9.2.2.2.4. Advantages and disadvantages of aminoglycoside antibiotics

There are advantages and disadvantages of aminoglycoside use;

a) **Advantages**

- Chemically stable, hydrophilic, easily soluble in water

- Demonstrate concentration-dependent killing of bacteria.

- Highly active against Gram negative bacilli

- Can be used in combination with other antibiotics for treatment of Gram positive bacteria.

- Active against *Francisella* spp (Tularemia), *Yersinia* spp (Plague), *Brucella* spp and *Mycobacterium* spp.

- Eliminated through the kidneys (90%, unchanged form) with minimal drug-related hepatic metabolism and toxicity.

b) **Disadvantages**

- No gastric absorption; must be administered intravenously or intramuscularly

- No activity against anaerobic bacteria or facultative anaerobes in anaerobic environment

- Significant toxicity issues (nephrotoxicity, ototoxicity and neuromuscular weakness)

- Complicated dosing in critically ill due to altered pharmacokinetics

- Occurrence of transient, reversible adaptive resistance in bacteria usually within 8 to 16 hours after drug administration due to decreased drug uptake by the organisms by down-regulation of the enzymes that are responsible for energy-dependent uptake of aminoglycosides [246, 247]; clinical significance of this effect is uncertain

1.9.3. *In vitro* and *in vivo* pharmacodynamics

The ability of aminoglycoside antibiotics to clear pathogenic bacteria has been evaluated in *in vitro* and *in vivo* pharmacodynamic models. These studies have confirmed the potent concentration-dependent bactericidal activity of aminoglycosides. Craig et al have described the efficacy of amikacin in mouse thigh and lung infection models [248]. In their studies, uranyl nitrate was used to induce transient impairment of renal function to study the *in vivo* pharmacodynamics properties of amikacin under pharmacokinetic conditions (i.e. more limited renal clearance) similar to that of humans. Amikacin was found to exhibit concentration-dependent killing and produced prolonged *in vivo* post-antibiotic effect (PAE) similar to other aminoglycosides. A key finding in their many studies was that aminoglycosides exhibit concentration-dependent killing both *in vivo* and *in vitro* [183, 202, 248] Gudmundsson et al reported from their *in vivo* studies in mice that beta-lactam/aminoglycoside combinations produced a prolonged post antibiotic effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [249]. Tsuji et al reported from their studies that single high dose of gentamicin in combination with daptomycin or vancomycin was useful in maximizing synergistic and bactericidal activity and may minimize toxicity [250]. Many publications have described various dosing strategies and their significance in treating different infections.

1.9.4. Conventional (multiple daily) dosing

Historically, aminoglycosides were dosed at 1–1.5 mg/kg every 8 or 12 hours (gentamicin) [202, 203, 221, 251]. Standard dosing of aminoglycosides involved intravenous administration, approximately 1.3 mg/kg every 8 hours (assuming normal renal function) of gentamicin/tobramycin/netilmicin [221, 252]. Maximum concentration in serum is achieved immediately following a standard 30 minute intravenous infusion. The protein binding of aminoglycosides is < 30% and elimination half-life ($T_{\frac{1}{2}}$) is 3 – 3.5 h in infants and 1.5 – 3 h in adults, but 36 – 70 h in end-stage renal disease patients [221, 253, 254].

For the effective treatment and successful outcome with gentamicin and tobramycin, the C_{max}: MIC ratio is expected to be 8 – 10 µg/ml for effectiveness and the trough concentration should be ≤ 2 µg/ml to limit toxicity [183, 212]. The standard multiple daily dose regimens could generally produce acceptable serum concentrations with sensitive organisms in healthy subjects. One notable disadvantage is that the trough concentrations may be elevated for prolonged periods leading to nephrotoxicity particularly in elderly and chronically ill patients [255, 256].

1.9.5. Once-daily dosing

Physicians have begun to take advantage of the unique pharmacodynamics properties of aminoglycosides (i.e. concentration-dependent killing and dominant trough related renal toxicity) by moving to once-daily dosing (e.g. 5 - 7 mg/kg gentamicin equivalent). This approach also has the advantage of ensuring early therapeutic drug concentrations in critically ill patients. With this strategy, the entire daily dose is given in a single dose rather than divided into 8 hours dosing intervals (1-1.5 mg/kg X 3 doses gentamicin) [252]. This

approach appears to yield at least equivalent and perhaps higher efficacy with some evidence of decreased renal toxicity [202, 203, 212, 257, 258]. Achievement of a C_{max} /MIC ratio of $\geq$ 10 µg/ml is associated with a shorter time to clinical response and greater clinical cure rate [183, 212]. Achievement of this target can be problematic with standard dosing but can be much more reliably attained with once-daily dosing even for relatively resistant pathogens.

Blaser et al have reported the superiority of once-daily netilmicin over continuous infusion and conventional (three times a day) dosing with faster killing of *E. coli, K. pneumoniae* and *S. aureus* in an *in vitro* pharmacodynamic model [259]. Kapusnik et al have described the superior bactericidal activity of single, large daily doses of tobramycin against Pseudomonas in an *in vitro* time kill model as well as in and *in vivo* lung infection model [245]. Nicolau et al reported once-daily gentamicin at 7 mg/ kg/ day dose by the intravenous route to be clinically effective with reduced incidence of nephrotoxicity in a clinical study involving 2,184 adult patients [260]. This dosing regimen was a cost-effective method for administration of aminoglycosides. Heininger et al concluded from their small clinical trial in 26 cystic fibrosis patients with Pseudomonas infection that, a single daily dose of aminoglycoside was as efficacious as conventional triple daily dose therapy with similar toxicity [261]. Prins et al after conducting a randomized controlled trial in 123 patients with serious bacterial infections concluded that once-daily aminoglycoside therapy was as effective as three times a day therapy but was advantageous because of decreased nephrotoxicity [262]. Carapetis et al conducted a randomized, controlled trial in children from 1 month to 12 years old with urinary tract infections and concluded that once a day dosing of gentamicin was safe and effective in treating the pediatric population [263]. Abdel-Bari et al from their clinical

trial in critically ill patients reported that once a day dosing of aminoglycosides was preferred over individualized multiple daily dosing [264].

Several meta-analyses have assessed the relative efficacy and toxicity of once versus 3X daily dosing of aminoglycosides. For the most part, these meta-analyses suggest improved clinical or bacteriologic efficacy with an inconsistent trend towards decreased toxicity [265, 266].

Doses beyond 7 mg/kg/q24 h (gentamicin equivalent) have not been evaluated, in part due to acute and chronic toxicity concerns.

1.9.6. Altered kinetics of aminoglycosides in septic and critically ill patients

In sepsis and septic shock, various pathophysiological changes occur, leading to alteration in the pharmacokinetics of the aminoglycosides as well as other drugs (**figure 14**). At the initial stages of sepsis there is an increase in the cardiac output causing increased clearance of aminoglycosides [180, 267]. In many forms of acute critical illness including sepsis, septic shock and burn wound infections, the volume of distribution (Vd) of aminoglycosides is markedly increased causing lower peak concentrations of the drug [268-272]. Increased Vd may be due to aggressive fluid resuscitation (often on the order of 30-50% of extracellular water volume) with associated interstitial edema in critically ill patients. This leads to the low overall plasma concentration of administered aminoglycosides and other water soluble antibiotics.

Given this increased Vd, standard, multiple daily dosing often results in sub-therapeutic initial serum levels, resulting in poor clinical responses from the very patients in whom effective therapy is most required [256, 269, 273-275]. In addition, suboptimal

aminoglycoside concentrations can also select for resistance in pathogenic bacterial strains [276]. On the other hand, multi-organ dysfunction is commonly observed in severe sepsis and, particularly, septic shock. Hepatic and renal clearance of antimicrobials and other drugs can decrease considerably. This may cause higher plasma concentrations than expected and can lead to significant toxicity, especially later in the course of illness when overt organ failure has manifested.

Critically ill patients with sepsis and septic shock typically also have lowered serum albumin levels [277, 278]. However, given the low protein binding of aminoglycosides, hypoalbuminemia in sepsis and septic shock has only a modest effect on the aminoglycoside pharmacokinetics [279].

1.10. Toxicity associated with aminoglycosides

Aminoglycosides are well known to be associated with sub-acute oto-vestibular toxicity and nephrotoxicity [223, 280]. These toxicities are typically observed when larger doses of aminoglycosides are administered for prolonged periods or usual doses are given to patients with underlying renal disease [281]. A relatively underappreciated toxicity associated with high C_{max} is neuromuscular weakness [282, 283].

1.10.1. Oto-vestibular toxicity

Aminoglycoside antibiotics may cause oto-vestibular toxicity when used for prolonged periods. Different aminoglycosides appear to carry varying propensities to different forms of toxicity. Gentamicin and tobramycin are more prone to induce vestibular toxicity, whereas amikacin, neomycin, and kanamycin tend to result in more oto- (cochlear) toxicity [284].

1.10.1.1.   Inner ear

A basic understanding of the inner ear and structures responsible for hearing and balance is useful in appreciating the basis of aminoglycoside-induced oto-vestibular toxicity. The inner ear comprises of membranous compartments and fluids enclosed in a bony shell. Within the temporal bone, just beyond the middle ear, small structures called the cochlea, semicircular canals and the vestibule are present. All these structures together form the labyrinth. The cochlea holds the receptors for hearing. The vestibule along with the semicircular canals harbors receptors for balance and equilibrium.

1.10.1.1.1. Cochlea:

The cochlea is a shell shaped structure resembling the shell of a snail. Membranes divide the cochlea into compartments called the basilar membrane and the Reissner's membrane. These compartments have the perilymph and endolymph [285]. The cochlea also harbors the auditory receptors called hair cells. These are of two types; outer hair cells and inner hair cells based on their alignment in the cochlea. The cochlea converts sound energy (mechanical) into electrical impulses that can be recognized by the brain via impulses from the hair cells. Problems in the basilar membrane or in the hair cells lead to hearing problems [286].

1.10.1.1.2. Vestibule:

The vestibule lies in between the cochlea and the semicircular canals in the ear. Within the vestibule are structures called the 'utricle' and 'saccule'. These structures contain sensory hair cells in them and are responsible for sensing linear acceleration and balance. The

sensory cells in the vestibule sense the movements of the head and they send the impulses to the brain through the vestibulocochlear nerve. Vestibular injury can lead to balance problems [287-290].

1.10.1.2. Molecular mechanism of ototoxicity

1.10.1.2.1. Cochlear (auditory) toxicity:

After prolonged systemic exposure, aminoglycosides accumulate in the outer and eventually the inner hair cells that are present at the base of the cochlea. Outer hair cell death occurs first; later, the inner hair cells follow. The dead hair cells do not regenerate spontaneously. Aminoglycosides accumulate in the organ of corti [291] as well as stria vascularis (comprise of capillaries and blood vessels that help in the production of endolymph for the cochlea) [292, 293]. Aminoglycosides are reported to induce morphological/structural changes in the stria vascularis region with associated hair cell loss [294, 295]. Kanamycin and streptomycin are found in the spiral ganglion after systemic administration in animals, not necessarily exceeding serum concentration [296, 297].

Aminoglycoside-induced ototoxicity appears to be mediated by mitochondrial protein synthesis inhibition, formation of free radicals and reactive oxygen species (ROS) that cause oxidative damage to biomolecules and over-activation of n-methyl-d-aspartate receptors (NMDA receptors) [298]. McFadden et al suggested that hair cell death is due to activation of the NMDA receptors and an excessive influx of calcium into the hair cells [299]. This causes apoptosis in the hair cells and leads to permanent hearing loss. The putative pathway to aminoglycoside-induced ROS production resulting in severe damage to the sensory cells and neurons within the inner ear is shown in **figure 15**. Aminoglycosides form ROS through an

interaction with iron and lipids. This has been confirmed by *in vitro* and *in vivo* experiments [300, 301]. Schacht et al and others, for example, have described the ability of aminoglycosides to react with metals such as iron to produce oxygen radicles that induce toxicity by activating caspases and inducing apoptosis in the hair cells [300, 302-307]. Aminoglycoside accumulation in the cytoplasm of the hair cells also causes decreased protein synthesis in mitochondria. This leads to stress and release of cytochrome c from the mitochondria resulting in caspase activation that induce apoptosis in the hair cells [308, 309].

Clinical hearing loss is usually observed after a few weeks to months of aminoglycoside therapy. Patients usually lose high frequency (>8 kHz) hearing ability at an early stage. Hearing loss is most common in patients under aminoglycoside therapy for prolonged durations [310].

On rare occasions, hearing loss has been reported immediately after a single dose of gentamicin. This may be due to a genetic predisposition of certain individuals to aminoglycoside-induced auditory toxicity [311]. For example, at the 1555 position of the mitochondrial 12S rRNA, an adenine (A) to guanine (G) mutation is reported in some groups with aminoglycoside auditory toxicity [311, 312]. This mutation causes a structural similarity of this 12S rRNA to that of the bacterial rRNA which promotes the binding of aminoglycoside antibiotics to this site. This results in inhibition of protein synthesis in mitochondria leading to apoptotic death of the hair cells causing hearing loss [313, 314].

1.10.1.2.2. Vestibular toxicity:

Vestibular toxicity is another side effect of aminoglycoside antibiotics [315, 316]. Vestibular toxicity occurs in up to 15 % of patients exposed to aminoglycoside antibiotics

[317]. This toxicity is also typically observed in patients who are exposed to aminoglycoside therapy for a prolonged period [318-320]. In their analysis of the literature, Ariano et al suggested that vestibular toxicity is not related to the concentration achieved in the serum or the dosing strategy, but is dependent only on the duration of exposure [321]. The central role of duration of aminoglycoside exposure as the primary determinant of vestibular toxicity is also supported by others [322].

Vestibular toxicity is mainly due to damage of the structure and function of labyrinthine hair cells and their connection through the eighth nerve to the central nervous system. Symptoms and signs include dizziness, ataxia, nystagmus and when severe, disequilibrium and oscillopsia [323, 324]. With aminoglycoside-induced vestibular toxicity, the most extensive hair cell damage is reported in the apex of the cristae and the striolar regions of the maculae in guinea pigs [325]. Mild to moderate degeneration in the cristae ampullaris and moderate to severe damage in the utriculus in association with vestibular dysfunction has been reported in gentamicin treated guinea pigs [326].

Streptomycin has been identified as most vestibulotoxic aminoglycoside [325]. Vestibular toxicity typically reverses after discontinuation of aminoglycoside exposure to some extent in animal models as well as in clinical trials of human toxicity [327-330]. Animals studies suggest hair cell regeneration although the nature of clinical recovery (whether microanatomic or strictly functional/adaptive) is uncertain.

1.10.1.3. Preventing / minimizing oto-vestibular toxicity

In several studies, safety and efficacy of once a day and multiple doses of

aminoglycosides were compared. Oto- and vestibular toxicity are both primarily due to the formation of free radicals resulting in hair cell damage and death. Many enzymes with antioxidant activity that appear to reduce ototoxicity are reported [331]. Animal experiments have indicated that both cochlear and vestibular toxicity can be attenuated using iron chelators and antioxidants [332, 333]. A protective effect of anti-free radical agents such as dihydroxybenzoate, [334, 335], alpha-lipoic acid [336], salicylate [337, 338] on ototoxicity has been proved in animal experiments. Animal studies indicate that oto-vestibular toxicity can be prevented by co-administering NMDA receptor antagonists [299].

In humans, gentamicin related toxicity can be limited by administering as an extended interval dose, thereby allowing a drug free (< 0.5 mg/L) period of at least 4 hours (washout period for kidneys) [255, 260, 339, 340]. Dosing of these antibiotics must be stopped once toxic symptoms appear in the patients. There is no consistent difference in the frequency of occurrence of auditory and vestibular toxicity between multiple daily and once-daily dosing regimens [341, 342]. Meta-analyses have confirmed the comparable toxicity between once and multiple daily dosing regimens in humans [342].

1.10.2. Nephrotoxicity:

Nephrotoxicity is one of the major side effects of aminoglycosides and is one of the main therapeutic limitations of these antibiotics. Many studies of risk factors for aminoglycoside-induced renal toxicity in the clinical environment have been performed. Risk factors consistently found to be associated with renal toxicity include dehydration, age, intravascular volume depletion, sepsis, pre-existing renal insufficiency, length of therapy, advanced age, concomitant use of other nephrotoxic drug, female sex, chronic renal failure

and liver disease [343-347]. The selection of aminoglycoside does not appear to be associated with the frequency of renal toxicity [281, 345]. Prins et al from their randomized clinical trial in patients with severe infection reported nephrotoxicity and ototoxicity associated with once a day, intravenous doses of netilmicin and gentamicin [348]. No advantage of netilmicin over gentamicin was found in regards to nephrotoxicity and ototoxicity. Pancorbo et al compared nephrotoxicity due to gentamicin and tobramycin in patients receiving individualized-pharmacokinetic dosing regimens [349]. Four percent of gentamicin treated patients and 2.6% of the tobramycin treated patients developed nephrotoxicity. There was no statistically significant difference between the two antibiotics in regards to the nephrotoxicity. However, one prospective comparative study of gentamicin and amikacin reported that gentamicin was significantly more nephrotoxic compared to amikacin [350].

Aminoglycoside antibiotics are almost entirely excreted in unchanged form through the urine with a minimal liver metabolism [351]. Animal studies and clinical trials of kidney toxicity show that aminoglycosides accumulate in the renal cortex with concomitant induction of morphological changes in that region [352]. The concentration achieved in the cortex of kidneys is greater than that in serum. The central aspect of aminoglycoside-induced nephrotoxicity is renal tubular damage that leads to a loss of integrity of the brush border membrane of the renal epithelial cells [353] followed by tubular epithelial cell apoptosis [354, 355].

1.10.2.1. Mechanisms of aminoglycoside-induced nephrotoxicity

Aminoglycoside-induced renal toxicity can be reproduced in animal studies. Tubular necrosis along with gross morphological changes in glomerular structures along with a

progressive increase in plasma creatinine is seen in rats after prolonged gentamicin administration [356]. Baylis et al reported that large doses of gentamicin administered to rats evoke decreased glomerular filtration rate (GFR) due to a decrease in renal plasma flow (RPF) [357]. The authors also reported that water permeability and surface area of the glomerulus are important determinants of GFR and that gentamicin has adverse effects on these in a dose-dependent manner. In these experiments, gentamicin was dosed subcutaneously (SC) at 60 mg/kg/day for 14 days in rats.

Mesangial cells also play an important role in the glomerular filtration rate. These cells are located at the core of the glomerular tuft between capillary loops. Mesangial tone is reported to exert a mechanical traction on the glomerular basal membrane and on the endothelial capillary lining. This determines the area of filtration surface and ultra-filtration coefficient and modulates glomerular vascular resistance. Reduction in GFR, glomerular surface and ultrafiltration co-efficiency due to contraction of mesangial cells is already reported in response to aminoglycosides [358, 359] (**figure 16**). Matinez-Salgado et al have also demonstrated the role of gentamicin in simultaneous mesangial proliferation and apoptosis in rats [360].

1.10.2.1.1. Accumulation of aminoglycosides in tubular epithelial cells

Figure 17 demonstrates the primary mechanisms and cell signaling pathways underlying the cytotoxic effect of gentamicin in kidneys Aminoglycoside accumulation in the tubular epithelial cells has been extensively studied in animals [361-364]. Renal tubular cells have a membrane endocytic complex, which involves two proteins, megalin and cubilin [364-367]. These cells internalize aminoglycosides in the tubules by pinococytosis with the

compound entering the cytoplasm in an endosome. These endosomes fuse with the lysosomes present in the cell forming endolysosomes. Aminoglycosides inhibit the phospholipases present in the lysosomes [368, 369], causing an alteration in lipid metabolism and phospholipidosis [369-371]. Eventually, endolysosomes become permeable and aminoglycosides along with other lysosomal contents enter the cell cytoplasm at a high concentration. The rupture of lysosomes causes the release of proteases and cathepsins into the cell cytoplasm [372]. These stimulate the proteolytic activation of caspase 3 and caspase 7 that induces apoptosis of the tubular epithelial cells [373]. After accumulating in the endothelial cells of the tubules, aminoglycosides (gentamicin in these studies) trigger the membrane-bound calcium sensing receptor (CaSR), an extra-cellular calcium sensitive G-protein-coupled receptor [374, 375]. In the proximal tubules, this CaSR receptor induces tubular cell death/apoptosis [376].

1.10.2.1.1.1. Targeting mitochondria of the tubular epithelial cells

Gentamicin is reported to accumulate in the cytosol of the tubular epithelial cells at high concentration after being released from the lysosomes resulting in impairment of mitochondrial respiration [377]. Gentamicin accumulated in the cytoplasm acts on the mitochondria to activate the apoptotic pathways [378, 379]. The increased cytoplasmic concentration of gentamicin has also been reported to cause oxidative stress and reduce ATP levels in the cells [379]. Oxidative stress is mediated by the hydroxyl free radicals from hydrogen peroxide as well as the superoxide anions from mitochondria [380]. Superoxide and hydrogen peroxide in turn react with elements like iron to form more reactive oxidants leading to further injury [381]. The primary iron source for this process appears to be ferritin

(the stored form of iron in mitochondria) which is affected by superoxides to release iron. Further reactions occur, leading to the formation of highly active free radicals which lead to the toxic effect [305]. These free radicals cause cellular damage and death of the epithelial cells by inhibiting the electron transport chain and suppression of cellular respiration limiting ATP production. This results in release of cytochrome c and apoptosis induction factor (AIF) into the cytoplasm from the mitochondria [369]. The free radicals inhibit the transmembrane sodium flow by inhibiting the sodium/potassium pump. This causes accumulation of sodium in the cells, leading to cell swelling and necrosis [382].

1.10.2.1.1.2.   Accumulation of aminoglycosides in endoplasmic reticulum (ER)

Gentamicin present in the lysosomes enters the Golgi and ER through the secretory pathway. Accumulation of gentamicin in the ER causes a stress response in the ER. Gentamicin binds to calreticulin and inhibits the correct posttranslational protein folding. This causes cell cycle arrest due to activation of unfolded protein response (UPR) [383, 384]. The UPR activation leads to activation of caspases and results in apoptosis of the tubular epithelial cells [373]. There is a difference in the accumulation and activation of apoptosis by different aminoglycoside molecules; for example, gentamicin and netilmicin accumulate in the kidneys at higher concentrations compared to tobramycin and amikacin. Activation of apoptosis is high with gentamicin and tobramycin and is comparatively low with amikacin and netilmicin [354, 368]. The proximal tubular cells regenerate with time and hence the nephrotoxicity is reversible to some extent [370, 385]. Animal studies have reported a reduction of tissue injury and renal dysfunction by using antioxidants along with gentamicin [386-390].

1.10.2.2. Preventing / minimizing nephrotoxicity

Although there is some disagreement, available evidence suggests that the active transport process that drives the uptake of aminoglycosides into the proximal tubular cells can be saturated [391]. Once the transport mechanism is saturated, higher concentration of aminoglycosides in the lumen due to higher doses does not lead to increased uptake and an increasing adverse effect on the tubules is not apparent. In humans, accumulation with once-daily doses of gentamicin and netilmicin is reduced by 30 to 50% when compared to continuous infusion [392]. The exact relation between the concentration of aminoglycosides in blood and nephrotoxicity are not clear. However, adequate information is available indicating nephrotoxicity to be associated with higher trough concentrations rather than the peak concentration of aminoglycosides [344, 393, 394]. This limits renal toxicity associated with peak levels [344].

Clinically, nephrotoxicity of aminoglycosides can be minimized by preventing prolonged treatment with aminoglycosides. Serum creatinine and, if necessary, creatinine clearance rate, can be monitored from time to time to estimate the renal injury [395, 396]. Renal injury biomarkers such as beta-2-microglobulin measurement may be useful [397], but, this assay is currently not available for clinical use with patients; this may change in the future. A prolonged maintenance of trough concentration of aminoglycosides (> 2.5 mg/L), and the absence of windows during which blood concentration of aminoglycosides is unmeasurable may present a risk of renal toxicity.

As has been noted, aminoglycoside-induced renal toxicity is mediated in part via a saturable transport mechanism. Once a day dosing of aminoglycosides may be a favorable alternative to decrease potential [391, 398]. Once there is a preliminary indication of

nephrotoxicity, aminoglycoside dosing should be terminated. Experimental animal studies have reported that free radical scavengers and iron cheaters can be used to prevent free radical mediated toxicity [399]. However, these approaches remain strictly experimental in nature.

1.10.3. Neuromuscular weakness

Acute neuromuscular weakness is one of the rarely reported side effects of aminoglycoside dosing. This side effect is sometimes observed after a single dose of aminoglycosides [282, 400]. In a case report, Warner et al indicated that patients with low calcium may exhibit particular sensitivity to neuromuscular weakness and ventilator dependency with aminoglycoside administration [401]. Neuromuscular weakness may occur with increased frequency when aminoglycosides are administered intravenously along with loop diuretics, non-steroid anti-inflammatory drugs, anti-malarial drugs, other neuromuscular blocking drugs and/ or anesthetic agents [282, 402]. Neuromuscular weakness appears to be due to a block of the release of acetylcholine in response to neuronal stimulation and also by decreased post junctional sensitivity [400, 402, 403]. There is some evidence that aminoglycoside-induced neuromuscular weakness can be partially reversed by administering calcium [403].

1.10.4. Potential of ultra-high dose aminoglycoside therapy

As previously noted, antimicrobial strategies that might substantially increase antimicrobial efficacy, but carry a risk significant toxicity have not been assessed. Aminoglycoside antibiotics are well known and studied antibiotics that have been in clinical use for the past 50 years. These antibiotics have a broad spectrum of activity and eliminate bacteria in a concentration-dependent manner [404]. Available data suggest that single ultra-

high doses should exhibit a rapid clearance of pathogenic bacteria. We have confirmed this possibility in an *in vitro* pharmacodynamic model of aminoglycoside therapy in a wide range of pathogens (unpublished). In this study, we found that most susceptible and moderately resistant Gram positive and Gram negative bacterial strains were cleared to below the detection limit within minutes after initiation of treatment. Given that accelerated pathogen clearance may be a key to improving survival in septic shock patients, ultra-high dose therapy could potentially be useful in this condition assuming that toxicity concerns can be adequately addressed.

If an ultra-high dose aminoglycoside therapy approach can be validated in animal models, there is a substantial probability that it could be translated to the bedside to improve outcome in critically ill patients with septic shock including those with organisms that might otherwise be considered to be resistant to antibiotics. This approach may also be highly effective in life-threatening Gram positive infections since many major Gram positive bacteria remain sensitive to aminoglycosides even when resistant to other standard agents. Further, this approach may be useful as a single dose therapy for sensitive biowarfare/bioterrorism agents, many of which remain sensitive to these drugs. Given that mortality of septic shock exceed 50 - 70% and that an estimated 210,000 sepsis-related deaths occur every year in North America alone [6, 22, 405], an inexpensive antimicrobial agent that produce a measurable increase in survival probability while generating significant though manageable side effects could be an entirely acceptable option.

1.10.5. Preliminary studies

In preliminary, unpublished studies by our group, we determined that substantially higher than normal aminoglycoside doses (15 - 20 mg/kg single dose versus 5 – 7 mg/kg gentamicin) can be used in a rat model of *E. coli* Bort induced peritonitis/septic shock with marked increase in the blood bacterial kill rate. This accelerated bacterial clearance is an effect that has been associated with an improvement in mortality in a parallel mouse survival model of *E. coli* Bort induced septic shock [145]. We have also determined that even higher ("ultra-high") doses of gentamicin (80 mg/kg single dose), which normally cause immediate death, are well tolerated if the animal is ventilated for 15 - 45 minutes immediately following administration. In the absence of mechanical ventilation, death appears to be due to acute neuromuscular paralysis, an effect that has been previously described on rare occasions in the pharmacological literature [400].

1.10.6. Knowledge Gaps:

1) Do experimental animals treated with single, ultra-high dose aminoglycoside therapy exhibit acute neuromuscular paralysis and if so, of what duration? Is this neuromuscular paralysis reversible?

2) Do experimental animals treated with single ultra-high dose aminoglycoside therapy exhibit renal and/or ototoxicity?

3) Can single ultra-high dose aminoglycoside therapy accelerate clearance of *E. coli* Bort (a sensitive bacterial strain) from the blood stream in a septic shock model?

4) Can the single ultra-high dose aminoglycoside therapy clear the bacterial load from the vital organs in a septic shock model induced by *E. coli* Bort?

CHAPTER 2

2. Hypothesis and Objectives

2.1. Hypothesis

Basing on the preliminary studies conducted, we hypothesize that, a single dose of gentamicin/tobramycin at 80 and 160 mg/kg, intravenously, will effectively clear bacteremia and reduce inflammatory cytokine response and lactic acid production in a rat model of *E. coli* induced peritonitis/septic shock.

2.2. Objectives

2.2.1. Objective 1

Examine the effect of a single, ultra-high dose of 80 and 160 mg/kg of gentamicin and tobramycin on short-term (9 h) neuromuscular function and renal function in normal healthy rats.

2.2.1.1. Rationale

As described earlier, aminoglycoside antibiotics exert significant toxicity when administered at higher doses. Before conducting the primary septic shock study, the ability of Sprague-Dawley rats (SD rats) to tolerate the ultra-high doses of aminoglycosides must be ascertained. They must fully recover from any neuromuscular paralysis after support with artificial ventilation without evidence of substantial renal injury. Neuromuscular weakness is well recognized manifestation of acute aminoglycoside toxicity and can lead to respiratory failure and death from asphyxia if the animals are not supported by artificial ventilation.

Determination of the duration of neuromuscular weakness/paralysis and viability of the transient ventilator support of the animals is critical as all further studies are dependent on effective management of this issue. This study will also identify the potential presence of acute nephrotoxicity following a single, ultra-high dose of gentamicin and tobramycin. If the animals are able to tolerate the single, ultra-high doses with manageable toxicity, then further studies are warranted.

2.2.2. Objective 2.

Examine the effect of single, ultra-high dose of 80 and 160 mg/kg of gentamicin and tobramycin on long-term (6 months) auditory, vestibular and renal function in normal healthy rats.

2.2.2.1. Rationale:

Once short-term tolerance and survival is demonstrated, the absence of significant longer term oto-vestibular toxicity and nephrotoxicity associated with proposed 80 and 160 mg/kg doses of gentamicin and tobramycin must be demonstrated. Clinically, oto-vestibular toxicity is observed days to months after completion of aminoglycoside therapy. Auditory brain stem response studies, vestibular toxicity studies (behavioral studies) and nephrotoxicity studies will be performed up to 6 months (180 days). If significant oto-vestibular and nephrotoxicity are not observed, further studies in septic shock model will proceed.

2.2.3. Objective 3.

Examine the effect of single intravenous 5, 10, 20, 40, 80 and 160 mg/kg doses

of gentamicin on blood bacterial kill, organ bacterial burden and serum cytokine/lactate levels in a rat model of *E. coli* induced septic shock.

2.2.3.1. Rationale:

In septic shock, the total bacterial load may be a central driver for progression of septic shock. If we are able to accelerate bacterial clearance, then hemodynamic and inflammatory responses may resolve more quickly. Dose proportional clearance of bacteria was observed in *in vitro* models using concentrations of aminoglycosides comparable to those in this study.

Ultra-high doses have not been assessed in *in vivo* models due to potential toxicity concerns. Assuming the previous phases of this study program show tolerability of this approach, the ability of single, ultra-high doses (80 mg/kg and 160 mg/kg) of gentamicin to accelerate clearance of bacteria compared to other lower doses will be assessed. Sequential serum cytokine and lactate levels will also be determined from all the animals. We anticipate the potential for complete clearance of bacterial counts from the blood in ultra-high gentamicin dosed animals compared to the untreated control (UTC) animals as well as animals treated with lower gentamicin doses. We also anticipate substantial reductions in organ bacterial counts at the end of the acute study.

CHAPTER 3

3. Materials and general methods

3.1. Antibiotics

All antibiotics used in the study were procured commercially and stored at 4ºC. Gentamicin and tobramycin were obtained from Sandoz, Canada. Cefotaxime was obtained from Sanofi Aventis.

3.2. Other chemicals/ drugs

Ketamine (Wyeth, Canada), acepromazine (Ayerst, Canada), buprenorphine (Schering-Plough, UK), bupivacaine (AstraZeneca, Canada), atropine (Rafter, Canada), heparin (Pharmaceutical Partners of Canada, Canada), epinephrine (Pharmaceutical Partners of Canada, Canada), pentobarbital sodium (CEVA, France), and isoflurane (Baxter, Canada) were also obtained commercially. Controlled drugs, including ketamine and buprenorphine were procured after obtaining approval from Health Canada.

3.3. Bacteria

E. coli ATCC 700973 Serotype O18:K1:H7 (Bort) was obtained from the American Type Culture Collection (ATCC). This strain was selected for our studies based on its virulence properties and its ability to cause septic shock in mice in previous studies [145]. The organism was originally isolated from the blood of a neonate with bacterial meningitis [406]. *E. coli* ATCC 700973 Serotype O18:K1:H7 usually causes extra-intestinal diseases such as urinary tract infections with bloodstream infection [407]. The principal virulence

factors of this strain are the lipopolysaccharide, capsule production and the type 1 pili [408].

Virulence factors such as adhesins (e.g., Pap, Sfa, and Dra), hemolysin (Hly), cytotoxic

necrotizing factor-1 (CNF-1), and the aerobactin (Aer) iron-sequestration systems are specific

for this strain and are not found in the non-virulent fecal *E. coli* [408]. In this thesis, *E. coli*

ATCC 700973 Serotype O18:K1:H7 will be addressed as *E. coli* Bort for convenience.

3.4. Culture media

Cation adjusted Mueller-Hinton Broth (Ca-MHB) (Difco, USA), Mueller-Hinton broth

(MHB) (Difco, USA), Sodium Chloride; Trypticase Soya Agar (TSA) plates (HiMedia,

India), Agar (HiMedia, India) were used for culturing bacteria and determining bacterial

counts.

3.5. *In vitro stud*ies: Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution

method following the Clinical and Laboratory Standard Institute (CLSI) guidelines [409]**.**

MICs of gentamicin, tobramycin and cefotaxime were determined for *E. coli* Bort used in this

study. Antibiotic stock solutions were prepared following the CLSI M7-A7 method. *E. coli*

Bort was sub-cultured in MHB (Difco, USA), overnight and the optical density (OD) of the

inoculum was adjusted to 0.080 at 625 nm using a spectrophotometer (Spectronics-20,

Genesys). The inoculum was diluted 1: 100 and 50 µL of inoculum was dispensed into wells

containing 50 µL of cation adjusted CaMHB with the desired concentration of antibiotic to

attain a bacterial concentration of 5 x 10^4 colony forming units (CFU)/well. The bacterial

counts were confirmed by serial dilution in normal saline and plating on MHA plates.

Antibiotic concentrations tested were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 µg. Plates were incubated at 37^0 C for 18 – 24 hours to determine the MICs. The lowest concentration of antibiotic at which visible growth of bacteria was inhibited was assessed as the MIC of that antibiotic against the *E. coli* Bort. MICs of both gentamicin and tobramycin along with cefotaxime against *E. coli* Bort were determined in triplicate on separate days.

3.6. Ethics

All animal experiments were approved by the animal ethics committee, University of Manitoba.

3.7. Animals

Sprague-Dawley rats, females, weighing 300 ± 10 grams were used for all the animal experiments. All rats were purchased from the veterinary facility, University of Manitoba. Animals were housed individually in transparent cages in rooms with 12 h day and night cycles where the room temperature was maintained at 23 ± 2^0 C. Animals were supplied with food and water ad libitum.

Toxicity studies were performed using a small group of animals (3 animals/ dose/antibiotic) to determine the ability of the animals to tolerate the ultra-high doses of aminoglycoside (gentamicin and tobramycin) as well as to evaluate the possible side effects of these doses. These studies were comprised of two major experiments; an invasive, short-term study (9 hours) and non-invasive long-term study (180 days). For the both studies, gentamicin and tobramycin at 80 and 160 mg/kg doses were used. A total of 6 animals for each ultra-high dose of aminoglycoside was used (3 animals from gentamicin group + 3

animals from tobramycin group) for the invasive study. In the non-invasive study, 4 animals (2 animals from gentamicin group + 2 animals from tobramycin group) were used for determining the renal, oto- and vestibular toxicity (auditory and vestibular studies).

The other key study involved an animal infection model. Septic shock was established in SD rats using *E. coli* Bort and the dose proportional reduction of bacterial count from blood and vital organs was determined. The ability of ultra-high doses of gentamicin at 80 mg/kg and 160 mg/kg doses to eliminate the bacterial load from the blood and vital organs were assessed. For this study, 6 animals/ group were used. Cefotaxime was used as a comparator (explained in detail in further below section). Tobramycin was not assessed in the septic shock model.

3.7.1. Procedures on animals

3.7.1.1. Anesthesia: Animals were anesthetized with an intramuscular injection of ketamine/acepromazine (100 mg/kg/ 2.5 mg/kg) in toxicity studies and using 5% isoflurane in the septic shock model. 2 - 3 % Isoflurane was used as maintenance anesthesia.

3.7.1.2. Body temperature monitoring:

Anesthetized animals were placed on a homeothermic blanket (Harvard Apparatus inc., Massachusetts, USA) to maintain body temperature. Body temperature was monitored via a digital rectal thermometer (Cole Parmer Inc., Illinois, USA) at regular intervals and maintained at 37^0 C.

3.7.1.3. Fluid resuscitation

During the early hours of septic shock (11 – 12 h post-infection), the jugular vein and carotid artery of the animals were cannulated. Animals were administered normal saline and were allowed to stabilize for 20-30 minutes. Gentamicin (in 2 mL saline) was administered as a slow infusion through the jugular vein over 20 minutes. Additional saline was intermittently administered to maintain the mean arterial pressure (MAP) of animals above 60 - 65 mmHg throughout the experiment (normal MAP of SD rats is 90 mmHg).

3.7.1.4. Intubation for ventilator support

Once fully anesthetized, the animals were administered an intravenous (IV) injection of atropine (0.05 mg/kg) to reduce salivary/bronchial secretions and maintain heart rate. In the invasive, short-term study and in the septic shock study, the trachea was exposed by blunt dissection. An incision was made in between the tracheal rings in the proximal part of the trachea. A 2 inch, 14 gauge polyethylene catheter (Terumo Medical Corporation, New Jersey, USA) was inserted carefully into the trachea, 2 cm towards the lungs and the tubing was secured to the trachea using 4 - 0 silk sutures (Ethicon Inc., Ohio, USA). In the non-invasive, long-term study, animals were tracheally intubated by the oral route.

Animals were allowed to breathe spontaneously through the catheter while being electrocardiographically and oxygen saturation monitored. After administering ultra-high doses of aminoglycoside antibiotics, animals were given ventilator support (Columbus instruments Inc., Ohio, USA) at a rate of 80 breaths per minute (brpm) (2 mL tidal volume) for the duration of neuromuscular paralysis. Once the animals recovered with demonstrated evidence of spontaneous breathing, ventilator support was withdrawn. At that point, spontaneous ventilation occurred through the tracheostomy catheter. The invasive study and

septic shock model were termination studies where the animals were euthanized after completion of the experiment. In the non-invasive study, the oral intubation tube was carefully removed once the animal was stable. In the non-invasive study (long-term study), animals were observed for 180 days after administration of ultra-high doses of aminoglycosides.

3.7.1.5. Cannulation of urinary bladder

A 3 french urinary catheter (Braintree Scientific Inc., Massachusetts, USA) was placed into the bladder of the anesthetized animals to collect urine at specific time points as per the experimental requirement (time points are addressed in the procedures described in other sections of the methods).

3.7.1.6. Carotid artery cannulation

During invasive studies, animals were monitored for heart rate, systolic pressure, diastolic pressure, mean arterial pressure and other physiological parameters using a heart pressure analyzer (Digimed, HPA 400, Micro-Med Inc., Kentucky, USA). For these invasive observations, the carotid artery of the animals was cannulated [410].

The carotid artery was exposed by blunt dissection and was carefully separated from the adjacent connective tissues. The artery was ligated away from the heart using 4-0 silk suture (Ethicon Inc., Ohio, USA). A second ligature was placed one centimeter proximal to the already ligated area (towards the heart) but the ligature was not initially tightened. The carotid artery was clamped proximal to the second loose ligature using a vascular clamp close to the heart. An incision was made using a micro-scissors and a 3 french polyurethane

catheter (Instech Laboratories Inc., Pennsylvania, USA) was carefully placed towards the heart through the carotid artery and slid up to the clamp. The second ligature was then tightened and a second vascular clamp (loose fitting) was placed on the artery over the tube to keep the tube from exiting. The proximal vascular clamp was then released and the tubing was further threaded 2 cm into the artery. The second ligature was tightened more (but not enough to cut off the flow). The flow in the catheter was tested with a syringe of saline and the blood pressure monitor was checked (to see if it was reading the blood pressure correctly). The second ligature was then knotted. The skin was sutured and the catheter was secured to the skin with surgical tape. The catheter was filled with 10% heparinized saline to prevent clotting of blood and the other end of the catheter was connected to the blood pressure transducer (TXD – 310, Micro-Med Inc., Kentucky, USA) which was in turn connected to pre-calibrated heart pressure analyzer. This analyzer was in turn connected to the computer (Dell Inc. , USA) and the software (DMSI-400/4, Version 3.4, Micro-Med Inc., Kentucky, USA) provided by the manufacturer was used to record the readings from heart continuously from the start of the experiment. All readings were saved for further analysis.

3.7.1.7. Jugular vein cannulation:

The jugular vein was cannulated using 3 french polyurethane catheter (Instech Laboratories Inc., Pennsylvania, USA) [410]. The jugular vein was exposed by blunt dissection and separated from the surrounding connective tissue. The remaining procedures were similar to that of carotid artery cannulation. A small incision was made on the jugular vein and a catheter prefilled with 10% heparin was carefully inserted into the jugular vein towards the heart. The catheter was inserted 2 cm into the jugular vein and then stabilized

and sutured to the vein using silk sutures. Jugular vein cannulation was necessary to administer the ultra-high gentamicin and tobramycin doses as well as to administer intermittent fluid support.

3.7.1.8. Blood Collection and processing

Approximately $200 - 300$ μL of blood was collected at specified time points from each animal (time points are mentioned in methods for each procedure). Equal volumes of normal saline were administered to maintain intravascular volume and blood pressure. Immediately after collection, blood was centrifuged at 14000 rpm for 10 minutes and serum was separated and stored in $- 80^0$ C freezer (So-low, Environmental Equipment Inc., Ohio, USA) until assayed. In the septic shock model, blood samples were also used to determine bacterial counts from treated and control animals as well as to determine the serum aminoglycoside, creatinine, lactate and cytokine concentrations.

3.7.1.9. Non-invasive procedures

A pulse oximeter (STARR Scientific Corp., Pennsylvania, USA) was affixed to the paw of the study rat for non-invasive observation of heart rate, breath rate, and arterial O_2 saturation. The pulse oximeter was in turn connected to the computer and all values were saved on the hard drive for further analysis. A CODA tail cuff analyzer (Kent Scientific Co., Connecticut, USA) was used to determine pharmacodynamic parameters non-invasively).

3.7.2. Assays
3.7.2.1. Serum creatinine levels

Renal injury due to ultra-high doses of aminoglycosides can be assessed by measuring serum creatinine levels in animals [411]. After instrumentation, aminoglycoside antibiotics at 80 and 160 mg/kg (gentamicin and tobramycin) were infused over 20 minutes using a calibrated infusion pump (Harvard Apparatus Inc., Massachusetts, USA) into the jugular vein. Blood and urine samples were collected at different time points based on the particular experiment. Kidney (renal clearance) function was determined by measuring serial serum creatinine levels using a commercial kit (ARBOR Assays - KB02-H2). The lower limit of detection using this kit was 0.081 mg/dL.

3.7.2.2. Neutrophil Gelatinase Associated Lipocalin 2 (NGAL) Assay

Urinary NGAL was assayed using a rat NGAL Enzyme-Linked Immunosorbent Assay (ELISA) kit from Abcam Inc. (ab119602). The range of detection using this kit is 156 pg/mL – 10,000 pg/mL.

3.7.2.3. TUNEL Assay:

Apoptotic cells can be detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in which the free 3′-OH terminal of fragmented DNA are labeled with modified nucleotides [412, 413]. TumorTACS^{TM} Kits (Trevigen Inc, 4815-30-K) were used for identifying apoptosis. All TUNEL-positive cells will be pyknotic, with condensed chromatin and a brown nucleus. The number of tunel positive cells in the ultra-high dose aminoglycoside treated samples was compared with the positive control (provided by the kit) as well as the untreated control (negative control). Depending upon the number of apoptotic cells in the test samples and controls, the toxicity of the doses was determined. This assay

was performed in collaboration with Dr. Sabine Hombach-Klonisch, Department of Anatomy, University of Manitoba.

3.7.2.4. Gentamicin assay:

Serum gentamicin concentrations were determined by using 'CEIDA Gentamicin II Assay kit' from Thermo Scientific. This work was done in collaboration with the Clinical Chemistry Department, Diagnostic Services Manitoba, Health Sciences Center, Winnipeg, Manitoba. The range of detection by this kit is 0.2 – 12 mg/L.

3.7.2.5. Cytokine Assay

Key cytokine levels involved in septic shock were determined from experimental animal serum using a multiplex ELISA kit from QUANSYS (Rat cytokine - inflammation (9-plex) – 110451RT). The cytokine assay was performed in collaboration with Dr. Steven Opal, Department of Medicine, Brown University, USA. The upper and lower limit of quantification of different cytokines along with the limit of detection is presented in **table 7.**

3.7.2.6. Lactate Assay

Serum lactate levels were determined using L-Lactate assay kit (Abcam – ab65330). The sensitivity of this kit is > 0.001 mM and the range of detection is 0.001 mM – 10 mM.

3.7.2.7. Histology studies

Following the final study time point, all animals were euthanized by 100 mg/kg pentobarbital. Kidneys and other organs were harvested and stored in 10% buffered formalin

bottles for later analysis (submerged fixation). The temporal bones from all the animals were dissected out and the complete cochlea was isolated and was stored in 10% buffered formalin. All histology studies were performed by an experienced technician from the Department of Anatomy, University of Manitoba. The tissue samples were assessed by Dr. Hombach, Assistant Professor, Department of Anatomy, University of Manitoba.

The cochlear samples were decalcified using a commercially available bone decalcification kit (Cal-Ex, CS510-1D, Fisher Scientific) and were fixed in 10% buffered formalin. These samples along with kidney samples were subsequently embedded in paraffin blocks and 4 um sections were cut. The paraffin sections were then stained with Haematoxylin and Eosin staining (H & E staining) to assess for necrosis or apoptosis along with other toxic morphological changes in the endothelial cells of kidney as well as in the organ of corti.

3.7.3. Preliminary pilot electromyogram (EMG) study

Before initiating our research program, we had to determine if the animals were transiently paralyzed by ultra-high dose aminoglycosides and whether they could recover if supported by a ventilator during the course of paralysis. We also wanted to document the loss in neuromuscular activity by electromyogram study (EMG study). This work was performed in collaboration with Dr. Phillip Gardiner, Department of Physiology, University of Manitoba. Preliminary data were generated in a single SD rat weighing 300 ± 10 grams. The animal was anesthetized by ketamine/xylazine anesthesia (100mg/kg and10 mg/kg) by intraperitoneal route. Atropine was administered intraperitoneally at 0.05 mg/kg to prevent secretions in the trachea. Animals were intubated using a 14 G catheter by tracheotomy. The

jugular vein was catheterized using a polyurethane catheter. The animal was secured to the

dissection table using surgical tape on the paws and thighs. The hair around the

gastrocnemius muscle was removed using clippers and disinfected using 70% alcohol and

iodine. Following skin incision and blunt dissection, the gastrocnemius muscle was exposed.

Bipolar, ball silver electrodes were placed on the surface of the gastrocnemius muscle and the

sciatic nerve was stimulated using a bipolar silver electrode, using 1 ms square-wave pulses at

supra-max voltage, at 1/sz [414]. A baseline EMG was recorded using custom-made EMG

amplifiers. Gentamicin, at 80 mg/kg dose, was administered as a 20 minute infusion through

the jugular vein. The animals were ventilated using an animal ventilator (Columbus

Instruments, Columbus, OH) at 2 mL tidal volumes (70 – 80 breaths/minute). From this point

forward, for every 5 minutes, an EMG wave was recorded. The animals were observed for 60

minutes. The animal was euthanized by intravenous administration of 100 mg/kg dose of

pentobarbital at the end of the study.

3.7.4. Invasive, short-term toxicity study: Time course of paralysis and renal injury with ultra-high dose aminoglycoside therapy in normal, healthy SD rats

We wanted to examine the effect of a single ultra-high dose of 80 and 160 mg/kg of

aminoglycoside with respect to neuromuscular paralysis (as measured by return of

spontaneous ventilation), hemodynamic stability and renal function (serum creatinine) as well

as measure serial aminoglycoside levels in normal healthy anesthetized and ventilated rats

over a 9 hour period (**figure 18**).

Groups of 3 SD rats/dose/antibiotic were used in this study. Animals were

anesthetized with an intramuscular injection of ketamine/acepromazine (100 mg/kg + 2.5

mg/kg). Urinary bladder catheterization, body temperature monitoring and non-invasive hemodynamic monitoring were performed. The jugular vein catheter and carotid artery catheter were placed and the physiological parameters were recorded throughout the experiment using heart performance analyzers (HPA) (Digimed, HPA 400, Micro-Med Inc., Kentucky, USA).

After instrumentation, aminoglycoside antibiotics at 80 and 160 mg/kg (3 animals gentamicin per dose and 3 animals tobramycin per dose = 6 animals per dose) were infused over 20 minutes using a calibrated infusion pump (Harvard Apparatus Inc., Massachusetts, USA) into the jugular vein. The animals were directly observed to ascertain the duration of aminoglycoside-induced neuromuscular paralysis based on ventilator dependency and return of spontaneous respiration. Blood and urine samples were collected at baseline (10 minutes post dosing designated as time 0), 0.75 h, 1.5 h, 3 h, 6 h and 9 h and processed as described earlier. After the respiratory muscle function was re-established, ventilatory support was withdrawn and animals were allowed to breathe spontaneously through the tracheostomy tube. Animals were maintained under anesthetic plane by using a supplemental dose of anesthesia (ketamine 10 mg/kg - subcutaneous (SC) route) for up to 9 hours. Animals were euthanized after 9 hours and organs collected as previously specified. Serial blood and urine samples were assayed for serum creatinine and NGAL respectively.

3.7.5.   Non-invasive, long-term toxicity study (180 days): Assessment of oto-vestibular toxicity (auditory brainstem responses – cochlear and vestibular toxicity) and renal toxicity associated with ultra-high dose aminoglycoside therapy in normal, healthy SD rats:

The ability of the animals to thrive after an ultra-high dose of aminoglycoside antibiotics and ototoxicity and renal toxicity associated with these doses were evaluated. In our study, single, ultra-high doses of 80 and 160 mg/kg aminoglycoside antibiotics were administered intravenously to 4 (2 gentamicin + 2 tobramycin) SD rats per group. Auditory brain stem response (ABR) studies [415] were performed (courtesy of Dr. Brian Blakley, University of Manitoba, Winnipeg, Manitoba, Canada) to determine the hearing loss in the animals at the serial time points up to 180 days (6 months). Nephrotoxicity was assessed by measuring the serum creatinine concentrations as well as urinary NGAL levels at these times points as well. Vestibular toxicity was determined by behavioral studies at baseline and at 180 days. TUNEL assay was performed to determine the apoptosis in the endothelial cells of the kidneys using organs harvested at study termination (180 days).

Animals were anesthetized using ketamine and acepromazine (100 mg/kg and 10 mg/kg) and underwent tracheal intubation via the oral route for ventilator support. Ultra-high doses of aminoglycosides were administered by the intravenous route to a group of 4 SD rats per dose. Supplemental doses of anesthesia (ketamine – 10 mg/kg) were given by the SC route as needed. Once the animals became ventilator independent following resolution of paralysis, they were extubated and allowed to recover in a recovery cage.

Blood was collected from the tail vein and urine samples were obtained at Baseline day 0 (1 day before treatment), day 1 (1 day after treatment), day 7, day 30, day 60 and day 180. Urine samples were obtained by bladder catheterization on day 0 time point only and on all the remaining time points animals were placed in metabolic cages for 24 hours and urine was collected by passive drainage. Anesthesia was not required at these time points.

3.7.5.1. Auditory brain stem response study

At baseline (day before dosing) and on day 30, day 60 and 180 days, animals

underwent auditory brainstem response (ABR) testing. ABR testing was performed using the

intelligent hearing system. Animals were tested for their hearing ability at four different

frequencies ranging from 3000 Hz to 24,000 Hz. Three subcutaneous needle electrodes were

placed: a) active electrode under the test ear (positive) b) a reference electrode near the

contra-lateral ear (negative) and c) a third electrode in the left flank as a ground or neutral

electrode [416, 417]. Needle insertion and testing was performed while under

ketamine/acepromazine (100 mg/kg + 2.5 mg/kg intramuscularly) anesthesia with the animal

on a warming blanket to maintain body temperature at 35 - 37°C, in an Eckel AB2000 sound

booth (Eckel Industries of Canada Ltd., Ontario, Canada) with added copper electrical

shielding, grounding and lining with echo-reducing carpet. The general anesthetic was

needed so that the animal remains motionless during the 20 minutes procedure. Using high-

frequency transducers from Intelligent Hearing Systems (Intelligent Hearing Systems,

Florida, USA), 500 click stimuli were delivered at 21 sec^{-1} while averaging the 10 ms evoked

EEG waveform. The responses were band-pass filtered from 30-3000 Hz. using the system II

PC computer-based auditory research system from Tucker-Davis Technologies. (Gainesville,

Florida, USA) and stored on the hard drive for analysis. An ER10B+ microphone/dual sound

probe unit (Etymotic Research Inc., Illinois, USA) was placed in the treated ear of the animal

for stimulus delivery. Using differential recording, the pre-amplifier was used to amplify the

two channels X 100 before attenuation to optimize digitization before analog to digital

conversion. The auditory threshold was the highest level at which the AEP waveform

disappearance was recorded. A threshold shift of approximately 40 dB of sound pressure

level (SPL) at the specific frequency was considered as a significant hearing loss with aminoglycoside antibiotics [418]. Results of ultra-high aminoglycoside dosed animals were compared to saline administered control animals.

3.7.5.2. Vestibular toxicity

The disturbance of vestibular function was determined using a battery of tests that were developed for rodents. These tests have been successfully used to detect vestibular toxicity associated with various chemical and surgical procedures [419-422]. A set of 3 different behavioral studies was performed on day 0 (one day before dosing) and on the 180th day to determine the vestibular toxicity in ultra-high aminoglycoside dosed animals. Each test was performed 3 times with a gap of 2 hours and the mean value was recorded. All the three tests were performed on aminoglycoside treated animals, as well as saline-treated control animals [423-425]. The battery of behavioral tests used to determine vestibular functioning include;

        A.   Tail hanging reflex test

        B.   Contact inhibition of righting reflex test and

        C.   Air-righting reflex test

A.   Tail hanging reflex test:

For this test, animals were lifted by the tail. Normal animals tend to extend their forelimbs when they land on a surface, but the rats with impaired or damaged vestibular function bend vertically or try to crawl towards their tail leading to occipital landing.   In this experiment, the animals are scored from 0 – 2. Where,

0 – straight body posture with forelimbs extended towards the ground –

normal animal.

1 – Bending body slightly in a vertical direction - intermediate response.

2 – Bending the body continuously as well as crawling towards the tail –

severe response

B. Contact inhibition of righting reflex test:

For this test, the animals were held in supine posture and were dropped on a foam surface from a height of 20 – 30 cm. The responses are scored from 0 – 2. Where,

0 - Normal animals tend to right themselves in the air and land on their feet

1 – Animals fall sideward indicating intermediate response

2 – Animals completely fail in righting and tend to fall on their back – severe

response

C. Air-righting reflex test:

For this test, animals were placed in supine position and a hard, horizontal surface was placed in contact with their limbs and toes. The responses are scored from 0 – 2. Where,

0 – Animal rights itself once it comes in contact with the surface board – normal

animal

1 – Animal tries to right itself to some extent - intermediate response

2 – Animals tend to walk on their back - severe response

The final score was determined by adding the scores from each test. Animals exhibiting a final score of 5 - 6 were considered to have developed high level vestibular toxicity.

Animals with a score of 3 - 4 were considered to have partial/ minimal toxicity and those with a 0 - 2 score were considered normal or no toxicity.

3.7.5.3. Renal toxicity associated with ultra-high dose aminoglycoside therapy in normal, healthy rats

Serum creatinine and urinary Neutrophil Gelatinase Associated Lipocalin 2 (NGAL) are biomarkers of aminoglycoside-induced renal toxicity. Levels of both these biomarkers were measured on Day 0, 1, 7, 30, 60 and 180. Histology studies were performed on the kidneys at the end of the study (day 180) to determine the morphological changes. The histology sections were also used for 'Tunel assay' to determine apoptosis of the endothelial cells of the kidney which is an indication of renal toxicity [426].

3.7.6. Infection Studies

3.7.6.1. Standardization of septic shock model

The purpose of the study was to establish a peritonitis-induced septic shock model in SD rats using *E. coli* Bort in order to mimic human severe sepsis/septic shock. Three SD rats were used per each group. After induction of anesthesia as described below, animals were implanted with α-cellulose filled gelatin capsules holding a fibrin clot with 1×10^6 CFU of *E. coli* Bort. Group 1 animals were implanted with a single capsule, group 2 animals were implanted with 2 capsules and group 3 animals were implanted with 4 capsules. Animals were followed for up to 48 hours in order to assess suitability of the model for further work. Blood samples, 200 – 300 μl, were collected from the tail vein at multiple time points for quantitative blood culture.

Animals were closely monitored by veterinary staff who were independently responsible for the decision to euthanize animals if ethically required.

3.7.6.2. Impact of ultra-high dose gentamicin in a rat model of *E. coli* Bort-induced peritonitis-associated severe sepsis/septic shock model:

This study was designed to characterize serial hemodynamic responses, blood bacterial kill, serum lactate levels and serum cytokine concentrations as well as organ bacterial clearance after single doses of gentamicin in an experimental *E. coli* Bort induced septic shock model in rats. **Figure 19** indicates the overall sequence and timing of procedures. Animals were peritoneally implanted with *E. coli* Bort in a α-cellulose/fibrin clot encased with a gelatin capsule. These animals develop peritonitis and septic shock over a variable period of time depending on the number of implanted organisms and the use of supportive and antibacterial therapy.

The primary experimental groups were animals dosed with single doses of 5, 10, 20, 40, 80 and 160 mg/kg gentamicin. Cefotaxime was dosed at 15 and 60 mg/kg doses (single) as a comparator and a group of animals were used as untreated control. Another group of animals was used as a sham capsule (α- cellulose + fibrin clot devoid of bacteria) infected controls. Concentration-dependent elimination of bacteria by aminoglycosides was assessed in relation to the comparator and control groups.

Cefotaxime is dosed three times a day in severe infections [427]. In our study, we dosed cefotaxime at 60 mg/kg in rats, which is equivalent to 15 mg/kg dose in humans based on differences in rat and human hepatic/renal clearance [428, 429]. Since the total duration of

our experiment after dosing antibiotics was 9 h, only a single dose of cefotaxime was administered.

3.7.6.2.1. Capsule Preparation

3.7.6.2.1.1. Gelatin capsules

Size 4 gelatin capsules were obtained from Qualicaps Inc., USA. These capsules were sterilized by gamma irradiation (one million rads) using a cobalt source at the Manitoba Agriculture, Food and Rural Initiative, Veterinary Services Branch, Animal Health Center, Winnipeg, MB, Canada. Sterilized capsules were stored in the refrigerator until use.

3.7.6.2.1.2. 3.7.6.2.1.2. Inoculum

E. coli Bort was cultured overnight in 250 mL of sterile MHB in a shaker incubator (VWR International, Ontario, Canada) at 37^0 C. After sub-culturing in MHB and further incubation for 4 h at 37^0 C in the shaker incubator, the inoculum was prepared in 4% fibrinogen (Sigma, F8630). The optical density (OD) of the culture was adjusted to 0.080 – 0.085 at 625 nm using a spectrophotometer (Genesys, Thermo Scientific). The bacterial density at this OD yields 1×10^8 CFU/mL. This inoculum was further diluted 1:1 in 4% fibrinogen (Sigma, F8630) yielding 5×10^7 CFU/mL of inoculum. Bacterial counts were confirmed by serial dilution and plating on MHA plates. Plates were incubated for 24 h at 37^0 C and counts were recorded.

3.7.6.2.1.3. Procedure

Four percent fibrinogen (Sigma, F8630) was prepared in sterile saline. Thrombin (Sigma, T6634) was prepared in distilled water at a concentration of 10 IU/20 µL. Sterile capsules were opened up in the biosafety cabinet (sterile environment) and the base of the capsules was filled with α-cellulose (Sigma, C8002) (approximately 25 – 50 mg). 100 µL of inoculum containing 5×10^6 CFU was added to the α-cellulose matrix and immediately 20 µL of thrombin was added on to the inoculum forming a fibrin clot entrapping the organism. The capsules were recapped immediately and placed in 1.5 mL centrifuge tubes (Eppendorf Inc.) and transferred into liquid nitrogen, thereby flash freezing them. A set of 100 capsules was prepared at a time and stored in -80^0 C freezer for use as required.

3.7.6.2.1.4. ification of inoculum from capsule

The final experimental inoculum from the capsules was quantified to determine the $\log_{10}$ CFUs for consistency on one day after the preparation, 1 week, 1 month and every 3 months thereafter. A single capsule was thawed in 1 mL normal saline and serially diluted and plated on MHA plates in duplicate. The plates were incubated at 37^0 C for 18 – 24 hours and $\log_{10}$ CFUs were determined. Each capsule yields a bacterial load of 1×10^6 CFU (20% of the initial load) indicating a loss of viable bacterial count due to freeze-thawing as expected from previous studies [145].

3.7.6.2.2. Capsule implantation

Six female SD rats were used for each aminoglycoside dose and for each control group for this experiment. The animals were infected using 2 gelatin capsules containing a 4%

fibrinogen clot in an α-cellulose matrix laced with 1 x 10^6 CFU of *E. coli* Bort per capsule (ie

total 2 x 10^6 CFU of *E. coli* Bort per animal). Animals were anesthetized by using 5%

isoflurane. The hair at the surgical site on the abdomen was shaved using a clipper. The

surgical site was disinfected using approved disinfectants such as 70% alcohol and iodine.

Gelatin capsules (premade) containing the *E. coli* Bort were surgically implanted through a 0.75

cm incision into the peritoneum. The incision was closed in two steps using 4 - 0 sutures. The

rats received buprenorphine at 0.03 mg/kg SC at the end of capsule implantation surgery and

subsequent doses every 6 hours at 0.01 mg/kg. Animals were maintained in the anesthetic plane

by using 2% isoflurane throughout the implant process.

3.7.6.2.3. Observation and sample collection:

After infection, the animals were placed in empty cages with a heating pad below and

were allowed to recover from anesthesia. After complete recovery, the animals were transferred

back to their own cages and were supplied with feed and water *ad libitum*. Animals were

observed carefully for their behavior after implantation of capsules under the supervision of

experienced veterinarian Dr. Valerie Smid, Animal Facility, University of Manitoba, Winnipeg,

Manitoba. Animals were monitored for clinical signs of sepsis by a qualified veterinarian and

animal house technicians. General signs of severe infection/ sepsis such as piloerection,

hunchback posture, hypothermia, decreased or absent spontaneous movement, movement of the

animal only after being provoked etc. were observed. Early increased body temperature (>

38.2^0 C), tachycardia and tachypnea were other signs of severe sepsis and / or septic shock.

Hypothermia and bradycardia are noted later. Blood samples, 200 – 300 µl, were collected

from the tail vein at each time point (explained below) from all the animals. The decision to euthanize the animals humanely at any time point was independently made by the veterinarians.

3.7.6.2.4. Surgery and cannulations

Based on previous unpublished studies, after an implant of 2 x 10^6 CFU of *E. coli* Bort strain animals begin to enter early septic shock at about 12-14 hours post-implant. Therefore, 10-12 hours post-implant (sepsis induction), animals were studied. They were re-anaesthetized using 5% isoflurane and transferred onto a surgical table with a heating pad to maintain the body temperature at 35 ± 2^0 C throughout the study. The maintenance dose of inhalant anesthesia, isoflurane, was adjusted to 2%. After 5 minutes, the animals were checked for pedal reflex to determine the depth of anesthesia. Once the animals were on the anesthetic plane they were dosed with 0.03 mg/kg dose of atropine intravenously to reduce the mucous secretions in the trachea. The animals were observed for hemodynamic parameters such as heart rate, ventilatory rate, O_2 saturation etc. using a pulse oximeter (STARR Scientific Corp., Pennsylvania, USA) connected to the foot of the animals. Once the animal was in a surgical plane, blunt dissection was performed in the thoracic region, exposing the trachea. A tracheotomy was performed carefully and animals were intubated using 14 gauge polyethylene catheter (Terumo Medical Corporation, New Jersey, USA). The tubing was fixed at a position in the trachea by suturing it to the tracheal rings on the digital and proximal ends. The animals were ventilated using animal ventilator (Columbus instruments Inc., Ohio, USA) at 80 brpm. Here, the level of isoflurane anesthesia was reduced to 1.5 - 2%. Animals were surgically instrumented with carotid and jugular catheters (3 French polyurethane catheters (Instech Laboratories Inc., Pennsylvania, USA)). The urinary bladder was

catheterized using a 3 french urinary catheter (Braintree Scientific Inc., Massachusetts, USA). A rectal thermometer (Cole Parmer Inc., Illinois, USA) was placed to determine the body temperature. All these procedures were performed within 30 – 45 minutes. Animals were administered 0.01 mg/kg of epinephrine intramuscularly and then allowed to stabilize for 5 – 10 minutes. Intermittent doses of epinephrine were used whenever required to maintain the blood pressure and heart rate throughout the study. Relevant hemodynamic parameters (heart rate, systolic pressure, diastolic pressure and mean blood pressure) were measured continuously through a HPA (Digimed, HPA 400, Micro-Med Inc., Kentucky, USA) and pulse oximeter (STARR Scientific Corp., Pennsylvania, USA) and were recorded using software provided by the manufacturers.

3.7.6.2.5. Treatment

Gentamicin at 5, 10, 20, 40, 80 and 160 mg/kg doses and cefotaxime at 15 and 60 mg/kg doses were administered to animals through the jugular vein over 20 minutes using an infusion pump (Harvard Apparatus Inc., Massachusetts, USA). Untreated control animals and sham capsule infected animals were administered heparinized normal saline. Baseline hemodynamics (heart rate, systolic pressure, diastolic blood pressure and mean blood pressure) were assessed. Blood and urine samples (approximately 300 µL) were collected at baseline (20 minutes before treatment), 0 h (10 minutes after dosing), 0.75 h (45 minutes), 1.5 h, 3 h, 6 h and 9 h intervals. Animals were euthanized by intravenous injection of 100 mg/kg of pentobarbital following the final blood and urine collection at 9 hours after the initiation of dosing.

3.7.6.2.6. Qualitative culture of blood and tissue samples

Once the animals were euthanized, the peritoneum (the site of infection) was opened up and peritoneal fluid was collected using a sterile syringe. The *E. coli* Bort count was determined by serial dilution and plating in duplicates on MHA plates. The remaining vital organs such as kidneys, spleen, liver and lungs were collected, washed thrice in 10 mL normal saline. A portion of all the organs was placed in 10% buffered formalin to be used for histological studies. One gram of each organ was homogenized in 1 mL of sterile normal saline using tissue homogenizer (Thermo Fisher scientific Inc., Massachusetts, USA). The homogenates were serially diluted (for 6 – 8 dilutions) in normal saline with 100 μL and 10 μL of the homogenates plated in duplicate on MHA plates. A direct sample (100 μL) of the homogenate was also spread on the MHA plates. Bacterial counts were enumerated by incubating the plates at 37^0 C for 18 – 24 hours. The limit of detection of bacterial counts from tissues was 100 CFU/gram of tissue.

A portion of the blood sample was used for determination of bacterial counts. Blood samples were washed 3 X in 1 mL saline by centrifugation to remove/ wash out gentamicin from the blood. After the final wash, the supernatant was discarded and the volume was made up to the original volume by adding saline. The blood was serially diluted in normal saline (6 – 8 dilutions) and 100 μL and 10 μL of each diluent was plated on MHA plates. Direct blood samples (100 μL) were spread on the MHA plates to determine the counts from blood. The plates were incubated for 18 – 24 hours at 37^0 C and the mean bacterial counts were determined. Limit of detection of bacterial counts was 100 CFU/mL of blood.

3.7.6.2.7. Assay for biomarkers:

Serum creatinine levels were determined from all the treated and untreated animals using the serum creatinine detection assay kit from ARBOR Assays (KB02-H1). Lactate levels in the serum were determined using L-Lactate assay kit (Abcam – ab65330), cytokine levels in serum were determined by a multiplex ELISA kit (Rat cytokine – inflammation 9-plex [Cat # 110451RT] Quansys Biosciences, Logan UT, USA) [430]

3.7.6.2.8. Hemodynamic parameters

A HPA and pulse oximeter (STARR Scientific Corp., Pennsylvania, USA) were used to determine the hemodynamic parameters in all the treated and control animals as described earlier. The values obtained from these analyzers were recorded and saved for later analysis.

3.7.6.2.9. Antibiotic concentrations and pharmacokinetic parameters

Gentamicin concentrations were determined from the serum as described earlier. Based on the concentrations obtained from the serum samples, pharmacokinetic parameters such as AUC, C_{max}, clearance and Vd were calculated using appropriate formulae as described below;

Pharmacokinetic parameters were calculated by using the following formulae;

1) Area under the concentration curve (AUC) was calculated by the trapezoid rule [431, 432].

2) The elimination rate (Ke) was determined from the slope of the linear-concentration vs time decay curves for each of the animals

3) Clearance (Cl) = Dose / Area under the curve (AUC)

4) Volume of distribution (Vd) (area) = Clearance (Cl) / Elimination rate (Ke)

5) Time taken to eliminate half of the drug from the body (T $_{1/2}$) = Linear-concentrations / Elimination rate (Ke)

The maximum concentration achieved in serum after 10 minutes of intravenous dosing was designated as the C_{max}.

Gentamicin concentrations were determined from animals in both the toxicity studies and infected animal studies as previously described. As expected, an aminoglycoside dose-dependent increase in the AUCs and Cmax was observed. Other (dose-independent) parameters such as clearance, volume of distribution, half-life and elimination rate were very similar at both 80 and 160 mg/kg doses in non-septic animals in the toxicity studies.

Notably, a difference in some key pharmacokinetic parameters was noted between normal animals and septic shock induced animals. At 80 and 160 mg/kg doses, there was a decrease in elimination rate and peak concentrations achieved in the septic shock animals compared to non-septic shock animals **(table 12 & table 16)**. There was a moderate increase in half-life and volume of distribution of the drug in septic shock animals compared to non-septic shock animals. There was no major difference in the AUC and clearance for similar doses of gentamicin in septic shock and non-septic shock animals. At the 160 mg/kg dose of gentamicin, the total time taken to achieve concentrations below the MIC of *E. coli* Bort (1 μg/mL) was approximately 14.3 h and 9.8 h in septic shock-induced and normal animals respectively (calculated based on the elimination half-life of gentamicin in these animals (i.e. approximately 9 half-lives)). This indicates that, by using 80 and 160 mg/kg doses of gentamicin in septic shock animals, serum concentrations fall below the recommended trough levels of 2 μg/mL within 11 and 12.9 h after dosing. Complete elimination of aminoglycosides from the rat can be anticipated within 24 h after dosing with the highest dose

(160 mg/kg) proposed. This suggests a very low probability for the trough concentration to be maintained for a prolonged period after dosing limiting the potential for nephrotoxicity.

In addition, it is worth noting the remarkable AUC/MIC and Cmax/MIC values generated with this method. These values are extremely high with either 80 or 160 mg/kg dosing scheme and are approximately 400 and 600 respectively. If these high concentrations of aminoglycosides can be shown to accelerate pathogen (e.g *E. coli* in this model) clearance rates in infected animals or humans, the potential for clinical benefit may exist [138].

3.7.6.2.10. etermination of cefotaxime concentrations by bioassay

Cefotaxime concentrations were determined using bioassay [433]. *M. luteus* ATCC 4698 was used as the test organism with a lower limit of quantification of 0.06 mg/L. A linear concentration range from 0.03 to 32 mg/L was used for assessment. The agar-well method was used to determine the zone diameters of *M. luteus* using standard concentrations of cefotaxime as well as from the serum samples collected at different time points from cefotaxime treated animals. The concentration of cefotaxime was determined at various time points for each dose by comparing the zone diameters from the treated groups with that of the standard zone of inhibition diameters at the different concentrations. Based on these concentrations, area under the concentration curve (AUC) / MIC (mg. h /L), peak concentration (C_{max})/ MIC (µg/mL) were calculated for the bacteria strain tested using appropriate formulae.

3.7.6.2.11. ata analysis

The occurrence of neuromuscular paralysis and respiratory depression after high doses of aminoglycosides has previously been reported [282, 283, 434]. In our invasive, short-term study, we wanted to determine if the animals enter neuromuscular paralysis and survive when supported by ventilator for the duration of paralysis. The sample size calculation was based on dichotomous data describing the occurrence or absence of neuromuscular paralysis after ultra-high dosing of aminoglycoside. In our studies, the sample size required was determined by following formula;

$$n = \frac{\log \beta}{\log p},$$

Where,

$1 - \beta$ is the power and is usually set at 80 % by researchers and β is usually 0.20. For our experiment we considered β to be 0.05 and the power to be 95 %.

p – represents the proportion of animals which did not have neuromuscular paralysis after ultra-high aminoglycoside doses.

Previous reported studies indicated that the majority of animals entered neuromuscular paralysis after high doses of aminoglycosides [282, 283, 403]. In our study, we assumed 70 % of the animals would enter neuromuscular paralysis after ultra-high doses of aminoglycosides. The number of animals required for a 95% chance to detect neuromuscular paralysis is as follows;

n = log 0.05/ log 0.3

= - 1.3010/ - 0.5228

= 2.48 = 3 animals per dose.

Potential differences between gentamicin and tobramycin at similar doses was assessed using t-test. GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA was used for calculating statisitics. A p-value of $\leq$ 0.05 was considered significant. Animals from the gentamicin and tobramycin ultra-high dose groups were combined together (3 + 3= 6 animals) to determine the duration of neuromuscular paralysis at specific dose.

In our long-term, non-invasive studies, for the serum creatinine and urinary NGAL levels, blood and urine were collected repeatedly at different time points from both the treated and control animals. Repeated measures analysis of variance (ANOVA) was the statistical test selected as more than three groups (doses) were compared to analyze the differences between group means and their associated variance. The results obtained from treated groups were compared to control and to standard gentamicin dose (20 mg/kg). Dunnett's post hoc test was used along with repeated measures ANOVA for comparison of treated groups with the control and standard dose group for significance. GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA was used for statistical analysis for all of our experiments except for ABR studies. Results from treated and untreated animals were compared for statistical significance. Statistical significance was calculated using repeated measures analysis of variance (ANOVA) followed by Dunnett's test. The p-value of $\leq$ 0.05 was considered significant.

Data on auditory brainstem responses were analyzed by the particular frequency level being tested (3000 Hz, 6000 Hz, 12000 Hz and 24000 Hz). Left and right ears were

considered clustered (i.e., repeated) observations of a case. Generalized estimating equations (GEE) with an identity link and normal distribution were run on the data using SAS version 9.1 (Cary, NC, USA) with saline administered controls, 80 mg/kg and 160 mg/kg of aminoglycoside doses as a between-subjects factor and day (0,180) as a repeated measure, as well as the interaction between dose and day. GEE models account for the clustered nature of multiple observations (i.e., left and right ear) and also provide robust standard errors of effects, for a more accurate estimate of effect significance. A significant interaction would indicate that the change over time depended on which dose an animal was administered. A p-value of ≤ 0.01 was considered significant.

Ultra-high dose aminoglycoside treatment is a novel strategy in treating septic shock animals. There is no data available/ reported on the antimicrobial efficacy of the ultra-high dosing aminoglycoside regimen in any experimental septic shock/ infection model. However, our previous published studies suggest that the highest standard deviation of 0.8 log CFU/mL in bacterial count reduction in our murine septic shock model with ampicillin treatment [145]. To be conservative, the standard deviation for log CFU/mL reduction was estimated to be 1.3 (0.5 log more) for our studies in rats for purposes of calculating the sample size. For the septic shock model, the sample size for comparing treated groups with infected control group with a 95 % confidence limit was determined by following formula [435];

$$n = 1 + 2C\left(\frac{s}{d}\right)^2$$

Where;

s – standard deviation

d – magnitude of difference the researcher wants to detect, also called the 'effect'.

C – constant dependent on value of α and β selected.

Note that for $\alpha = 0.05$ and $1-\beta = 0.9$, C is 10.51 and 2C would be 21

In our study, standard deviation (s) is considered to be 1.5 log CFU/mL and the magnitude of difference (d) expected to be achieved is 3 log CFU/mL.

Therefore,

$$n = 1 + 21 \, (1.3/3)^2$$

$$= 1 + 21(0.43)^2$$

$$= 1 + 3.8829$$

$$= 4.8829 \text{ or } 5 \text{ animals per group}$$

As we are working with a septic shock model, there is a risk of losing animals prematurely. To compensate, 1 more animal was added to each group.

n = 6 animals per group

In the septic shock model, the primary endpoint was the number of surviving bacteria in the blood and organs of the treated and untreated control animals expressed as $\log_{10}$ CFU/mL and $\log_{10}$ CFU/gram respectively. The efficacies of the different doses were compared by repeated measures ANOVA followed by a Dunnett's test for comparison of treated groups with untreated control for the blood bacterial counts. For bacterial counts in the vital organs,

ANOVA followed by Dunnett's test was used to determine the significance. p-value of $\leq$ 0.01 was considered significant between the groups in all the experiments. GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA was used for stastical analysis.

All data in graphs is depicted as mean ± standard error. Data in tables and text is described as mean ± standard deviation.

CHAPTER 4

4.   Results

4.1. *In vitro* studies: Determination of MIC by broth micro-dilution method

The minimum inhibitory concentrations of gentamicin, tobramycin and cefotaxime for

E. coli Bort (ATCC 700973) were determined. The MICs are tabulated in **table 8**.

4.2. *In vivo* studies

4.2.1.   Pilot electromyogram (EMG) study:

In the pilot toxicity study, the normal functioning and response of gastrocnemius

muscle to electrical stimulus was recorded using EMG. The baseline EMG wave was

recorded after stimulating the sciatic nerve and recording the muscle response. In the EMG

wave recording, the initial electrical stimulus appeared as a single sharp peak, which is

considered as stimulus artifact followed by an M - wave and H-reflex. At baseline, the peak

of M-wave was 1000 mV after the stimulus and H-reflex was 500 mV. Once half the total

dose of gentamicin 80 mg/kg dose (i.e. 40 mg/kg) was infused, the sciatic nerve was

stimulated and a decrease in the amplitude of M-wave as well as the H-reflex was observed,

indicating the onset of neuromuscular dysfunction. The peak of the M-wave was 500 mV

after stimulus at this point (10 minutes post-infusion) and the H-reflex was approximately 50

mV. The animal was able to breathe on its own. The M-wave and H-reflex disappeared

entirely after complete infusion of 80 mg/kg dose, indicating a complete absence of response

from the muscle after stimulation consistent with complete neuromuscular paralysis. The

animal required ventilator support by this time point (20 min after infusion). The M-wave

and H-reflex remained absent at 5 minutes, 10 minutes and 15 minutes after dosing. But, at

20 minutes after dosing, there was a small response from the muscle when stimulated. At this point, the peak of M-wave was 350 mV and H-reflex was restored to normal. Even though the muscles started to respond to stimulus, the animal was still ventilator dependent. At 30 minutes post-infusion, there was > 50% response from the muscle compared to baseline. The M-wave peak at this time point was approximately 650 mV and the H-reflex response was normal. The animal was able to breath by itself and ventilator support was withdrawn. The EMG waves, heart rate and blood pressure at baseline, 10 minutes after initiation of gentamicin 80 mg/kg infusion (10 minutes after start), after completion of 80 mg/kg dose (20 minutes after start), 10 minutes post dosing, 20 minutes post dosing and 30 minutes post dosing are presented in **figure 20a & 20b**. The corresponding heart rate and blood pressure are also recorded and tabulated. This experiment confirms that a) ultra-high dose aminoglycoside generates neuromuscular paralysis b) that this neuromuscular paralysis is transient and c) the animal will survive if ventilator support is provided during neuromuscular paralysis.

4.2.2. Invasive short-term (9 h) toxicity study:

4.2.2.1. Neuromuscular weakness and ventilator dependency

Animals were found to become ventilator dependent after single, ultra-high dose of aminoglycosides in invasive (and non-invasive) studies. At 80 mg/kg doses of both the gentamicin and tobramycin, the animals entered neuromuscular paralysis during or immediately after dosing. Animals entered neuromuscular paralysis within 10 minutes after initiation of 160 mg/kg dose with both the aminoglycoside antibiotics.

There was no statistically significant difference in the time duration of neuromuscular paralysis between equivalent doses (80 and 160 mg/kg) of gentamicin and tobramycin (**table 9a**). For each individual aminoglycoside, there was a trend towards a longer duration of paralysis with the 160 mg/kg compared to the 80 mg/kg dose (gentamicin, p=0.015; tobramycin p=0.0591, Students t-test). The mean time duration of paralysis and ventilator dependency at 80 mg/kg dose of aminoglycoside gentamicin + tobramycin) antibiotics was 32.16 ± 9.04 minutes and was 47.1 ± 3.54 minutes with 160 mg/kg dose (p=0.0361, Students t-test) (**table 9b**). The average duration of neuromuscular paralysis and ventilator dependency with both aminoglycoside antibiotics at each ultra-high dose are recorded (**table 9a. & 9b.**). As expected, there was no neuromuscular paralysis and ventilator dependency observed with untreated/saline administered control animals.

4.2.2.2. Renal injury: Serum creatinine levels

Sequential serum creatinine levels after both 80 and 160 mg/kg dose of aminoglycosides (3 animals of gentamicin + 3 animals of tobramycin) up to 9 h in comparison to saline-treated controls are presented in **figure 21**. . There was no statistically significant difference in serum creatinine between the aminoglycoside dose of 80 mg/kg compared to the control. There was a statistically significant difference between control and 160 mg/kg of aminoglycoside ($p < 0.01$). Despite this, all mean values remained well within the normal limits of 0.30 to 1.4 mg/dL (in rats) throughout the study. The long-term study examined serum creatinine at 24 hours to 180 days.

4.2.2.3. Hemodynamic parameters

Hemodynamic parameters were followed to ascertain any change in physiological stability due to ultra-high doses of aminoglycosides. A HPA was used to follow the mean arterial pressure, maximum (systolic) pressure, minimum (diastolic) pressure etc. Serial hemodynamic data are tabulated in **table 10 and table 11**. A pulse oximeter recorded heart rate, respiratory rate, % O_2 saturation of blood, ventilatory distention and pulse distention. These data after 80 and 160 mg/kg doses of aminoglycosides are presented in **figure 22 and 23**.

There was a statistically insignificant decrease in the heart rate immediately after intravenous infusion of 80 and 160 mg/kg doses of aminoglycoside. The heart rate tends to increase from 45 minutes (0.75 h) to 1.5 h after dosing and then normalize. The mean arterial pressure was usually maintained above 60 mmHg up to 4 to 6 h after dosing at both the doses and then fell below 60 mmHg. Neuromuscular paralysis was confirmed through ventilator dependency and a slight decrease in %O_2 saturation before ventilator dependency.

4.2.2.4. Gentamicin concentrations from serum

Maximum serum concentration (C_{max}) achieved by gentamicin at 80 and 160 mg/kg doses in normal animals were 423 ± 21 µg/mL and 616 ± 36.6 µg/mL respectively. The area under the concentration curve (AUC_{tot}) was 426 ± 58.5 mg*h/Liter and 725 ± 50.5 mg*h/Liter at 80 mg/kg and 160 mg/kg respectively (**figure 24a**). However, the clearance, elimination rate, $T_{1/2}$ and volume of distribution remained same at both the doses (**figure 24b**). All the pharmacokinetic parameters were calculated and tabulated in **table 12.**

4.2.2.5. Histology of kidneys

To determine the possibility of nephrotoxicity within 9 h after dosing, kidneys were collected at the end point for histological examination. H & E staining demonstrated no evidence of toxic effects such as tubular necrosis, mononuclear infiltration, inter-tubular hemorrhages, and congestion and swelling of the glomerulus in ultra-high dose aminoglycoside treated animals. Results indicated that there was no overt renal toxicity associated with this experimental therapy (**figure 25 a - e**).

4.2.3. Non-invasive long-term (180 days) toxicity study

Ultra-high aminoglycoside dosed animals were observed for neuromuscular weakness/paralysis for the first few hours after single, ultra-high aminoglycoside doses. Evidence of oto-vestibular toxicity and renal injury associated with these doses was assessed over 180 days after initiation of therapy.

4.2.3.1. Neuromuscular weakness

Animals were observed for neuromuscular paralysis and ventilator dependency after ultra-high doses of aminoglycoside antibiotics. The animals exhibited a similar duration of neuromuscular paralysis as obtained from the invasive study confirming the previous results.

4.2.3.2. Ototoxicity
4.2.3.2.1. Auditory brain stem response study

The hearing sensitivity of ultra-high dose aminoglycoside administered animals was serially assessed at different frequencies (in comparison to baseline values) over 180 days (**figure 26 (a – c)**).

At 3000 Hz, the only significant effect was the main effect of dose ($p<0.01$), with doses of 80 mg/kg and 160 mg/kg having lower mean values than controls.   There was no effect of duration of time since dosing and the interaction between dose and day was not significant ($p=0.67$).   Post-hoc tests, however, indicated that the dose-effect may only be significant at day 0 ($p=0.03$) and not at day 180 ($p=0.58$). There is no significant difference in hearing ability of animals at this frequency tested on day 0 compared to day 180 at both the ultra-high aminoglycoside doses.

At 6000 Hz, the only significant effect was the main effect of dose ($p<0.01$), with doses of 80 mg/kg and 160 mg/kg having lower mean values than controls.   There was no effect of duration of time from dosing and the interaction between dose and day was not significant ($p=0.03$) (a statistically significant difference will only be considered when $p < 0.01$ due to multiple tests).   Post-hoc tests, however, indicated that there was a dose effect at 80 mg/kg with a $p <0.01$ but this effect was not observed with 160 mg/kg dose of aminoglycosides ($p = 0.420$). There was a significant difference in hearing ability of animals at this frequency tested on day 0 compared to day 180 with the 80 mg/kg dose, but this was not observed with the 160 mg/kg dose.

At 12000 Hz, the only significant effect was the main effect of dose ($p<0.01$) with doses of 80 mg/kg and 160 mg/kg having lower mean values than controls.   There was no effect of duration since dosing and the interaction between dose and day was not significant ($p=0.207$).   Post-hoc tests, however, indicated that the dose effect may only be significant at day 0 ($p<0.01$) and not at day 180 ($p=0.22$). There is no significant difference in hearing ability of animals at this frequency tested on day 0 compared to day 180 at both the ultra-high aminoglycoside doses.

At 24000 hZ, the only significant effect was the main effect of dose (p<0.01), with doses of 80 mg/kg and 160 mg/kg having lower mean values than controls.   There was no effect of duration since dosing and the interaction between dose and day was not significant (p=0.742).   Post-hoc tests, however, indicated that there was no dose effect on day 0 (p=0.120) and day 180 (p=0.402). There is no significant difference in hearing ability of animals at this frequency tested on day 0 compared to day 180 at both the ultra-high aminoglycoside doses.

Overall, no statistically significant difference was found in the hearing ability of the animals between the control group and single, ultra-high aminoglycoside groups at all the frequencies except for 80 mg/kg dose at 6000 Hz.

However, the possibility of auditory injury occurring at 6000 Hz at 80 mg/kg dose without having toxicity at higher frequencies is unlikely. Available data uniformly suggest hearing loss at higher frequencies first, followed by hearing loss at lower frequencies [436]. In addition, a similar hearing loss was not observed with 160 mg/kg dose at all the frequencies tested. This indicates that the toxicity observed at 80 mg/kg dose of aminoglycosides is most probably due to type I error.

4.2.3.2.2. Vestibular toxicity/ behavioral studies

The studies were performed in triplicate with a 2 hour gap between each test on day 0 and day 180. All the animals were tested for vestibular related toxicity by performing the tail hanging reflex test, air righting reflex and contact inhibition of righting reflex test.

All animals exhibited normal behavior on day 0 without any signs of vestibular related problems. On day 180, one animal in the gentamicin 160 mg/kg group exhibited a consistent

vertical bending (3 out of 3 times) in the tail hanging reflex test. The mean score was 1 for this test animal. One animal in the tobramycin 80 mg/kg dose group exhibited vertical bending twice in a set of three so the mean value was recorded as 0.67. The final scores in these animals were in the range of 0 – 2 hence are considered to be normal. All the animals exhibited normal behavior in all the three tests on day 180. The final scores are presented in the **table 13**.

Overall, there was no difference between the single, ultra-high aminoglycoside treated animals compared to that of controls by the end of the experiment. By combining the animals at 80 and 160 mg/kg doses, respectively, we have 4 animals at each dose for each aminoglycoside in total. Assessing the mean results of the 4 animals per each single, ultra-high dose, there was no obvious vestibular dysfunction with responses that were comparable to that of the saline treated control animals.

4.2.3.2.3. Histology of cochlea

The sections of cochlea were cut and stained using H& E staining. Inner ear fixation with formalin was insufficient (lack of perfusion fixation) and did not allow for the assessment of cellular and structural integrity of the organ of corti and the cochlear ganglion cells from H&E stained sections. The H & E stained cochlea are presented in **figure 27** (**A - E**). There were no morphological differences between the ultra-high dose aminoglycoside groups compared to the untreated control groups. Due to the inadequate fixation of the cochlea and low number of samples, further studies are required in a much larger group of animals per dose to definitively confirm these results.

4.2.3.3. Renal injury

4.2.3.3.1. Estimation of serum creatinine levels

Serum creatinine levels were determined from the animals at serial time points up to 180 days after aminoglycoside administration. The results from the experiment are presented in **figure 28**. Serum creatinine levels in all the treated and untreated animals were in the normal range throughout the 180 days. The aminoglycoside 160 mg/kg dosed animals exhibited a slight elevation in serum creatinine levels on 180th day (0.63 mg/dL) compared to other animals, but the creatinine levels were well in the normal range (0.35 – 1.4 mg/dL) [437]. There was no statistically significant difference between the control and treated groups measured by repeated measures ANOVA followed by Dunnett's test.

4.2.3.3.2. Estimation of NGAL levels from urine for acute renal toxicity

NGAL levels were determined from the urine of rats treated with ultra-high doses of gentamicin and tobramycin as well as from the control animals. Results are shown in **figure 29**. Urinary NGAL levels increased from 56.3 ± 22.4 ng/mL at baseline to 75.56 ± 23.8 ng/mL at 24 h post 80 mg/kg dose of aminoglycosides. With 160 mg/kg dose, at baseline, the urinary NGAL levels were 41.4 ± 10.8 ng/mL and after 24 h of therapy the levels were 54.2 ± 17.5 ng/mL. In the saline-treated control animals, NGAL levels at baseline were 33.9 ± 7.9 ng/mL and by 24 h time point the levels were 43.3 ± 14.8 ng/mL. By day 7 at 80 mg/kg and day 60 at 160 mg/kg dose the NGAL levels became similar to baseline. NGAL levels did not vary more than 2 fold throughout the experiment consistent with the absence of AKI. There was a small increase in early urinary NGAL in the ultra-high dose aminoglycoside dosing groups. However, there was no statistically significant difference between the control and

treated groups over the time points assessed (in part because the control group also showed a modest early increase in NGAL).

4.2.3.3.3. Histology of kidneys from non-invasive long-term toxicity study

The collected kidneys from the 180-day study were used for histology studies. The H & E stain revealed that there was no gross histological changes between the ultra-high dose aminoglycoside treated animals and untreated controls at day 180. Photomicrographs are not shown as they are entirely unremarkable.

4.2.3.3.3.1. NEL Assay

The kidney samples used for the H & E staining were also used for the TUNEL assay. A very low percentage of apoptosis was observed in both untreated controls and ultra-high aminoglycoside dosed groups. There was no significant difference between the number of apoptotic cells observed in control and both the single, ultra-high aminoglycoside dosed groups. Based on the limited sample size, qualitatively, we can infer that there was no significant apoptosis of endothelial cells due to ultra-high doses of aminoglycosides. Positive control slides indicate the apoptotic cells (the brown colored nucleus) for comparison. TUNEL assay photomicrographs are presented in **figure 30 (a – f).**

4.2.3.4. Hemodynamic parameters

Vital parameters such as heart rate, % oxygen saturation, ventilatory rate, ventilatory distention and pulse distention were recorded for the first 2 hours using a pulse oximeter after initiation of ultra-high dose aminoglycosides in the non-invasive, 180 day toxicity study. All

values remained within normal range for the duration of observation in all groups. Results are presented in **figure 31 (A - C)**.

In this experiment, the animals underwent tracheal intubation via the oral route. Once animals recovered from neuromuscular paralysis, they were extubated and followed for 2 hours. Subsequently, animals were observed for 180 days for ototoxicity and renal toxicity.

4.2.4. Standardization of septic shock in SD rats

The *E. coli* Bort is a serum resistant encapsulated strain and is highly virulent [406]. After surgical implantation of the capsules containing the *E. coli* Bort, the animals recovered within several hours and exhibited normal behavior initially. Symptoms of sepsis and septic shock occurred sequentially depending upon the number of capsules surgically implanted (number of organisms) into the peritoneum of the animal. Within few hours after infection, the animals exhibited an increase in body temperature $> 38^0$ C indicative of sepsis. Within hours, heart rate was found to increase followed by morbid state with decreased to absent movement in the cage and the movement of the animal only after being provoked were observed. Late symptoms of sepsis such as ruffled fur, piloerection, hunchback posture, hypothermia, morbid state and limited then absent spontaneous movement with increased respiratory rate were observed.

Group 1 animals implanted with one capsule exhibited the above symptoms after 18th hour. In this group, the animals had a blood bacterial density of 3 x 10^5 CFU/mL by 18th hour. These animals entered septic shock (based on inability to obtain an oximeter signal and the use of a tail cuff plethysmograph) at approximately 24 hours post implant with a blood bacterial

density of 2×10^6 CFU/mL. Due to their moribund state, animals were euthanized at about 36 hours.

Group 2 animals were implanted with 2 capsules and these animals exhibited symptoms of sepsis by 6-8 h post-infection with a blood bacterial concentration of 6×10^5 CFU/mL by the 9th hour. The animals entered septic shock with a blood bacterial concentration of 2×10^6 CFU/mL at 11 - 12 hours post implant. These animals were observed and euthanized at 20 hours post implant with a blood count of 8×10^7 CFU/mL.

Group 3 animals were implanted with 4 capsules and these animals entered sepsis within $4 - 6$ hours post infection with a blood bacterial concentration of 8×10^5 CFU/mL at the 6th hour. Septic shock occurred within 8 - 10 h post infection. These animals had a blood bacterial concentration of 2×10^8 CFU/mL by 12 hours post implant. Animals required euthanization by this time point due to their moribund state. The sequential blood bacterial concentration in the 3 groups is presented in **table 14 a - c**.

Group 1 and group 3 animals developed septic shock at about 24 h and 8 h after implantation of capsules respectively. Both these are impractical time points for logistic reasons. All groups exhibited a consistent increase in bacterial load from the start of the infection to the final assessment and were eventually euthanized for humane reasons. Group 2 animals, specifically exhibited consistent bacterial growth in blood with appropriate signs and symptoms of septic shock over time and, in addition, exhibited an ideal disease trajectory for study logistic purposes. Veterinary staff estimated the animals would have died due to septic shock by 20 to 24 hours (which was confirmed in our septic shock model where fully anesthetized untreated control animals died within 8 h after the start of the experiment i.e. 20 h after infection). All animals were autopsied and organs harvested for histological and analysis.

4.2.4.1. Histology of all organs from septic shock induced animals

Lungs, kidney, spleen and liver of the infected animals (with 2 capsule insertions) were harvested and used for histology studies. Tissue was fixed and H& E staining performed as described elsewhere All the samples were observed and are shown in **figure 32 (i – iv)**. A considerable number of bacilli were observed in the lungs, spleen, liver and kidneys with a large amount of neutrophil infiltrations into these organs consistent with disseminated bacterial infection.

4.2.4.2. Consolidated observations:

a) **Clinical observations:**

- Body temperature above 38.2^0 C (after 6 - 8 hours of infection).

- Tachycardia and tachypnea present (after 6-8 hours).

- Bradycardia observed (after 12 - 14 hours after infection) and confirmed with the inability of the pulse oximeter to identify the signals.

- Hypothermia observed (12-14 hours after infection).

b) **Microbiological observations:**

- Bacterial counts from peritoneal fluid (site of infection) confirmed to be *E. coli* Bort.

- Blood bacterial count was determined and organism confirmed as *E. coli* Bort.

- Bacterial counts from all the vital organs determined and confirmed as *E. coli* Bort.

c) **Histological observations:**

- Perivascular edema due to capillary leakage.

- large number of neutrophils, macrophages and rod-shaped bacteria (*E. coli* Bort, confirmed by culturing) in peritoneal fluid.

- Extensive neutrophil infiltration along with rod-shaped bacteria observed in the spleen, liver, lungs and kidneys of H & E stained organ sections.

Based on the progression of infection, signs and symptoms of severe sepsis/septic shock and the histology finding from the organs of these animals, we concluded that a 2 capsule (approximately 2 x10^6 organisms) implant model was appropriate for further study with ultra-high dose aminoglycosides.

4.2.5.   Gentamicin in *E. coli* Bort induced septic shock SD rats

4.2.5.1. Bacterial load and log$_{10}$ CFU reduction in blood

The bacterial concentration in the blood of all the animals at the time of antibiotic dosing (12 h post infection) was 6.26 ± 0.11 log$_{10}$ CFU/mL. The bacterial concentration in untreated controls at the end (9 h post sham dosing) of the experiment was 8.91 ± 0.37 log$_{10}$ CFUs/mL of blood (**figure 33**). Log$_{10}$ CFUs in blood after 9 hours of 5, 10, 20, 40, 80 and 160 mg/kg doses of gentamicin treatment were 5.98 ± 0.62, 4.9 ± 0.53, 4.38 ± 0.95, 2.33 ± 0.34, 0.90 ± 1.3 and below the detection limit at the end of the experiment respectively. The cefotaxime (15 and 60 mg/kg) dosed groups exhibited 3.25 ± 0.58 and 2.65 ± 0.53 log$_{10}$ CFU/mL in blood by the end of the experiment. The maximum log$_{10}$ CFU reduction observed with the 5 and 10 mg/kg doses of gentamicin was 3.79 and 3.92 within 1.5 h and 45 minutes (0.75 h) after dosing.   With the 20 and 40 m/kg dose the maximum log$_{10}$ CFU reduction observed was 5.48 and 5.98 within minutes after completion of dosing. At 80 and

160 mg/kg doses of gentamicin the bacteria from the blood were below the detection limit immediately after completion of intravenous infusion (> 6.26 $\log_{10}$ CFU reduction).

At 5, 10, 20, and 40 mg/kg doses of gentamicin the bacteria exhibited regrowth before the end of the study period and with 80 mg/kg dose there was modest regrowth (< 1 $\log_{10}$ CFU) at the end of the experiment (**figure 34**). At 160 mg/kg dose, the bacteria were below the detection limit from the blood throughout to the end of the study. The $\log_{10}$ CFU reduction compared to UTC after 9 h of dosing (endpoint) was 2.9, 4, 4.5, 6.6, 8.01 and 8.9 at 5, 10, 20, 40, 80 and 160 mg/kg doses of gentamicin respectively. Maximum $\log_{10}$ CFU reduction with cefotaxime at 15 and 60 mg/kg doses was 3.98 and 4.90 by 3 h and 1.5 h respectively. However, the animals from cefotaxime group died within 8 hours after treatment in both the groups.

With increasing gentamicin, there was a dose proportional decrease in $\log_{10}$ CFUs in the blood across time points ($p<0.001$). With ultra-high doses (80 and 160 mg/kg) of gentamicin, the bacteria were below the detection limit within minutes after dosing in contrast to the cefotaxime and standard dose aminoglycoside groups. There was a statistically significant difference between all the treated groups compared to UTC with a p-value < 0.01 by the end of the experiment. There was a statistically significant difference between the 20 mg/kg dose of gentamicin compared to both the ultra-high doses of 80 and 160 mg/kg of gentamicin with a p-value < 0.01.

4.2.5.2. Bacterial load and $\log_{10}$ CFU reduction in organs and peritoneal fluid
4.2.5.2.1. Bacterial load from kidneys

Gentamicin is eliminated almost entirely through the kidneys. It accumulates in the kidneys at higher concentrations than serum and other organs [438]. The bacterial count from kidneys was determined from UTCs as well as from treated animals 9 h after aminoglycoside dosing (**figure 35**). UTC exhibited 8.9 ± 0.46 $\log_{10}$ CFU/ gram of kidney at the end of the experiment (9 h). $\log_{10}$ CFU reduction of *E. coli* Bort from kidneys of animals treated with 5, 10, 20 and 40 mg/kg doses was 3.53, 4, 5.44 and 8 $\log_{10}$ CFU compared to UTC at the end of the experiment. Bacteria were below the detection limit from the kidneys after single, ultra-high doses of gentamicin after 80 and 160 mg/kg (≥ 8.9 $\log_{10}$ CFU compared to control) by the end of the experiment. $\log_{10}$ CFU reduction at 15 and 60 mg/kg of cefotaxime groups was 5.00 and 6.07 $\log_{10}$ CFU compared to UTC. A statistically significant difference was observed between treated and untreated controls (UTC) with a p-value < 0.01 by one-way ANOVA followed by Dunnett's multiple comparison test. There was a statistically significant difference between gentamicin 20 mg/kg compared to ultra-high (80 and 160 mg/kg) gentamicin doses with a p-value of < 0.01.

4.2.5.2.2. Bacterial load from spleen

E. coli Bort counts per spleen from UTC were 9.12 ± 0.75 $\log_{10}$ CFU at the end of the experiment (9 h post-dosing). A substantial $\log_{10}$ CFU reduction was observed with gentamicin doses compared to UTC (**figure 36**). Gentamicin at 80 and 160 mg/kg as well as cefotaxime at 15 and 60 mg/kg doses exhibited > 3 $\log_{10}$ CFU reduction in *E. coli* Bort from spleen. There was a $\log_{10}$ CFU reduction of 2.1, 2.40, 2.42, 2.86, 3.17 and 3.6 with 5, 10, 20, 40, 80 and 160 mg/kg doses compared to UTC by the end of the experiment. With

cefotaxime 15 and 60 mg/kg doses, there was a 3.55 $\log_{10}$ CFU reduction at both the doses compared to UTC.

A statistically significant difference was observed between treated and untreated controls (UTC) with a p-value of < 0.01 by one-way ANOVA followed by Dunnett's multiple comparison test. There was a no statistically significant difference between gentamicin 20 mg/kg compared to 40 and 80 mg/kg doses. A statistically significant difference was observed with a p-value of < 0.01 between gentamicin 20 mg/kg and 160 mg/kg groups.

4.2.5.2.3. Bacterial load from the liver

Untreated control animals exhibited a 9.7 ± 0.7 $\log_{10}$ CFU of *E. coli* Bort per gram of liver by the end point (**figure 37**). There was a > 3 $\log_{10}$ CFU reduction with 5 and 10 mg/kg doses by 9 h post dosing compared to UTC. The $\log_{10}$ CFU reduction with 20, 40, 80 and 160 mg/kg doses was 3.9, 4, 4.47 and 4.64 $\log_{10}$ CFU compared to UTC by the end of the study (9 h post dosing). $\log_{10}$ CFU reduction with cefotaxime doses at 15 and 60 mg/kg were 4.47 and 5.38. A statistically significant difference was observed between treated and untreated controls (UTC) with a p-value < 0.01 by one-way ANOVA followed by Dunnett's multiple comparison test. No significant difference was observed between gentamicin 20 mg/kg and single, ultra-high gentamicin doses (80 and 160 mg/kg).

4.2.5.2.4. Bacterial load from lungs

Bacterial counts from the lungs of untreated control animals were 9.2 ± 0.84 $\log_{10}$ CFU/gram of lung (**figure 38**). Compared to UTC, gentamicin at 5, 10, 20, 40, 80 and 160 mg/kg doses exhibited 3.4, 4, 4.3, 4.4, 4.86 and 4.81 $\log_{10}$ CFU reductions by the end of the study (9 h post-dosing). Cefotaxime exhibited a 3.43 and 5.48 $\log_{10}$ CFU reduction at 15 and

60 mg/kg doses compared to UTC. A statistically significant difference was observed between treated and untreated controls (UTC) with a p-value < 0.01 by one-way ANOVA followed by Dunnett's multiple comparison test. There was no statistically significant difference between gentamicin 20 mg/kg compared to ultra-high gentamicin doses (80 and 160 mg/kg).

4.2.5.2.5. Bacterial load from peritoneal fluid

Bacterial counts from the peritoneal fluid of untreated control animal exhibited   9.86 $\pm$ 0.56 $\log_{10}$ CFU/mL by end of the experiment. Gentamicin exhibited a dose proportional decrease in bacterial load from the peritoneal fluid (site of infection) compared to UTC with a p-value < 0.001 (**figure 39**). The $\log_{10}$ CFU reductions at 5, 10, 20, 40, 80 and 160 mg/kg doses of gentamicin were 0.5, 2.5, 2.9, 4.4, 5.25 and 5.85 compared to UTC. Cefotaxime at 15 and 60 mg/kg doses exhibited a $\log_{10}$ CFU reduction of 3.6 and 5 compared to UTC at the end of the experiment. There was a dose-dependent eradication of bacteria from the site of infection by the ultra-high dose gentamicin therapy. One-way ANOVA followed by Dunnett's multiple comparison tests revealed that there was a significant difference between the treated animals compared to an untreated control group with a p-value of <0.01 except for the 5 mg/kg dose. There was a significant difference between ultra-high gentamicin (80 and 160 mg/kg) dosed animals compared to gentamicin 20 mg/kg dose with p-value <0.01 by one-way ANOVA followed by Dunnett's multiple comparison test.

4.2.5.3. Serum creatinine levels

Serial serum creatinine levels were determined from UTC as well as from the treated and sham animals. In treated (except 10 mg/kg gentamicin) and sham animals, the serum

creatinine levels were well within the normal range of 0.35 to 1.4 mg/dL during the course of the study (**figure 40**). Serum creatinine levels at the 10 mg/kg dose of gentamicin and UTC at the start of the experiment were 0.84 mg/dL and 0.9 mg/dL and 1.5 mg/dL and 1.94 mg/dL by the end of the experiment respectively (indicating significant renal dysfunction in these animals due to septic shock). There was an increase in the serum creatinine levels in gentamicin 40 mg/kg dose, but was still below the normal levels in serum. The serum creatinine levels in the 20, 40, 80 and 160 mg/kg dosed gentamicin animals as well as sham capsule infected animals at the start of the experiment were in the range of 0.6 to 1.1 mg/dL and were 0.80 to 1.2 mg/dL by the end of the experiment. The serum creatinine levels in cefotaxime 60 mg/kg doses animals was 0.63 mg/dL at the start of the experiment and 1.04 mg/dL at the end of the experiment.

The serum creatinine levels in gentamicin treated groups were similar to that of cefotaxime dosed animals, indicating that there was no renal toxicity induced by gentamicin at all the doses tested in this model. There was a significant difference in the serum creatinine levels between UTC compared to 20, 40 80 and 160 mg/kg doses of gentamicin, cefotaxime 60 mg/kg dose and sham animals with a p-value of < 0.01 determined by repeated measures ANOVA followed by Dunnett's multiple comparison test. There was no statistically significant difference between UTC and 10 mg/kg dose of gentamicin.

4.2.5.4. Antibiotic concentrations and pharmacokinetic parameters

Cefotaxime antibiotic pharmacodynamic parameters are tabulated in **table 15.** Gentamicin pharmacokinetic parameters such as AUC, C_{max}, clearance and volume of distribution are tabulated in **table 16**. A dose-dependent increase in C_{max} and AUC of

gentamicin was observed. The other parameters such as volume of distribution, half-life (T ½) of gentamicin and clearance rate did not change.

4.2.5.5. Serum lactate levels:

Serum lactate levels increased by 1.25 to 1.5 times at baseline (before antibiotic administration) compared to before infection. Serum lactate levels usually increase after 12 h of initiation of infection in this model (in our previous experience). In our study, the mean serum lactate levels generally just exceed the upper limit of normal in all groups of animals after 11 - 12 hours of infection. After the initiation of gentamicin therapy, the serum lactate levels started to go down and were maintained within the standard limits in all the treated groups. In the untreated control group, the serum lactate levels were found to increase and were far above the standard limits up to the end of the experiment. The serum lactate levels at all the doses are presented in **figure 41**.

4.2.5.6. Serum cytokine levels

In UTC, the pro-inflammatory cytokine levels (peak) of IL-1β, TNF-α, IL-6, IL-12, IFNγ increased by approximately 126, 15, 12, 9, 22 times, whereas the anti-inflammatory cytokine levels (peak) of IL-4 and IL-10 increased by 2.5 and 60 times by the end of the experiment compared to the levels before infection (**figures 42 (a - e) and figure 43 (a -b))**. In the sham animals, there was no significant increase in either pro or anti-inflammatory cytokine levels. In gentamicin treated animals, the TNF-α levels were considerably lower compared to that of UTC by the end of the experiment (9 h post dosing). There was a significant difference in the levels of IL-6 and IL-10 cytokines in UTC compared to all the

treated and sham animals. The levels of other cytokines were also high in UTC compared to treated and sham animals.

Most cytokine levels in UTC increased gradually and reached maximum concentrations in the serum by the end of the experiment. In the treated groups, the cytokine levels were moderately high by 0.75 h to 1.5 h and started to go down below the baseline levels by the end of the experiment. The cytokine levels in all the groups of animals are presented in the **figures 42 (a - e) and figure 43 (a -b)**.

4.2.5.7.   Hemodynamic parameters

4.2.5.7.1.   Heart performance analyzer (HPA)

Serial hemodynamic parameters were recorded and are tabulated in **table 17 (a – d)**. Heart rate (HR), mean arterial pressure (MAP), and other parameters at all gentamicin dosing groups, the 15 and 60 mg/kg dosing groups of cefotaxime as well as UTC and sham animals were recorded. In all the animals, the HR was low at the start of the experiment. The heart rate in the UTC dropped significantly by the end of the experiment compared to the sham animals. The HR of the animals in the treated group tends to increase after 45 minutes to 1.5 h of initiation of therapy and was maintained well above the baseline values. In the gentamicin 5 mg/kg group, the HR dropped lower than that of the baseline value from 8th hour. In cefotaxime 15 mg/kg group, the heart rate was well below the baseline value after the start of the experiment. There was a statistically significant difference in the heart rate between 5, 10, 20, 160 mg/kg compared to UTC with a p-value of < 0.01. There was no statistically significant difference between UTC and remaining doses.

The mean arterial pressure in UTC and gentamicin 5 mg/kg group dropped down lower than 65 mm Hg within 3 h and 5 h respectively consistent with shock. However, in the remaining groups, the mean arterial pressure was well above 60 - 65 mmHg for more than 6 h after initiation of therapy. There was a statistically significant difference in the mean arterial pressure between the UTC and 80 and 160 mg/kg of gentamicin treated groups with a p-value of < 0.01. There was no statistically significant difference between the remaining antibiotic doses and UTC **(table 17 (a – d)).**

4.2.5.7.2. Pulse oximeter:

Heart rate, respiratory rate, % O_2 saturation, ventilatory distention and pulse distention were measured non-invasively using a pulse oximeter. Heart rate, pulse distention were low in UTC compared to rest of the groups. There was no significant difference in mean % O_2 saturation in UTC compared to all other groups indicating effective ventilation and oxygenation. Overall, there was a highly significant change in heart rate over time in all the groups ($p < 0.01$, ANOVA). The change in heart rate was statistically significant between the UTC and gentamicin 5, 80, 160 mg/kg groups, the UTC and cefotaxime 15 mg/kg group as well as the UTC and sham groups ($P < 0.01$, repeated ANOVA followed by Dunnett's). The change in heart rate was not significant with the other groups. There was a statistically lower pulse distention in the UTC (consistent with shock) compared to gentamicin 160 mg/kg and cefotaxime 15 mg/kg groups with a p-value for each comparison of < 0.01. Ventilatory distention between all the animals was very similar with no significant difference between the UTC and treated as well as sham groups. All the results are presented in **table**s **18 (a – e).**

CHAPTER 5

5. Discussion

The incidence of sepsis has been found to have increased rapidly in the past three decades [12]. Health Canada has reported approximately 10.9% of all deaths that occurred in hospitals throughout Canada (excluding Quebec), during 2008 – 2009, were related to sepsis [4]. The prevalence of sepsis in Canada is estimated to be between 27,000 and 30,000 cases per year with an overall mortality rate of 30.5% [4]. The economic burden for treating sepsis patients in American ICUs and wards is approximately $20,528 and $12,422 respectively (total of approximately $32,000) [11]. The treatment cost depends on the age of the patient, the frequency of other co-morbidities and the number of organ failures along with the degree of supportive care required. The total cost for treating septic and septic shock patients exceeds $ 20 billion dollars per year in the United States of America (USA) alone [12].

5.1. Treatment options and failures in septic shock patients

The pathophysiology of septic shock is still not well understood after many animal studies and clinical trials. Despite this, specific guidelines for treating septic shock patients are in place based on available clinical studies [149]. Irrespective of guidelines, many patients with septic shock, perhaps as many as 50%, fail to recover. Death occurs despite aggressive supportive care, such as support of cardiac output and arterial pressure with vasopressors and respiratory failure/ARDS with advanced ventilator support. Renal failure is common, but is rarely fatal as dialysis is an available option [439]. Death is often due to overwhelming infection associated with failure to efficiently eliminate pathogens [138, 139,

147, 148, 440]. This may be due to resistant pathogens or due to suboptimal antimicrobial dosing [138, 440-443].

Despite more than 40 years of extensive research, novel therapeutic approaches for sepsis have not been successfully translated to the clinical setting. Clinical trials of experimental treatment options for sepsis and septic shock have almost uniformly failed [444-446]. Drotrecogin-alfa (activated) a form of activated protein C, inhibitors of the coagulation cascade, against TNF-α, IL-6, statins, immune-stimulants have shown no significant benefit in survival of sepsis and septic shock patients [111, 164, 444-448]. None-the-less, the mortality of sepsis and septic shock has fallen in the last 20 years [449].

In my opinion, many mechanisms and cascades of the immune system are involved in sepsis and septic shock. Targeting a specific component of the immune system may not be helpful in attaining a good clinical outcome. This is apparent with the reported failures of the clinical trial involving antibodies against different cytokines, antibodies against the endotoxin and other mediator-targeted studies. All these failures indicate the complexity of pathogenesis of septic shock.

5.2. Antibiotic therapy

Accelerated elimination of pathogens has been proposed to be a critical factor in effective therapy of sepsis [138]. This position is supported by the fact that only antimicrobial therapy, among drug therapies, has been shown to improve the outcome in septic shock [22, 138, 167]. As mentioned in the previous section of this thesis, inappropriate antimicrobial therapy leads to 5 fold reduction in survival of septic shock patients [22]. Delay in dosing appropriate antibiotics also increases the mortality rate in sepsis and septic shock [2,

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167, 450]. Antimicrobial strategies that accelerate pathogen clearance in septic shock, such as combination therapy [190, 191] and pharmacokinetic optimization (increased time above MIC for β-lactams [207, 209, 451] and greater peak concentration or area under the curve to MIC ratio for aminoglycosides and fluoroquinolones [183]) are also associated with improved clinical outcomes and survival.

For optimal antimicrobial treatment, physiological changes that occur in critically ill and septic shock patients require consideration. An increase in volume of distribution of drugs is reported in septic shock patients [221, 273, 275, 452, 453]. This leads to treatment failure as optimal therapeutic concentrations are not achieved with normal empirical doses [268, 269]. A requirement for higher than normal loading doses has been reported with aminoglycosides and beta-lactam antibiotics in treating septic shock patients [256, 269, 272, 273, 275, 454-457]. In cystic fibrosis patients, tobramycin has been used at higher than normal doses for a prolonged time to clear pseudomonas infection without any adverse effects [458, 459]. Optimization of antimicrobial therapy is required for accelerated elimination of infection causing pathogens.

As explained earlier, aminoglycoside antibiotics are well-studied molecules with activity against most Gram negative pathogens and are used in combination with other antibiotics to treat Gram positive bacteria. Notably, aminoglycosides retain activity against Gram positive pathogens, but are generally not used as monotherapy. In recent years, aminoglycosides such as gentamicin and tobramycin have been typically administered intravenously at 5 – 7 mg/kg as a single dose per day (a change from previous use when doses of 1 - 1.5 mg/kg 3 times/day were used [221, 252, 265, 266]. Doses higher than 7 mg/kg (gentamicin equivalent) have not been used due to toxicity concerns. In septic shock, when a

patient is at very high risk of death, utilizing these molecules as a single, higher than normal dose may be a feasible option if toxicity can be managed and the strategy potentially improves the probability of survival.

5.3. Ultra-high dose aminoglycoside antibiotics

Single, ultra-high dose aminoglycoside therapy (80 mg/kg and 160 mg/kg) would be a novel approach for treating septic shock patients. There are no reports of utilizing such a high dose for treating any infection. In our research, we wanted to determine the potential efficacy (with respect to bacterial clearance) and safety of ultra-high doses of aminoglycosides at 80 and 160 mg/kg doses in a murine model of septic shock.

To evaluate the potential utility of ultra-high aminoglycoside doses in a septic shock model, three aspects of the study require specific assessment:

i.   Validity of the model as a reflection of septic shock

ii. Tolerability of toxicity associated with ultra-high dose aminoglycosides

iii. Efficacy of microbial clearance and resolution of inflammatory cytokines/metabolic dysfunction markers with ultra-high dose aminoglycosides

With respect to the above issues, our series of studies indicates that:

1) Our septic shock model adequately reflects key physiologic, immunologic and microbiologic responses to sepsis and septic shock

2) Pharmacological toxicities are either absent or tolerable in both short-term, 9 h studies (neuromuscular paralysis and renal injury) and in a long-term, 180-day study (chronic ototoxicity and nephrotoxicity) in healthy rats.

3) *E. coli* clearance in both the blood and solid organs of rats with experimental *E. coli* septic shock is substantially accelerated relative to standard aminoglycoside dosing and relative cefotaxime. In addition, it appears there are parallel improvements in inflammatory/metabolic injury markers in the septic shock model in response to ultra-high dose aminoglycosides

5.3.1. Animal model

There are many different experimental animal models of septic shock that can be utilized for sepsis studies. Fink, however, has emphasized the importance of the appropriateness of the animal model used to mimic septic shock in sepsis research [460]. In determining an appropriate model, Seok et al noted the variable susceptibility of animals to infection and variation in inflammatory responses to injury relative to humans [461]. Various sepsis and septic shock models have been described [462-466].

Models that replicate some of the signs and symptoms and the laboratory findings observed in human septic shock include; endotoxemia, cecal ligation and puncture, bacterial peritonitis (fibrin clot implant), pneumonia, soft tissue infection and meningitis models [467, 468]. These models have been established in a variety of experimental animals such as mice, rats, rabbits, sheep, dogs and primates [467, 469-474]. Most notable among the commonly used septic shock models are endotoxic/live organism infusion shock, cecal-ligation/perforation and the peritoneal clot implantation model. Each has its own advantages and disadvantages.

We choose to use fibrin clot containing *E. coli* Bort to induce peritonitis/ septic shock model that our group previously developed in rodents [145, 475]. In particular, the advantages of our model include:

1) High degree of reproducibility

2) Use of a serum-resistant, live, virulent organism for infection with replication within the body (unlike endotoxic shock and most live organism infusions where serum complement almost immediately lyses the low virulence organism)

3) Defined organism infection (unlike cecal ligation/perforation)

4) The absence of a major surgical insult (unlike cecal ligation/perforation)

5) Hemodynamic and inflammatory characteristics similar to the clinical human syndrome.

The choice of the pathogen is critical in a monomicrobial infection. We have chosen an *E. coli* strain (Bort) with confirmed virulence based on isolation from invasive human disease [406]. In addition, the organism is known to be encapsulated and serum-resistant, characteristic of virulent gram negative isolates [406, 476]. In contrast to the primary alternate option, the cecal ligation/perforation model, there is no surgical bowel interruption and the starting organism burden is precisely defined. In contrast to models of soft tissue infection, immunosuppression is not required and the development of hypotension/shock is well defined.

In our study, septic shock model was established by utilizing 2 gelatin capsules containing 5 x 10^5 CFU/capsule of *E. coli* Bort (total 10^6 CFU organisms were implanted). With respect to cardiovascular responses, hypotension and tachycardia are the hallmarks of septic shock [477]. Experimental animals with severe sepsis and septic shock typically

demonstrate tachypnea, lethargy, hypothermia and ruffled fur all of which were exhibited in this model [478, 479].

Our live infection model was appropriate to our needs and mimicked human sepsis/early septic shock. Animals became progressively more septic with increasing severity of illness culminating in mild-moderate hemodynamic instability in the hours following instrumentation (approximately 16-20 h) requiring occasional doses of subcutaneous catecholamines terminally. Early tachycardia, tachypnea and pyrexia (approximately 6 h after sepsis induction) with later bradycardia, and hypothermia (about 12 h after sepsis induction) were noted. A high-grade bacteremia with increased serum lactate and expression of pro-inflammatory cytokines was also noted.

Despite concerns regarding the validity of animal models of septic shock (primarily with respect to gene expression and cytokine profile[461]), we believe our model reasonably mimics human sepsis and septic shock with respect to pathogen growth and bacteremia, cardiovascular and other physiologic responses, and cytokine/immunologic aspects of infection progression.

5.4. Aminoglycoside toxicity with single, ultra-high doses

There exist remarkably little data on the nature of aminoglycoside toxicity at the doses examined in this study. For that reason, there is very little comparative data available.

5.4.1. Neuromuscular paralysis

In our pilot EMG study, we demonstrated the generation of a competitive neuromuscular blockade (similar to the non-depolarizing neuromuscular blocking agent,

pancuronium) and reversibility of this paralysis with time at 80 mg/kg dose of gentamicin. To our knowledge, the transient nature of this effect in intact animals is an entirely novel observation.

In our short-term and long-term studies, we demonstrated that gentamicin and tobramycin induce neuromuscular paralysis at single, ultra-high doses of 80 and 160 mg/kg. The animals survived when they were supported by mechanical ventilation for the duration of neuromuscular paralysis. The approximate 30-50 min duration of neuromuscular paralysis was similar in all our three experimental study designs including septic animals.

Several investigators have confirmed the occurrence of neuromuscular paralysis with aminoglycosides in both isolated muscle preparations and intact animals [282, 283, 403, 480-484]. Albiero et al compared the neuromuscular blocking effect of aminoglycoside antibiotics in rat sciatic-gastrocnemius muscle [485]. The authors reported a direct relationship between the neuromuscular blocking property and the LD_{50} of the aminoglycoside antibiotics. In studies on pigeons and rats, Brazil et al reported that gentamicin induces neuromuscular paralysis that was partially reversed by calcium administration [283]. Matsukawa et al demonstrated the neuromuscular blocking effects of sisomicin and micronomicin in rabbits [282]. In their experiments, 20 mg/kg and 40 mg/kg doses of sisomicin and 40 mg/kg and 80 mg/kg doses of micronomicin were used. The authors reported a dose-dependent reduction in muscle twitch tension. In addition, the neuromuscular blockade was partially antagonized by neostigmine or calcium. Singh et al evaluated the neuromuscular blocking effect and reversibility of 16 different antibiotics in their experiments on isolated mouse phrenic nerve-hemi-diaphragm preparations [403]. The authors described a similar neuromuscular blocking effect of different aminoglycosides including neomycin, gentamicin, streptomycin and

kanamycin which resembles the effect of magnesium. Magnesium decreases the release of acetylcholine and also decreases the post-junctional sensitivity thereby causing neuromuscular paralysis. The blockade of acetylcholine release by magnesium is antagonized by calcium. Hence, it can be inferred that aminoglycoside antibiotics exhibit both pre- and post-junctional neuromuscular blocking effect [403]. Mingji Liu et al described the neuromuscular blocking effect of arbekacin, astromicin, isepamicin and netilmicin on the diaphragm and limb muscles of rabbits [486]. Paradelis et al described the neuromuscular blocking effect of gentamicin using isolated rat diaphragms [487]. The same authors in another study described that the neuromuscular blocking potency of different aminoglycosides as gentamicin > streptomycin >amikacin > sisomicin > kanamycin = tobramycin > kanendomycin = dibekacin [488]. Aminoglycosides have long been known to cause neuromuscular weakness in human patients, particularly when used with steroids or with standard paralytic agents [489].

Our data clearly demonstrate that the primary dose-limiting toxicity of ultra-high dose aminoglycoside therapy is transient in nature and can be easily addressed with mechanical ventilation. One particularly interesting aspect of our observations is that neuromuscular paralysis/ weakness occurs immediately after rapid infusion of the drug with a duration that is proportional to the total dose of the drug (Table 9b). This suggests that it is the high peak concentrations of aminoglycosides that drives this toxicity. Renal toxicity has been suggested to be associated with high trough levels [490] while generation of auditory and vestibular toxicity is not related to dose or concentration in serum but appears to be a time-dependent process [321].

The primary purpose of our studies is to determine whether ultra-high dose aminoglycoside therapy might have any potential with respect to critically ill humans with

high risk of death due to overwhelming infection particularly when septic shock is present. A substantial majority of septic shock patients (approximately 70-80%) require ventilator support within 24 hours of admission [22, 491]. Given that, transient neuromuscular paralysis due to ultra-high aminoglycoside doses is not a disqualifying toxicity problem in the potential treatment of septic shock patients.

5.4.2. Cardiovascular Toxicity

Minor adverse cardiovascular effects attributable to ultra-high dose aminoglycosides were present. In our non-invasive long-term (180 days) study, hemodynamic parameters were recorded using a pulse oximeter from the time the animals were anesthetized to the time the animals became ventilator independent after completing the dosing. In this study, the hemodynamic parameters were observed for the first 2 hours only. There was a steady drop in heart rate after initiation of dosing of aminoglycoside antibiotics. This drop in HR was observed until the animal was ventilator dependent. A similar transient drop in heart rate immediately after infusion of ultra-high doses of aminoglycosides was also noted in the invasive studies.

The cardiovascular depressant effects of neomycin and gentamicin have been described in rhesus monkeys [492]. They also noted a transient hypotension and cardiovascular depressant effect in the animals treated with neomycin. The same author also described similar cardiovascular depressant effects of aminoglycoside antibiotics in cats, dogs, rhesus monkeys, squirrels, owls and dog-faced baboons [493]. Other authors have reported the reversal of this depression by administering calcium and 4-amino-pyridine [494]. Our invasive animal model did exhibit some hemodynamic instability, particularly late in the study period. In the short-term invasive toxicity study, the mean blood pressure was maintained with saline

administration above 55 mmHg up to 6th hour although it could drift downward thereafter. However, this was not reproduced in the non-invasive study and is unlikely to be related to aminoglycoside toxicity. The reason for the inconsistent mean arterial pressure drop after 6th hour of the invasive study may be due to blood loss from the surgical site during the prolonged post-surgical period.

Our results and those of other investigators suggest that adverse cardiovascular effects are not dose-limiting factors with respect to the use of ultra-high dose aminoglycoside therapy in experimental animals.

5.4.3. Nephrotoxicity

In our short-term invasive studies in healthy animals, administration of a single ultra-high dose of aminoglycoside was associated with a modest increase in serum creatinine levels compared to baseline, but these increases were well within the normal range of $0.3 - 1.4$ mg/dL in rats. Similarly, the long-term study demonstrated that serum creatinine levels were well in the normal range after 24 h of dosing (as predicted in the invasive short-term study). In addition, levels stayed well within the normal range at all assessed points up to day-180 indicating that there was no long-term effect on kidney functioning associated with single, ultra-high doses of aminoglycosides. Statistically, there was no significant difference in the serum creatinine levels between controls and treated groups in the long-term study (180 day).

NGAL also known as Lipocalin-2 is a very good biomarker of acute renal injury [495-497]. Levels of NGAL are reported to be elevated in animals with renal toxicity. With AKI, high levels of NGAL are secreted into the blood and urine. As NGAL is a very small protein, it can be detected in high concentrations within 2 hours after a kidney injury. In the long-

term study, urinary NGAL levels were measured at baseline (1 day before the start of the experiment) - day 0, day 1, day 7, day 30, day 60 and day 180 to assess for evidence of tubular injury associated with single, ultra-high doses of aminoglycosides at two different doses. NGAL levels did not increase more than twofold throughout the experiment indicating that there was no acute tubular injury. In addition, there was no statistically significant difference in NGAL levels between the treated and untreated (saline) controls. The major difference between previously reported studies and our renal toxicity analysis is that other researchers studied renal toxicity association with standard doses and durations of aminoglycosides. In our research, we were trying to exploit the concentration-dependent activity of aminoglycosides to treat critically ill animals with septic shock by using a single ultra-high dose (80 and 160 mg/kg) of aminoglycoside to accelerate pathogen clearance. The goal of our renal toxicity study was not to determine if any renal toxicity existed, but rather to determine whether a disqualifying severe toxicity could be demonstrated that would preclude further development of this experimental approach in higher animals.

In the short-term (9 h) invasive study, serum creatinine levels were well in the normal range for the first 9 h post dosing despite some fluctuation in the serum creatinine levels in both the ultra-high dosing groups. In the long-term toxicity study, a total of 4 animals (2 animals for gentamicin + 2 animals for tobramycin) per dose were used. Serum creatinine levels were well within the normal range for 180 days after administering single, ultra-high doses of gentamicin and tobramycin. Based on this, renal toxicity sufficient to justify the abandonment of this approach in higher animals was not apparent. However, given the small number of animals in these studies, a larger group of animals would have to be studied with

each ultra-high dose regimen in order to determine whether any significant renal toxicity occurs with single, ultra-high dose aminoglycoside administration.

In the septic shock model, serum creatinine levels over the course of the study rose substantially above the normal limits only in untreated infected control animals (UTC) although increases in the 10 mg/kg group gentamicin group marginally reached significance. In all the remaining groups, the serum creatinine levels remained well within the normal limits. The increase in UTC animals is likely a consequence of the progressive shock they experienced. We believe the modest increase in the 10 mg/kg gentamicin group is likely a random event as one would expect the range of doses of gentamicin would have shown a progressive effect if aminoglycoside-related toxicity was present.

As noted, NGAL is another marker for acute renal toxicity [495]. It is a very small, protease-resistant protein encoded by the LCN2 gene in human beings. It plays an important role in the innate immune system. This protein sequesters iron that is required for bacterial growth and helps in limiting the growth of bacteria during infections [498-500]. NGAL is expressed in neutrophils and at low levels in kidneys, respiratory epithelium and alimentary tract. Normally, a small amount of this protein is filtered through the kidneys, but is reabsorbed by the proximal renal tubules [501, 502]. Zhou et al evaluated the performance of 4 urinary biomarkers, including neutrophil gelatinase-associated lipocalin (NGAL), total protein, and N-acetyl-β-D-glucosaminidase (NAG) in beagle dogs with acute kidney injury induced by gentamicin [503]. In their study, beagle dogs were administered gentamicin at 80 mg/kg/day intramuscularly for 9 days. Expression of NGAL mRNA was found to be upregulated from day 6 onwards after administration of gentamicin. A 5.3 and 5.4 fold increase in NGAL mRNA expression on day 6 and day 9 compared to control was observed.

NGAL is well recognized as a marker of acute kidney injury after other kinds of renal insult as well. Bennett et al reported NGAL as the suitable post-surgical biomarker for acute kidney injury (AKI) from their clinical trial in 196 patients (children) undergoing cardiopulmonary bypass surgery [504]. The authors reported that the urinary NGAL levels increase by 15 fold within 2-hours and 25 to 26 fold in 4 to 6 hours after surgery, respectively, indicative of AKI. The 2 hour urinary NGAL levels correlated with severity and duration of acute kidney injury. Mishra et al suggested serum and urinary NGAL as sensitive and highly predictive early biomarkers for AKI after cardiac surgery from their clinical trials in 71 children undergoing cardiac surgery [505]. From the clinical trial, the authors reported that children who developed AKI immediately after cardiac surgery exhibited an elevation in urinary NGAL from a mean of 1.6 µg/L at baseline to 147 µg/L at 2 h post-surgery. This is a 91 times increase in urinary NGAL levels compared to baseline.

An extensive body of literature demonstrates nephrotoxicity in animal models after extended use of aminoglycosides particularly in association with prolonged elevation of trough concentrations. There is relatively little data on renal function alteration in response to the kinds of doses used in this study. Provoost et al determined the nephrotoxicity of gentamicin and amikacin in young and adult rats [506]. In their studies, gentamicin was dosed at 20 and 60 mg/kg and amikacin were dosed at 60 and 180 mg/kg for 14 days. They reported that the concentration of aminoglycosides was significantly lower in the renal cortex in young rats compared to adult rats indicating decreased nephrotoxicity in young rats. In addition, gentamicin appeared to exert more severe renal toxicity than did amikacin. This was evident in higher serum creatinine levels in gentamicin treated animals in their experiments. Moreira et al have reported gentamicin-induced nephrotoxicity with a significant increase in

serum creatinine levels after dosing gentamicin at 100 mg/kg/ day for 7 days by intraperitoneal route in rats [507]. The authors also reported proximal tubular damage associated with gentamicin dosing regimen. Sahu et al reported a significant increase in serum creatinine levels after dosing 100 mg/kg of gentamicin by intraperitoneal route for 7 days in Sprague-Dawley rats [508]. In this study, gentamicin dose-related histological changes in the kidney with tubular degeneration, massive necrosis and foci of inflammation containing neutrophils were reported. Sardana et al also reported a major increase in serum creatinine levels after dosing gentamicin at 100 mg/kg by intraperitoneal route for 14 days [411]. Severe pathological changes in kidneys such as degeneration in glomerular wall and mild hypertrophy as well as pathological changes in the tubules such as mononuclear cell infiltration, degeneration in epithelial layer, inter-tubular hemorrhage and hyaline casts from the gentamicin treated animals was also noted. Baylis et al reported that large doses of gentamicin (60 mg/kg for 14 days by subcutaneous route) administered to rats evoke decreased glomerular filtration rate (GFR) which is due to decrease in renal plasma flow (RPF) [357]. Reduction in GFR, glomerular surface and ultra-filtration co-efficiency due to contraction of mesangial cells is also reported [509, 510]. Matinez-Salgado et al have implicated gentamicin-associated toxicity in simultaneous mesangial proliferation and apoptosis in rats dosed with 100 mg/kg/day for 6 days administered by the subcutaneous route [360].

Most the animal studies mentioned in above paragraphs reported some evidence of aminoglycoside-induced proximal tubular damage and elevation in serum creatinine levels with elevated urinary NGAL levels. The studies were designed to understand the mechanisms involved in gentamicin/aminoglycoside-induced nephrotoxicity rather than the antimicrobial

efficacy. No reports/ studies are available on nephrotoxicity of aminoglycosides when used as a single, ultra-high dose. Clinical trials in humans showed nephrotoxicity with elevated serum creatinine concentrations only after dosing aminoglycosides for prolonged periods.

As mentioned in the previous paragraph, the elevation in NGAL levels was very high compared to baseline in patients with acute kidney injury. In our study, the NGAL levels increased less than 2 fold and fell to normal within few days after dosing at both the ultra-high doses indicating no renal tubular damage due to these doses. Given the marginal increase in serum creatinine in the 9 h invasive study and the lack of a significant increase in the long-term non-invasive study, the modest acute increase in creatinine (albeit remaining within the normal range) with ultra-high dose aminoglycoside doses appears to be of minimal relevance to the experimental animal's outcome.

In summary, laboratory markers of renal injury, including NGAL and serum creatinine indicated minimal or absence of nephrotoxicity with single, ultra-high doses of aminoglycosides, likely due to an absence of prolonged elevation of trough concentrations of the drug using this approach. But, with a limited number of animals in our study, it is recommended to perform another experiment with a larger number of animals per dose to confirm these results.

5.4.3.1. Histology of kidneys

From the long-term non-invasive study, kidney histology indicated an absence of overt toxicity in single, ultra-high dosed animals compared to saline administered controls. The H & E stain revealed that the glomerular structures were intact with clear Bowman's capsules. The proximal and distal tubules were clear with no signs of toxicity. H & E staining revealed the same results in the short-term invasive study. Despite a transient and modest increase in

serum creatinine in this 9 h study, major histological changes would be unexpected at this time point. The kidney sections of non-invasive study were used for Tunel assay to assess for apoptosis in renal tubular cells and other cells of renal tissue due to ultra-high dose aminoglycosides. Although there were a few random apoptotic cells present, there was no difference in frequency in the treated and untreated control groups, indicating an absence of nephrotoxicity associated with ultra-high dose aminoglycoside administration.

Despite the limited sample numbers available, qualitatively, we can determine that there was no overt apoptosis of renal tubular cells and other cells of renal tissue due to ultra-high doses of aminoglycosides. However, the overall sample size was too small to fully assess more modest potential toxicity. Another study using larger group of animals per dose is recommended to confirm these results.

5.4.4. Oto-vestibular toxicity

In humans, oto-vestibular toxicity typically occurs after a few days to weeks after receiving a normal dose regimen of aminoglycosides. As we are assessing the potential of single, ultra-high doses, it is important for us to determine if this dosing strategy may cause long-term oto-vestibular toxicity.

Most published reports examining this issue have used doses of 100 mg/kg and higher gentamicin-equivalent by intraperitoneal, intramuscular or subcutaneous routes for 7 – 14 days to induce toxicity. In most of the studies, ABR and vestibular toxicity studies were performed at baseline and immediately after a prolonged dosing course, as well as after a few weeks (mostly usually 3-5 weeks) of completion of dosing. We evaluated the long-term (6 months) oto-vestibular toxic effects of a single, ultra-high dose of an aminoglycoside (gentamicin and tobramycin) at 80 mg/kg and 160 mg/kg in normal healthy rats. This is a

novel study in this area of research as far as the duration of follow-up. None of the animal research performed previously has followed subject animals for over a 6 month period for toxicity.

5.4.4.1. Auditory toxicity

Virtually all animal studies of aminoglycoside-induced auditory toxicity have involved administration of high-dose therapy for 10-14 days or more. For example, Polony et al and Murillo-Cuesta et al reported auditory toxicity in mice after administering kanamycin at 800 mg/kg, twice daily for two weeks (14 days) by the intramuscular routes [418, 511]. Sequential ABR studies demonstrated kanamycin induced auditory toxicity, starting from the third week. ABR studies indicated an increased hearing loss at higher more than lower frequencies. Le Prelle et al in their research using guinea pigs also reported gentamicin-induced auditory toxicity using ABR analysis and histologic assessment of inner and outer hair cell survival/apoptosis [416]. In this study, gentamicin was administered by subcutaneous route at 140 mg/kg/day (single dose) for 14-16 days. Ye et al reported the protective effect of melatonin on gentamicin-induced auditory toxicity [417]. In their experiments, guinea pigs were administered gentamicin at 120 mg/kg/day for 17 days to induce auditory toxicity. Ojano et al reported gentamicin-induced auditory toxicity in albino guinea pigs. In their studies gentamicin was administered at 130 mg/kg dose for 10 days by the subcutaneous route [512].

In all the above studies, aminoglycoside antibiotics were administered at high doses for a prolonged time (10-14 days minimum). In our studies, aminoglycosides were dosed at 80 mg/kg and 160 mg/kg by intravenous route as a single intravenous dose. The animals were exposed to high peak concentrations for a very short period of time, which may explain

the absence of toxicity in the model. Animals were observed for 180 days (6 months) and did not exhibit any notable adverse auditory toxicity.

High dose aminoglycoside regimens are known to induce a deficit in the threshold hearing ability of the animals at different frequencies as determined by ABR studies. Investigators have confirmed a 30 – 60 decibel threshold shift at different frequencies along with a considerable amount of hair cell death in several ototoxicity studies of aminoglycosides [335, 338, 513].

In contrast, our ABR studies did not demonstrate a > 30 decibel difference on day 30, 60 and 180 compared to day 0 (before antibiotic dosing) at all the frequencies tested with both the single, ultra-high aminoglycoside doses. At the end of the 180 day study, the cochlear samples were fixed by immersion fixation method. Unfortunately, the tissues did not fix properly and we were unable to assess hair cell survival/death in our cochlear samples.

Although there was no significant hearing loss at the high frequencies of 12000 Hz and 24000 Hz as well as at lower frequency of 3000 Hz in our study, there was a significant hearing loss observed at 6000 Hz with the 80 mg/kg dose but not at 160 mg/kg dose. We believe this is likely to represent random variation rather than a true marker of aminoglycoside auditory toxicity. As mentioned earlier, hearing loss due to aminoglycosides initially appears at higher frequencies [335, 338, 418, 511, 513]. It would seem unlikely that the hearing loss would occur only at the 6000 Hz range without some involvement at higher frequencies. In addition, to be more convincing as a toxic effect, it would also be expected to be observed at the higher 160 mg/kg dose.

Using Generalized Estimating Equations (GEE) (SAS version 9.1 (Cary, NC, USA), an inordinately large numbers of animals would need to be studied to achieve significance if

the relatively small effects at all the frequencies tested except at 6000 Hz represented true

auditory toxicity. A post-hoc power analysis found that we would need

- 960 rats (320 in each group) for 3000 Hz

- 9 rats (3 in each group) for 6000 Hz (although this is probably based on a type

  I error in the current study; assuming that is the case, the real number required is

  probably much larger.)

- 120 rats (40 in each group) for 12000 Hz

- 1059 rats (353 in each group) for 24000 Hz

These numbers are for the most part much larger than what can be run in the typical

study. From this post-hoc power analysis, we can state that auditory toxicity is very unlikely

in rats using this dosing regimen. Future studies would need to concentrate on this as the

primary outcome to verify if there is any degree of toxicity.

5.4.4.2. Vestibular toxicity

Along with auditory toxicity, vestibular damage is another toxic effect of aminoglycosides.

Angunsri et al in their experiments used explanted cultures of mouse utricles to demonstrate

the occurrence of aminoglycoside-induced vestibular hair cell death [514]. Fetoni et al

reported that gentamicin dosed at 100 mg/kg/day by the subcutaneous route for 14 days

causes severe auditory and vestibular toxicity in guinea pigs [513]. The authors determined

the vestibular toxicity by studying the vestibule-ocular reflexes. Auditory toxicity was

determined by electro-cochleographic recordings at different frequencies. Ylikoski et al

reported gentamicin-induced vestibular and auditory toxicity in guinea pigs administered

gentamicin at 120 mg/kg for 14 days by the subcutaneous route [515]. In these experiments,

the authors concluded that blocking the C-Jun N-terminal kinase pathway partially protects

hair cells by preventing apoptosis. Matsui et al used long leghorn chickens to show that streptomycin induces auditory and vestibular damage when administered by the intramuscular route at 1200 mg/kg dose of 3 to 5 days [516]. The authors showed that local administration of caspase inhibitors appears to offer some protection to the outer hair cells in the presence of toxic insult.

For the most part, the studies reviewed show that aminoglycosides cause vestibular toxicity when utilized at relatively high doses for prolonged periods. In all the above-mentioned studies, animals were dosed by the intraperitoneal, subcutaneous or intramuscular routes which would lead to lower peak concentrations and longer elimination than achieved when administered intravenously as in our study.

We studied vestibular toxicity using behavior assessment. Although vestibular evoked potentials can be assessed in experimental animals, their routine use in small animals is currently restricted to a very small number of laboratories with a high level of expertise. We were unable to produce reliable baseline data using this approach in pilot studies and elected to focus on behavioral analysis. Behavioral studies indicated an absence of vestibular toxicity with both the single, ultra-high aminoglycoside doses.

We conclude that aminoglycosides (gentamicin and tobramycin) at single, intravenous, ultra-high doses of 80 and 160 mg/kg do not cause major auditory or vestibular in animals despite the propensity to do so with prolonged dosing approaches. However, considering the low number of animals used per dose, further studies in a large number of animals are required to confirm these and to rule out the possibility of more subtle ototoxicity.

5.4.5. Efficacy of microbial clearance and impact on inflammatory mediators/organ injury markers of ultra-high dose aminoglycosides

Our aim of this study was to accelerate pathogen clearance in blood and organs in our septic shock model. Septic shock animals treated with incrementally increasing doses of gentamicin demonstrated dose proportional reduction of *E. coli* Bort from the blood across time points. In order to mimic human pharmacokinetic values, we had to take into account the species differences in drug clearance and metabolism. The renal clearance of most drugs by humans is about 4 times slower than that of animals [429, 517, 518]. The 20 mg/kg dose of gentamicin is expected to yield serum concentration profile similar to the 5 mg/kg dose in humans and the remaining 40, 80 and 160 mg/kg doses of gentamicin in rats are approximately equal to 10, 20 and 40 mg/kg doses in humans respectively. The 20 mg/kg dose was compared to the remaining ultra-high gentamicin doses to determine if there was any statistically significant difference.

Complete and persistent eradication of *E. coli* for the 9 h duration of the study was noted at the highest dose (160 mg/kg). At the next highest dose (80 mg/kg), there was a slight regrowth in the bacterial count by 9th hour. Complete elimination of the pathogen from blood occurred as quickly as the first assessed time point (20 min post-antibiotic administration) for both these doses. Bacterial clearance at 40 mg/kg and higher is accelerated relative to cefotaxime at 60 mg/kg.

Our goal of accelerated pathogen clearance was achieved with respect to blood using 80 and 160 mg/kg with clearance (i.e. a ≥ 6 $\log_{10}$ CFU reduction) within minutes after administration of the single, ultra-high doses compared to baseline. At 160 mg/kg dose of gentamicin, a ≥ 5 $\log_{10}$ CFU reduction was observed in the peritoneal fluid (site of infection)

compared to UTC and a ≥ 8 $\log_{10}$ CFU reduction of bacteria in the blood compared to UTC at the end of the experiment. Similarly, at the same dose, the bacterial reduction was ≥ 8.5 $\log_{10}$ CFU per gram of kidneys, ≥ 3 $\log_{10}$ CFU reductions per gram of spleen, ≥ 4 $\log_{10}$ CFU reductions per gram of liver and ≥ 4.5 $\log_{10}$ CFU per gram of lung compared to UTC at the end of the experiment. Post-gentamicin bacterial counts from kidneys were below the detection limit with the two highest doses.

Despite significant reductions in counts, pathogens do persist in organs such as liver, spleen and lungs even after ultra-high doses of gentamicin. We suspect one reason for this is the large number of macrophage line cells in these organs. These phagocytic cells likely hold large numbers of phagocytized intracellular pathogens that are released during the homogenization process for bacterial culture. The liver, for example, harbors 80% of the macrophages of the body as resident Kupffer cells [519]. In the lungs, alveolar macrophages form 95% of the cell fraction in broncho-alveolar lavage fluid [520]. The spleen also holds a large fraction of macrophages [521]. Aminoglycosides are highly hydrophilic molecules that do not penetrate well intracellularly. It is for this reason that aminoglycosides are considered excellent antimicrobials for bloodstream invasion, but less effective for intracellular pathogens. Note however, that incorporation of aminoglycosides into the phagocytes does occur when they are in contact with these antibiotics for prolonged periods beyond the 9 h period examined in this study [522-524].

The other possible reason for the higher number of organisms found in lung, liver and spleen is the 'anergy' observed in the macrophages along with other immune cells during sepsis and septic shock conditions. Due to immunological anergy, the macrophages and other phagocytic cells lose their ability to function properly as well their ability to generate

components that are required to effectively eliminate/kill the pathogens in the phagosomes is low. As a consequence, the bacteria harbored in the phagosomes of the macrophages present in these organs may not be eliminated as quickly as extracellular bacteria.

Pro-inflammatory cytokines are thought to be central in the pathogenesis of cellular and organ system dysfunction in sepsis. Experimental animal models of live organism infection suggest that cytokine expression is suppressed with early, appropriate antimicrobial therapy [145, 525]. To confirm if the levels of the cytokines go down after treatment with ultra-high dose aminoglycosides, several key serum cytokine levels were assayed in treated and untreated animals. Our model demonstrated a consistent increase in key serum cytokines from the start of the to the end of the experiment in untreated control animals while sham implant animals experienced no such increase. Cytokine levels before implant were uniformly normal in all animals. Similarly, in human septic shock, an increase in the levels of the pro and anti-inflammatory cytokines during the course of progressive sepsis/septic shock is almost uniform and represents the basis of the development of various anti-cytokine experimental therapies [93, 526].

Pro- and anti-inflammatory cytokines are known to have key roles in sepsis and septic shock in both experimental animal and spontaneous human disease. Vianna et al created septic shock by the cecal ligation and puncture (CLP) method in Swiss mice to evaluate the release of cytokines after antibiotic treatment [527]. Serum and peritoneal levels of TNFα and IL-6 were decreased at 6 and 24 hours with antimicrobial therapy. Peritoneal levels of the anti-inflammatory cytokine IL-10 were found to decrease in the early time points after treatment and tend to increase at 24 h after treatment. Antimicrobial therapy also improved survival in the model. Silverstein et al demonstrated the inflammatory cytokine response of

peritoneal macrophages against bacteria (either viable or killed) showing that responses were dependent on the specific bacterium and the host sensitivity [528].

TNF-α production is usually seen within a few hours of infection in animal models of septic shock [145] with TNF-α does not consistently correlate to outcome in human septic shock [529-531]. IL-6 elevations are more closely correlated to sepsis outcome in humans [532, 533]. IL-6 is usually produced by lymphocytes, fibroblasts and monocytes and plays a major role in activation of T and B – lymphocytes, induces the production of acute phase proteins in the liver. Along with these, IL-6 activates the coagulation system [534]. IL-6 levels are usually observed after 3 – 5 hours of onset of sepsis. In experimental endotoxic shock caused by *E. coli* or sub-lethal doses of endotoxin in baboons, peak TNF-α concentrations were observed within 1 – 2 hours, followed by IL-6 levels within 3 hours [535]. When lethal doses of endotoxin were administered to baboons in the same experiment, peak concentrations of TNF-α and IL-1β were observed within 2 – 5 hours, whereas the peak IL-6 levels were found after 6 – 8 h after injection. IL-6 is reported to suppress the production of of TNF-α and IL-1β [536, 537]. Blocking IL-6 using anti IL-6 antibodies or blocking IL-6 receptors provides some protection against lethal doses of TNF-α in a murine model [538]. Frequent blood sampling may be required during the early hours to determine the spike in TNF-α production in sepsis and septic shock. In our septic shock study, the serum TNF-α was measured at before infection/sepsis induction, 11 – 12 h after infection (at sepsis pre-antibiotic baseline) and at 45 min (0.75 h), 1.5 h, 3 h, 6 h and 9 h post-dosing of antibiotics. The chances of reduction in levels of TNF-α below the detection limit is increased at these time points. Our collaborator, Dr. Steven Opal (Department of Medicine, Brown University, USA) who has extensive experience with cytokine expression in animal

models suggested that SD rats show reduced TNF-α expression. On the other hand, our model does demonstrate a high level expression of IL-12 indicating the compensatory pro-inflammatory response in these animals.

The levels of the key serum cytokines started to go down after initiation of antibiotic therapy in treatment groups. Serum levels of major cytokines such as TNF-α, IL-6 and IL-12 were found to be decreased compared to the septic baseline levels in treated animals as well as when compare to the cytokine levels in UTC. The decrease in the level of pro-inflammatory cytokines after dosing ultra-high doses (80 and 160 mg/kg) of gentamicin suggest suppression of pro-inflammatory cytokine production in response due to elimination of infection causing bacteria from the blood and reduction of the bacterial burden within the animal. Again, these data indicate the efficacy of ultra-high dose aminoglycoside therapy with respect to attenuating the systemic inflammatory response associated with septic shock. There is also evidence to suggest that aminoglycosides may exert anti-inflammatory activity, particularly with regards to gram-negative pathogens, although the role of this effect in our model is unclear [539].

Serum lactate has long been known to be an important marker of tissue ischemia, shock and particularly septic shock. Serum lactate is closely associated with outcome in human septic shock [540-543]. In addition, many authors have shown increased lactate in various animal models of septic shock [145, 543]. For example, Kumar et al have reported increased serum lactate levels in blood circulation after induction of septic shock in a murine model of septic shock [145].

In our study, serum lactate levels were in the normal range throughout the experiment in the sham control animals. In untreated septic control animals, serum lactate levels were

modestly elevated at sepsis baseline (before antibiotic administration) to markedly elevated by the end of the experiment consistent with severe sepsis progressing to septic shock. Lactate levels were found to normalize after initiation of treatment and were in the normal range from 3rd hour onwards to the end of the experiment in all gentamicin treated groups.

We conclude that single, ultra-high doses of aminoglycoside antibiotics were well tolerated by the animals. These ultra-high doses appear to exert no overt renal and ototoxicity in the limited number of SD rats examined. However, larger numbers of experimental animals will have to be assessed in order to detect more subtle renal or oto-toxicity. Ultra-high doses eliminate the sensitive pathogenic *E. coli* (Bort strain) in our study from blood within minutes after administering the dose and demonstrate substantial reduction in organ and infection site pathogen burden by the end of the study when compared to untreated controls. Since there were some levels of bacteria still surviving in the organs, further studies are required to optimize the potential use of these single, ultra-high doses.

CHAPTER 6

6. Conclusion, limitations and future directions

6.1. Conclusion

The primary purpose of the studies described in this thesis was to assess the potential of single, ultra-high dose aminoglycoside (gentamicin and tobramycin) as a therapy for severe Gram negative infections using a rat model of peritonitis-induced septic shock. The underlying hypothesis was that such therapy could substantially accelerate pathogen clearance and ameliorate septic organ injury. We believe accelerated pathogen clearance is key to improving survival in human septic shock.

Because of the potential toxicity of ultra-high dose therapy of aminoglycosides, preliminary *in vivo* experiments were necessary to determine the ability of the animals to tolerate the proposed regimen. Hemodynamic parameters were observed in the treated and untreated animals from the start to the end of the experiment in the toxicity studies encompassed in this thesis. Even though there was an expected fluctuation of these parameters throughout the experiments, the HR, blood pressure and O_2 saturation remained within the normal range and similar to the baseline values throughout the acute toxicity studies. There was no substantial or sustained hemodynamic deterioration due to toxicity associated with single, ultra-high doses of aminoglycoside antibiotics.

The pilot EMG study indicated that there was a reversible neuromuscular paralysis in the animals with single, ultra-high dose of gentamicin at 80 mg/kg dose. From our invasive toxicity study (9 h), we were able to demonstrate that SD rats were able to tolerate the ultra-

high doses of gentamicin and tobramycin when administered as 20 min intravenous infusions and if the animals were ventilated for the duration of neuromuscular paralysis.

There was, at most, a modest transient adverse effect on kidney function as determined by the serum creatinine levels from the animals in invasive (9 h) study, although serum creatinine levels remained well within the normal range after the ultra-high doses of aminoglycoside antibiotics. Histological examination of the kidneys indicated that there was no morphological difference in the treated and untreated animals. The renal tubules and glomerulus were intact without any signs of aminoglycoside-induced toxic effect.

Based on the results from the short-term invasive studies, non-invasive, long-term toxicity studies (to 180 days) following single doses of varying doses of aminoglycosides were performed. Previous studies of aminoglycoside toxicity have used doses comparable to those used in our study, but they were administered by intramuscular and subcutaneous dosing (yielding lower peak serum concentrations) for prolonged periods. To our knowledge, no other studies have examined the toxic potential of single doses in excess of 80 mg/kg gentamicin-equivalent administered intravenously in any animal model.

Test animals were monitored for ototoxicity and renal toxicity for 180 days after intravenous dosing. Serum creatinine levels remained well within the normal range for the duration of the study at all the doses tested with no statistically significant difference between the untreated controls and ultra-high dosed animals. In addition, there was no statistically significant change in creatinine during the observation period in the ultra-high dose animals. The urinary biomarker, NGAL was shown to increase at both the maximum ultra-high doses tested, although levels were in the normal range by day 7 in 80 mg/kg dosed animals and by day 60 in 160 mg/kg dosed animals. In addition, although there were statistically significant

increases in NGAL levels, these levels were many-fold lower than those found in studies of clinical human and experimental animal AKI [503, 504]. H & E stained kidney sections demonstrated an absence of morphological changes between the saline-treated sham control and aminoglycoside treated animals in this long-term toxicity study. The 'TUNEL assay' confirmed that the amount of apoptosis observed in endothelial cells of the kidneys in the single, ultra-high aminoglycoside treated group was similar to that of the saline-treated control group indicating an absence of significant toxicity. The results from the ABR studies for hearing ability and behavioral studies for vestibular function demonstrated an absence of toxicity in these areas. All these data indicate an absence of overt long-term ototoxicity and nephrotoxicity associated with single, ultra-high aminoglycoside doses. However, experiments using a larger group of animals are required to confirm these results given the relatively small number of animals used for each ultra-high dose aminoglycoside in the toxicity experiment.

There are no studies assessing the antimicrobial efficacy of aminoglycosides when used as a single, ultra-high dose. In our septic shock studies, single, ultra-high doses ($\geq$ 80 mg/kg gentamicin-equivalent) were found to eliminate *E. coli* Bort to below the limit of detection from the blood within 5 – 10 minutes after dosing. The bacteria did not regrow up to 9 hours post treatment in the highest assessed dose. A dose-proportional $\log_{10}$ CFU reduction in blood bacterial burden was found with sequentially increasing doses across the range of doses tested from 5 mg/kg to 160 mg/kg of gentamicin with bacteria cleared more quickly with the highest single, ultra-high doses of gentamicin.

Septic shock animals treated with single, ultra-high doses of gentamicin showed no deterioration in renal function as indicated by serum creatinine levels that stayed within the

normal range throughout. Sham-treated animals also exhibited normal levels of serum

creatinine through the study period, but levels were significantly elevated in the UTC by the

end of the experiment.

Serum cytokine levels of IL-1β, IL-4, IL-6, IL-10, IL-12 and IFNγ in UTC were found

to be elevated over the course of the study. By the end of the 9 h observation period, all the

above-mentioned cytokines in treated septic animals were similar to the levels in UTC at the

start of the experiment (ie before sepsis induction). There was a time-dependent decrease in

the levels of the pro-inflammatory cytokines IL-1β and IL-6 at all the doses tested. The

decrease in the level of pro-inflammatory cytokines after dosing ultra-high doses of

gentamicin indicate reduced inflammatory response due to the elimination of infection

causing bacteria from the blood and tissues. Anti-inflammatory cytokines were also expressed

in serum in septic animals and were also reduced with antimicrobial therapy.

Similarly, serum lactate levels were elevated by the time antimicrobials were

administered (approximately 12 h after sepsis induction). However, lactate levels fell to

values similar to baseline (pre-sepsis) and similar to sham controls in septic animals treated

with all the doses of aminoglycosides. This again suggests efficacy of the aminoglycoside

dosing regimen.

These data support the proposal that the dose-antibacterial efficacy range of the

aminoglycoside antibiotic, gentamicin, extends far beyond the normal upper limit (7 mg/kg in

humans) of dosing. A single ultra-high dose of gentamicin (80-160 mg/kg) very rapidly and

effectively clears bacteremia and pathogen burden in the kidneys along with generation of

substantial reductions in solid organ pathogen burden of other organs. This blood bacterial

clearance is substantially accelerated compared to cefotaxime. In addition, ultra-high dose

aminoglycoside administration is associated with congruent reductions in pro-inflammatory cytokines and lactate levels. Available data suggests the absence of serious cardiovascular, oto-vestibular and renal toxicity using single, ultra-high dose aminoglycoside therapy.

While we studied sepsis due to *E. coli* (Bort strain), aminoglycosides have a wide range of activity against both Gram-positive and Gram-negative bacteria. Ultra-high doses may be administered as empirical therapy to speed clearance of bacteria potentially resulting in accelerated hemodynamic stability and improved survival. Ultra-high doses may then be followed by another relatively effective and less toxic antibiotic dosing regimen to clear the remaining bacteria from the vital organs. If this strategy is further tested and bacterial load is successfully cleared with faster attainment of hemodynamic stability, ultra-high doses of aminoglycosides can be considered for further studies in higher mammalian species. If similar results are obtained in the higher animal species with accelerated bacterial clearance from the blood and organs with acceptable toxicity, this treatment regimen could be translated from the 'lab to bedside' and could be used to treat septic shock patients.

6.1.1. Advantages of single, ultra-high doses of aminoglycosides

There are many advantages of utilizing single, ultra-high aminoglycoside doses in septic shock patients;

- Even with the changes in physiological parameters and alteration in the pharmacokinetics in septic shock patients, a high degree of early cidality can be achieved by using single, ultra-high aminoglycoside dosing.

- With ultra-high aminoglycoside doses, a C_{max}/MIC and AUC24/MIC far above the recommended minimum level of 8 - 10 and > 125 respectively for an acceptable

clinical/microbiologic response can be consistently achieved even for moderately resistant organisms.

- Elimination of sensitive aerobic bacteria, Gram positive or Gram negative, responsible for septic shock can substantially accelerated

- Single ultra-high dose aminoglycoside therapy can be followed up with another antibiotic belonging to a separate class. This will limit aminoglycoside-related nephrotoxicity toxicity and/or ototoxicity.

- Potential utility as high intensity (possibly short duration) therapy for biowarfare/bioterrorism bacterial pathogens, including plague (*Yersinia pestis*), tularemia (*Francisella tularensis*) and brucellosis (Brucella species)

6.1.2. Disadvantages single, ultra-high doses of aminoglycosides

- Careful monitoring may be required during neuromuscular paralysis.

- Follow-up of patients treated with ultra-high doses for renal and ototoxicity evaluation may be required.

6.2. Limitations

Although this model represents a clinically relevant model of septic shock, there are a few limitations:

- Species difference should be taken into account when extrapolating our findings to human disease. Further experiments should be performed in a large animal model more analogous to humans (dogs, sheep, pigs or, ideally, primates). A particular issue is the much higher glomerular filtration rate, as is evident by higher creatinine

clearance in rodents compared to humans. This necessitated using substantially higher doses in rats than might be contemplated in any future studies of the approach in human disease.

- The small animal number (4 animals/ultra-high dose) used in the non-invasive long-term toxicity study may engender type II error. Minimization of type II error requires use of a larger group of animals per dose to confirm the results obtained from this experiment. This study in rats is a preliminary study and was performed only to determine if there is evidence of overt toxicity that might preclude additional studies in larger animals. This study supports the use of larger mammalian species for further assessment of drug toxicity with the ultra-high dose regimen.

- Young, healthy adult animals were used to assess for our aminoglycoside toxicity studies and to assess the impact ultra-high dose aminoglycosides in severe sepsis/septic shock. However, in the clinical scenario, severe sepsis/septic shock is much more common in older patients with compromised cardiopulmonary conditions and other major co-morbidities. The risk of renal and oto-vestibular toxicity may be increased in such patients so any results using healthy animals should only be extended to human disease with great caution. Further experimental studies in rodents or higher animals may need to include older animals to more accurately reflect human disease.

- Along with single, ultra-high doses of aminoglycosides, additional antimicrobials and application of source control may be necessary to optimally mimic human septic shock treatment in experimental models. Such therapy may be additive to ultra-high dose aminoglycosides treatment potentially accelerating pathogen clearance and resolution of septic shock. The lack of examination of such therapies in our animal model further limits direct applicability to human disease therapy.

- Aminoglycosides and β-lactams have different propensities towards endotoxin release from Gram-negative pathogens. This has not been fully explored in this series of studies. Further assessment of serum endotoxin levels during antibiotic therapy in this model would be useful in understanding potential advantages of ultra-high dose therapy. Serum endotoxin evaluation from a larger sample of experimental animals in our future experiments may help us in understanding the potential role of endotoxin release in physiologic/ cardiovascular responses to ultra-high dose aminoglycoside therapy.

- The cochlea of single, ultra-high dose aminoglycoside treated animals as well as untreated control animals were not properly fixed in the 10% buffered formalin by immersion fixation method. Due to this, only H & E staining was performed and TUNEL assay for hair cell apoptosis was not recommended. In subsequent animal studies, we intend to use infusion fixation of all the organs to overcome this problem. Although the absence of adverse changes in ABRs argues against auditory toxicity, histology support would be useful.

- Similarly, despite multiple, prolonged efforts and recruitment of collaborators, we were unable to perform vestibular toxicity assessment to our satisfaction. Such testing is performed regularly in only a small number of labs worldwide. It was necessary to substitute behavioral testing for vestibular function which we believe is not as sensitive as vestibular evoked responses (although it may have more functional relevance). We hope to perform vestibular evoked responses testing in large animals in the next phase of our studies.

6.3. Future directions

- Evaluate the ability of single, ultra-high dose aminoglycoside antibiotics to clear *E. coli* (Bort) and other sensitive bacterial pathogens and improve survival (compared to standard antimicrobial therapy) in SD rats or mice with experimental septic shock (non-invasive septic shock study). Improvement in survival with correlative improvement in bacterial clearance would support the application of this dosing strategy to higher animals.

- Evaluate the toxicity of ultra-high dose aminoglycosides in a larger number of SD rats per group. In our study, only 2 animals/ dose/antibiotic were used for toxicity evaluation. Due to a small number of animals in our study, type II error cannot be avoided. Our results indicate that there was no overt toxicity associated with single, ultra-high doses of gentamicin. However, including a larger number of animals in the toxicity study would allow assessment of the possible existence of more subtle renal and oto-vestibular injury.

- Evaluate the antimicrobial efficacy and associated survival of a single, ultra-high dose gentamicin concurrently with a brief intramuscular dosing regimen of a β-lactam (with a different mode of antimicrobial action) against *E. coli* Bort and other sensitive bacterial pathogens in the same septic shock model in SD rats or mice. This will allow evaluation of efficacy of the ultra-high dosing strategy in a therapeutic regimen comparable to those used in humans with septic shock.

- Assess the potential of ultra-high, single and multiple dose aminoglycoside therapy with moderately resistant Gram-negative and Gram-positive pathogens. This study would allow assessment of the potential utility of the ultra-high aminoglycoside dosing strategy in resistant pathogens for which there are currently few therapeutic options

- Evaluate the antimicrobial efficacy (pathogen clearance) and toxicity of single, ultra-high dose fluoroquinolone therapy in normal healthy rats with experimental septic shock induced by *E. coli* Bort and other susceptible pathogens. Fluoroquinolones, like aminoglycosides, demonstrate concentration-dependent killing of a wide range of pathogens and are also generally cleared by the kidneys. An ultra-high dose strategy with these drugs may be similarly viable if neurotoxicity issues (the primary form of toxicity), can be addressed with neuroprotective agents (propofol/benzodiazepines, etc.).

- Assess antimicrobial efficacy, toxicity and survival with single, ultra-high doses of aminoglycosides during experimental live organism septic shock in higher animals, particularly primates. Ultimately, if our experiments in rodents are successful, it will be important to evaluate the ultra-high dosing regimen in higher animal species whose drug pharmacokinetics/pharmacodynamics, metabolism and toxicity risks are similar to humans. If the results from rodent models are reproduced in primates, this dosing regimen can be assessed in septic shock patients.

CHAPTER 7

7. References

[1] Kaukonen KM, Bailey M, Suzuki S, Pilcher D, Bellomo R. Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012. Jama. 2014;311:1308-16.

[2] Ferrer R, Martin-Loeches I, Phillips G, Osborn TM, Townsend S, Dellinger RP, et al. Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: results from a guideline-based performance improvement program. Critical care medicine. 2014;42:1749-55.

[3] Mayr FB, Yende S, Angus DC. Epidemiology of Severe Sepsis. Virulence. 2014;5:4-11.

[4] Husak L, Marcuzzi A, Herring J, Wen E, Yin L, Capan DD, et al. National analysis of sepsis hospitalizations and factors contributing to sepsis in-hospital mortality in Canada. Healthc Q. 2010;13 Spec No:35-41.

[5] Englert JA, Fink MP. The multiple organ dysfunction syndrome and late-phase mortality in sepsis. Current infectious disease reports. 2005;7:335-41.

[6] Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Critical care medicine. 2001;29:1303-10.

[7] Martin CM, Priestap F, Fisher H, Fowler RA, Heyland DK, Keenan SP, et al. A prospective, observational registry of patients with severe sepsis: the Canadian Sepsis Treatment and Response Registry. Critical care medicine. 2009;37:81-8.

[8] Brun-Buisson C, Meshaka P, Pinton P, Vallet B, Group ES. EPISEPSIS: a reappraisal of the epidemiology and outcome of severe sepsis in French intensive care units. Intensive care medicine. 2004;30:580-8.

[9] Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. The New England Journal of Medicine. 2003;348:1546-54.

[10] Adhikari NK, Fowler RA, Bhagwanjee S, Rubenfeld GD. Critical care and the global burden of critical illness in adults. Lancet. 2010;376:1339-46.

[11] Manns BJ, Lee H, Doig CJ, Johnson D, Donaldson C. An economic evaluation of activated protein C treatment for severe sepsis. The New England Journal of Medicine. 2002;347:993-1000.

[12] Dombrovskiy VY, Martin AA, Sunderram J, Paz HL. Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. Critical care medicine. 2007;35:1244-50.

[13] Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest. 1992;101:1644-55.

[14] Mokart D, Saillard C, Sannini A, Chow-Chine L, Brun JP, Faucher M, et al. Neutropenic cancer patients with severe sepsis: need for antibiotics in the first hour. Intensive care medicine. 2014;40:1173-4.

[15] Diskin CJ. Catheter-related sepsis in dialysis patients. QJM : Monthly Journal of the Association of Physicians. 2007;100:666-7.

[16] Fraimow HS, Tsigrelis C. Antimicrobial resistance in the intensive care unit: mechanisms, epidemiology, and management of specific resistant pathogens. Critical care clinics. 2011;27:163-205.

[17] De Gaudio AR, Rinaldi S, Chelazzi C, Borracci T. Pathophysiology of sepsis in the elderly: clinical impact and therapeutic considerations. Current drug targets. 2009;10:60-70.

[18] Sakr Y, Elia C, Mascia L, Barberis B, Cardellino S, Livigni S, et al. The influence of gender on the epidemiology of and outcome from severe sepsis. Critical care. 2013;17:R50.

[19] Schroder J, Kahlke V, Staubach KH, Zabel P, Stuber F. Gender differences in human sepsis. Archives of surgery. 1998;133:1200-5.

[20] Danai PA, Sinha S, Moss M, Haber MJ, Martin GS. Seasonal variation in the epidemiology of sepsis. Critical care medicine. 2007;35:410-5.

[21] Zahar JR, Timsit JF, Garrouste-Orgeas M, Francais A, Vesin A, Descorps-Declere A, et al. Outcomes in severe sepsis and patients with septic shock: pathogen species and infection sites are not associated with mortality. Critical care medicine. 2011;39:1886-95.

[22] Kumar A, Ellis P, Arabi Y, Roberts D, Light B, Parrillo JE, et al. Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock. Chest. 2009;136:1237-48.

[23] Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, et al. International study of the prevalence and outcomes of infection in intensive care units. Jama. 2009;302:2323-9.

[24] Opal SM, Garber GE, LaRosa SP, Maki DG, Freebairn RC, Kinasewitz GT, et al. Systemic host responses in severe sepsis analyzed by causative microorganism and treatment effects of drotrecogin alfa (activated). Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2003;37:50-8.

[25] Jordan JA, Durso MB. Real-time polymerase chain reaction for detecting bacterial DNA directly from blood of neonates being evaluated for sepsis. The Journal of molecular diagnostics : JMD. 2005;7:575-81.

[26] Visintin C, Mugglestone MA, Fields EJ, Jacklin P, Murphy MS, Pollard AJ, et al. Management of bacterial meningitis and meningococcal septicaemia in children and young people: summary of NICE guidance. Bmj. 2010;340:c3209.

[27] Baltimore RS, Huie SM, Meek JI, Schuchat A, O'Brien KL. Early-onset neonatal sepsis in the era of group B streptococcal prevention. Pediatrics. 2001;108:1094-8.

[28] Kethireddy S, Light RB, Mirzanejad Y, Maki D, Arabi Y, Lapinsky S, et al. Mycobacterium tuberculosis septic shock. Chest. 2013;144:474-82.

[29] Villinger F, Rollin PE, Brar SS, Chikkala NF, Winter J, Sundstrom JB, et al. Markedly elevated levels of interferon (IFN)-gamma, IFN-alpha, interleukin (IL)-2, IL-10, and tumor necrosis factor-alpha associated with fatal Ebola virus infection. The Journal of infectious diseases. 1999;179 Suppl 1:S188-91.

[30] Lum LC. Management of dengue haemorrhagic fever/dengue shock syndrome. The Malaysian journal of pathology. 1993;15:29-33.

[31] Muscedere J, Ofner M, Kumar A, Long J, Lamontagne F, Cook D, et al. The occurrence and impact of bacterial organisms complicating critical care illness associated with 2009 influenza A(H1N1) infection. Chest. 2013;144:39-47.

[32] Delaloye J, Calandra T. Invasive candidiasis as a cause of sepsis in the critically ill patient. Virulence. 2014;5:161-9.

[33] Pop-Vicas A, Opal SM. The clinical impact of multidrug-resistant gram-negative bacilli in the management of septic shock. Virulence. 2014;5:206-12.

[34] Bone RC. Sepsis, sepsis syndrome, and the systemic inflammatory response syndrome (SIRS). Gulliver in Laputa. Jama. 1995;273:155-6.

[35] Astiz M, Saha D, Lustbader D, Lin R, Rackow E. Monocyte response to bacterial toxins, expression of cell surface receptors, and release of anti-inflammatory cytokines during sepsis. The Journal of laboratory and clinical medicine. 1996;128:594-600.

[36] Anel RL, Kumar A. Experimental and emerging therapies for sepsis and septic shock. Expert opinion on investigational drugs. 2001;10:1471-85.

[37] Bosmann M, Ward PA. The inflammatory response in sepsis. Trends in immunology. 2013;34:129-36.

[38] Cai B, Deitch EA, Ulloa L. Novel insights for systemic inflammation in sepsis and hemorrhage. Mediators of inflammation. 2010;2010:642462.

[39] Decker T, Schneller F, Sparwasser T, Tretter T, Lipford GB, Wagner H, et al. Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells. Blood. 2000;95:999-1006.

[40] Sparwasser T, Koch ES, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. European journal of immunology. 1998;28:2045-54.

[41] D'Onofrio C, Paradisi F. The influence of bacterial exotoxins and endotoxins on the phagocytic activity of human macrophages in culture. Infection. 1983;11:137-43.

[42] Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. Science. 1990;248:1066.

[43] Eriksson BK, Andersson J, Holm SE, Norgren M. Invasive group A streptococcal infections: T1M1 isolates expressing pyrogenic exotoxins A and B in combination with selective lack of toxin-neutralizing antibodies are associated with increased risk of streptococcal toxic shock syndrome. The Journal of infectious diseases. 1999;180:410-8.

[44] Proft T, Sriskandan S, Yang L, Fraser JD. Superantigens and streptococcal toxic shock syndrome. Emerging infectious diseases. 2003;9:1211-8.

[45] Braunstein NS, Weber DA, Wang XC, Long EO, Karp D. Sequences in both class II major histocompatibility complex alpha and beta chains contribute to the binding of the superantigen toxic shock syndrome toxin 1. The Journal of experimental medicine. 1992;175:1301-5.

[46] Mourad W, Scholl P, Diaz A, Geha R, Chatila T. The staphylococcal toxic shock syndrome toxin 1 triggers B cell proliferation and differentiation via major histocompatibility complex-unrestricted cognate T/B cell interaction. The Journal of experimental medicine. 1989;170:2011-22.

[47] Arad G, Levy R, Nasie I, Hillman D, Rotfogel Z, Barash U, et al. Binding of superantigen toxins into the CD28 homodimer interface is essential for induction of cytokine genes that mediate lethal shock. PLoS biology. 2011;9:e1001149.

[48] Miethke T, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. The Journal of experimental medicine. 1992;175:91-8.

[49] Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140:805-20.

[50] Raetz CR, Ulevitch RJ, Wright SD, Sibley CH, Ding A, Nathan CF. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction.

FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1991;5:2652-60.

[51] Cai Y, Sugimoto C, Arainga M, Alvarez X, Didier ES, Kuroda MJ. In vivo characterization of alveolar and interstitial lung macrophages in rhesus macaques: implications for understanding lung disease in humans. J Immunol. 2014;192:2821-9.

[52] Karima R, Matsumoto S, Higashi H, Matsushima K. The molecular pathogenesis of endotoxic shock and organ failure. Molecular medicine today. 1999;5:123-32.

[53] Kiener PA, Marek F, Rodgers G, Lin PF, Warr G, Desiderio J. Induction of tumor necrosis factor, IFN-gamma, and acute lethality in mice by toxic and non-toxic forms of lipid A. J Immunol. 1988;141:870-4.

[54] Luderitz O, Galanos C, Lehmann V, Nurminen M, Rietschel ET, Rosenfelder G, et al. Lipid A: chemical structure and biological activity. The Journal of infectious diseases. 1973;128:Suppl:17-29.

[55] Khan SA, Everest P, Servos S, Foxwell N, Zahringer U, Brade H, et al. A lethal role for lipid A in Salmonella infections. Molecular microbiology. 1998;29:571-9.

[56] Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, et al. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. The Journal of clinical investigation. 2000;105:497-504.

[57] Zanoni I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, et al. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell. 2011;147:868-80.

[58] Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. The Journal of experimental medicine. 1999;189:1777-82.

[59] Sun SC, Ganchi PA, Ballard DW, Greene WC. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science. 1993;259:1912-5.

[60] Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol. 1999;162:3749-52.

[61] MacKenzie SA, Roher N, Boltana S, Goetz FW. Peptidoglycan, not endotoxin, is the key mediator of cytokine gene expression induced in rainbow trout macrophages by crude LPS. Molecular immunology. 2010;47:1450-7.

[62] Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001;410:1099-103.

[63] Ramachandran G. Gram-positive and gram-negative bacterial toxins in sepsis: a brief review. Virulence. 2014;5:213-8.

[64] Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, et al. Bacterial DNA causes septic shock. Nature. 1997;386:336-7.

[65] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000;408:740-5.

[66] Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol. 2002;169:10-4.

[67] Drage MG, Pecora ND, Hise AG, Febbraio M, Silverstein RL, Golenbock DT, et al. TLR2 and its co-receptors determine responses of macrophages and dendritic cells to lipoproteins of Mycobacterium tuberculosis. Cellular immunology. 2009;258:29-37.

[68] Cristofaro P, Opal SM. Role of Toll-like receptors in infection and immunity: clinical implications. Drugs. 2006;66:15-29.

[69] Takeda K, Akira S. Microbial recognition by Toll-like receptors. Journal of dermatological science. 2004;34:73-82.

[70] Weighardt H, Feterowski C, Veit M, Rump M, Wagner H, Holzmann B. Increased resistance against acute polymicrobial sepsis in mice challenged with immunostimulatory CpG oligodeoxynucleotides is related to an enhanced innate effector cell response. J Immunol. 2000;165:4537-43.

[71] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annual review of immunology. 2002;20:709-60.

[72] Chuang TH, Lee J, Kline L, Mathison JC, Ulevitch RJ. Toll-like receptor 9 mediates CpG-DNA signaling. Journal of leukocyte biology. 2002;71:538-44.

[73] Eidelman LA, Putterman D, Putterman C, Sprung CL. The spectrum of septic encephalopathy. Definitions, etiologies, and mortalities. Jama. 1996;275:470-3.

[74] Kumar A KA. Sepsis and Septic Shock. Gabrielli A, Layon JA, Yu M, Civetta, Taylor and Kirby's Critical Care 4th Ed Philadelphia: Lippincott, Williams and Wilkin Co. 2009:855-92.

[75] Sharma S, Kumar A. Septic shock, multiple organ failure, and acute respiratory distress syndrome. Current opinion in pulmonary medicine. 2003;9:199-209.

[76] Fourrier F, Chopin C, Goudemand J, Hendrycx S, Caron C, Rime A, et al. Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies. Chest. 1992;101:816-23.

[77] Esper AM, Moss M, Lewis CA, Nisbet R, Mannino DM, Martin GS. The role of infection and comorbidity: Factors that influence disparities in sepsis. Critical care medicine. 2006;34:2576-82.

[78] Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. Nature reviews Immunology. 2008;8:776-87.

[79] Cinel I, Opal SM. Molecular biology of inflammation and sepsis: a primer. Critical care medicine. 2009;37:291-304.

[80] Beutler B. Endotoxin, tumor necrosis factor, and related mediators: new approaches to shock. New Horiz. 1993;1:3-12.

[81] Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science, 1985, 229(4716):869-871. Classical article. J Immunol. 2008;181:7-9.

[82] Bernot D, Peiretti F, Canault M, Juhan-Vague I, Nalbone G. Upregulation of TNF-alpha-induced ICAM-1 surface expression by adenylate cyclase-dependent pathway in human endothelial cells. Journal of cellular physiology. 2005;202:434-41.

[83] Kumar A, Dhawan S, Hardegen NJ, Aggarwal BB. Curcumin (Diferuloylmethane) inhibition of tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppression of cell surface expression of adhesion molecules and of nuclear factor-kappaB activation. Biochemical pharmacology. 1998;55:775-83.

[84] Fortin CF, McDonald PP, Fulop T, Lesur O. Sepsis, leukocytes, and nitric oxide (NO): an intricate affair. Shock. 2010;33:344-52.

[85] Lee WL, Slutsky AS. Sepsis and endothelial permeability. The New England Journal of Medicine. 2010;363:689-91.

[86] Clark R, Kupper T. Old meets new: the interaction between innate and adaptive immunity. The Journal of investigative dermatology. 2005;125:629-37.

[87] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783-801.

[88] Eberle F, Sirin M, Binder M, Dalpke AH. Bacterial RNA is recognized by different sets of immunoreceptors. European journal of immunology. 2009;39:2537-47.

[89] Netea MG, Ferwerda G, van der Graaf CA, Van der Meer JW, Kullberg BJ. Recognition of fungal pathogens by toll-like receptors. Current pharmaceutical design. 2006;12:4195-201.

[90] Philpott DJ, Girardin SE. The role of Toll-like receptors and Nod proteins in bacterial infection. Molecular immunology. 2004;41:1099-108.

[91] Sabroe I, Jones EC, Usher LR, Whyte MK, Dower SK. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. J Immunol. 2002;168:4701-10.

[92] Koizumi Y, Toma C, Higa N, Nohara T, Nakasone N, Suzuki T. Inflammasome activation via intracellular NLRs triggered by bacterial infection. Cellular microbiology. 2012;14:149-54.

[93] Tamayo E, Fernandez A, Almansa R, Carrasco E, Heredia M, Lajo C, et al. Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. European cytokine network. 2011;22:82-7.

[94] Berends ET, Kuipers A, Ravesloot MM, Urbanus RT, Rooijakkers SH. Bacteria under stress by complement and coagulation. FEMS microbiology reviews. 2014:10.1111/574-6976.12080.

[95] Oh SJ, Kim JH, Chung DH. NOD2-mediated suppression of CD55 on neutrophils enhances C5a generation during polymicrobial sepsis. PLoS pathogens. 2013;9:e1003351.

[96] Ward PA. Role of the complement in experimental sepsis. Journal of leukocyte biology. 2008;83:467-70.

[97] Dumestre-Perard C, Doerr E, Colomb MG, Loos M. Involvement of complement pathways in patients with bacterial septicemia. Molecular immunology. 2007;44:1631-8.

[98] Bosmann M, Ward PA. Role of C3, C5 and anaphylatoxin receptors in acute lung injury and in sepsis. Advances in experimental medicine and biology. 2012;946:147-59.

[99] Furebring M, Hakansson LD, Venge P, Nilsson B, Sjolin J. Expression of the C5a receptor (CD88) on granulocytes and monocytes in patients with severe sepsis. Critical care. 2002;6:363-70.

[100] Huber-Lang MS, Younkin EM, Sarma JV, McGuire SR, Lu KT, Guo RF, et al. Complement-induced impairment of innate immunity during sepsis. J Immunol. 2002;169:3223-31.

[101] Solomkin JS, Jenkins MK, Nelson RD, Chenoweth D, Simmons RL. Neutrophil dysfunction in sepsis. II. Evidence for the role of complement activation products in cellular deactivation. Surgery. 1981;90:319-27.

[102] Czermak BJ, Sarma V, Pierson CL, Warner RL, Huber-Lang M, Bless NM, et al. Protective effects of C5a blockade in sepsis. Nature medicine. 1999;5:788-92.

[103] Van Epps DE, Simpson SJ, Johnson R. Relationship of C5a receptor modulation to the functional responsiveness of human polymorphonuclear leukocytes to C5a. J Immunol. 1993;150:246-52.

[104] Hopken U, Mohr M, Struber A, Montz H, Burchardi H, Gotze O, et al. Inhibition of interleukin-6 synthesis in an animal model of septic shock by anti-C5a monoclonal antibodies. European journal of immunology. 1996;26:1103-9.

[105] Hangen DH, Stevens JH, Satoh PS, Hall EW, O'Hanley PT, Raffin TA. Complement levels in septic primates treated with anti-C5a antibodies. The Journal of surgical research. 1989;46:195-9.

[106] Smedegard G, Cui LX, Hugli TE. Endotoxin-induced shock in the rat. A role for C5a. The American journal of pathology. 1989;135:489-97.

[107] Ward PA, Guo RF, Riedemann NC. Manipulation of the complement system for benefit in sepsis. Critical care research and practice. 2012;2012:427607.

[108] Logan TF, Virji MA, Gooding WE, Bontempo FA, Ernstoff MS, Kirkwood JM. The pathogenesis of disseminated intravascular coagulation in sepsis. Jama. 1994;271:427-8.

[109] Angus DC, van der Poll T. Severe sepsis and septic shock. The New England Journal of Medicine. 2013;369:840-51.

[110] Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. The New England Journal of Medicine. 2001;344:699-709.

[111] Ranieri VM, Thompson BT, Barie PS, Dhainaut JF, Douglas IS, Finfer S, et al. Drotrecogin alfa (activated) in adults with septic shock. The New England Journal of Medicine. 2012;366:2055-64.

[112] Boomer JS, Shuherk-Shaffer J, Hotchkiss RS, Green JM. A prospective analysis of lymphocyte phenotype and function over the course of acute sepsis. Critical care. 2012;16:R112.

[113] Chen X, Ye J, Ye J. Analysis of peripheral blood lymphocyte subsets and prognosis in patients with septic shock. Microbiology and immunology. 2011;55:736-42.

[114] Blaschitz C, Raffatellu M. Th17 cytokines and the gut mucosal barrier. Journal of clinical immunology. 2010;30:196-203.

[115] Prosser ME, Brown CE, Shami AF, Forman SJ, Jensen MC. Tumor PD-L1 co-stimulates primary human CD8(+) cytotoxic T cells modified to express a PD1:CD28 chimeric receptor. Molecular immunology. 2012;51:263-72.

[116] van der Poll T, van Deventer SJ. Cytokines and anticytokines in the pathogenesis of sepsis. Infectious disease clinics of North America. 1999;13:413-26, ix.

[117] Brunialti MK, Santos MC, Rigato O, Machado FR, Silva E, Salomao R. Increased percentages of T helper cells producing IL-17 and monocytes expressing markers of alternative activation in patients with sepsis. PloS one. 2012;7:e37393.

[118] Venet F, Chung CS, Kherouf H, Geeraert A, Malcus C, Poitevin F, et al. Increased circulating regulatory T cells (CD4(+)CD25 (+)CD127 (-)) contribute to lymphocyte anergy in septic shock patients. Intensive care medicine. 2009;35:678-86.

[119] Wang C, Hillsamer P, Kim CH. Phenotype, effector function, and tissue localization of PD-1-expressing human follicular helper T cell subsets. BMC immunology. 2011;12:53.

[120] Guignant C, Lepape A, Huang X, Kherouf H, Denis L, Poitevin F, et al. Programmed death-1 levels correlate with increased mortality, nosocomial infection and immune dysfunctions in septic shock patients. Critical care. 2011;15:R99.

[121] Antonelli M. Sepsis and septic shock: pro-inflammatory or anti-inflammatory state? Journal of chemotherapy. 1999;11:536-40.

[122] Williams MA, Withington S, Newland AC, Kelsey SM. Monocyte anergy in septic shock is associated with a predilection to apoptosis and is reversed by granulocyte-macrophage colony-stimulating factor ex vivo. The Journal of infectious diseases. 1998;178:1421-33.

[123] Heidecke CD, Hensler T, Weighardt H, Zantl N, Wagner H, Siewert JR, et al. Selective defects of T lymphocyte function in patients with lethal intraabdominal infection. American journal of surgery. 1999;178:288-92.

[124] Muenzer JT, Davis CG, Chang K, Schmidt RE, Dunne WM, Coopersmith CM, et al. Characterization and modulation of the immunosuppressive phase of sepsis. Infection and immunity. 2010;78:1582-92.

[125] Reddy RC, Chen GH, Tekchandani PK, Standiford TJ. Sepsis-induced immunosuppression: from bad to worse. Immunologic research. 2001;24:273-87.

[126] Kumar A, Parrillo J. Shock: classification, pathophysiology, and approach to management. Critical care medicine: Principles of diagnosis and management in adult. 2008; 3:377-422.

[127] De Kock I, Van Daele C, Poelaert J. Sepsis and septic shock: pathophysiological and cardiovascular background as basis for therapy. Acta clinica Belgica. 2010;65:323-9.

[128] De Backer D, Creteur J, Preiser JC, Dubois MJ, Vincent JL. Microvascular blood flow is altered in patients with sepsis. American journal of respiratory and critical care medicine. 2002;166:98-104.

[129] Beilman GJ. Selective inducible nitric oxide synthase inhibition in septic shock. Critical care medicine. 2001;29:2230-1.

[130] Doerschug KC, Delsing AS, Schmidt GA, Haynes WG. Impairments in microvascular reactivity are related to organ failure in human sepsis. American journal of physiology Heart and circulatory physiology. 2007;293:H1065-71.

[131] Sakr Y, Dubois MJ, De Backer D, Creteur J, Vincent JL. Persistent microcirculatory alterations are associated with organ failure and death in patients with septic shock. Critical care medicine. 2004;32:1825-31.

[132] Brun-Buisson C, Doyon F, Carlet J, Dellamonica P, Gouin F, Lepoutre A, et al. Incidence, risk factors, and outcome of severe sepsis and septic shock in adults. A multicenter prospective study in intensive care units. French ICU Group for Severe Sepsis. Jama. 1995;274:968-74.

[133] Paterson RL, Webster NR. Sepsis and the systemic inflammatory response syndrome. Journal of the Royal College of Surgeons of Edinburgh. 2000;45:178-82.

[134] Levin M, Quint PA, Goldstein B, Barton P, Bradley JS, Shemie SD, et al. Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. Lancet. 2000;356:961-7.

[135] Khatib R, Johnson LB, Fakih MG, Riederer K, Khosrovaneh A, Shamse Tabriz M, et al. Persistence in Staphylococcus aureus bacteremia: incidence, characteristics of patients and outcome. Scandinavian journal of infectious diseases. 2006;38:7-14.

[136] Hawkins C, Huang J, Jin N, Noskin GA, Zembower TR, Bolon M. Persistent Staphylococcus aureus bacteremia: an analysis of risk factors and outcomes. Archives of internal medicine. 2007;167:1861-7.

[137] Torgersen C, Moser P, Luckner G, Mayr V, Jochberger S, Hasibeder WR, et al. Macroscopic postmortem findings in 235 surgical intensive care patients with sepsis. Anesthesia and analgesia. 2009;108:1841-7.

[138] Kumar A. An alternate pathophysiologic paradigm of sepsis and septic shock: implications for optimizing antimicrobial therapy. Virulence. 2014;5:80-97.

[139] Otto GP, Sossdorf M, Claus RA, Rodel J, Menge K, Reinhart K, et al. The late phase of sepsis is characterized by an increased microbiological burden and death rate. Critical care. 2011;15:R183.

[140] Wiggers HC, Goldberg H, Roemhild F, Ingraham RC. Impending hemorrhagic shock and the course of events following administration of dibenamine. Circulation. 1950;2:179-85.

[141] Sampalis JS, Lavoie A, Williams JI, Mulder DS, Kalina M. Impact of on-Site Care, Prehospital Time, and Level of in-Hospital Care on Survival in Severely Injured Patients. J Trauma. 1993;34:252-61.

[142] De Luca G, Suryapranata H, Ottervanger JP, Antman EM. Time delay to treatment and mortality in primary angioplasty for acute myocardial infarction - Every minute of delay counts. Circulation. 2004;109:1223-5.

[143] Leligdowicz A, Dodek PM, Norena M, Wong H, Kumar A, Kumar A, et al. Association between source of infection and hospital mortality in patients who have septic shock. American journal of respiratory and critical care medicine. 2014;189:1204-13.

[144] Yagupsky P, Nolte FS. Quantitative aspects of septicemia. Clinical microbiology reviews. 1990;3:269-79.

[145] Kumar A, Haery C, Paladugu B, Kumar A, Symeoneides S, Taiberg L, et al. The duration of hypotension before the initiation of antibiotic treatment is a critical determinant of survival in a murine model of Escherichia coli septic shock: association with serum lactate and inflammatory cytokine levels. The Journal of infectious diseases. 2006;193:251-8.

[146] Proulx N, Frechette D, Toye B, Chan J, Kravcik S. Delays in the administration of antibiotics are associated with mortality from adult acute bacterial meningitis. QJM : Monthly Journal of the Association of Physicians. 2005;98:291-8.

[147] Chuang YC, Chang SC, Wang WK. Using the rate of bacterial clearance determined by real-time polymerase chain reaction as a timely surrogate marker to evaluate the appropriateness of antibiotic usage in critical patients with Acinetobacter baumannii bacteremia. Critical care medicine. 2012;40:2273-80.

[148] Werno AM, Anderson TP, Murdoch DR. Association between pneumococcal load and disease severity in adults with pneumonia. Journal of medical microbiology. 2012;61:1129-35.

[149] Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock, 2012. Intensive care medicine. 2013;39:165-228.

[150] Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, et al. Early goal-directed therapy in the treatment of severe sepsis and septic shock. The New England Journal of Medicine. 2001;345:1368-77.

[151] Yealy DM, Kellum JA, Huang DT, Barnato AE, Weissfeld LA, Pike F, et al. A randomized trial of protocol-based care for early septic shock. The New England Journal of Medicine. 2014;370:1683-93.

[152] Peake SL, Delaney A, Bailey M, Bellomo R, Cameron PA, Cooper DJ, et al. Goal-directed resuscitation for patients with early septic shock. The New England Journal of Medicine. 2014;371:1496-506.

[153] Olsen KM, Campbell GD, Jr. E5 monoclonal immunoglobulin M antibody for the treatment of gram-negative sepsis. DICP : the annals of pharmacotherapy. 1991;25:784-90.

[154] Ziegler EJ, Fisher CJ, Jr., Sprung CL, Straube RC, Sadoff JC, Foulke GE, et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group. The New England Journal of Medicine. 1991;324:429-36.

[155] Bone RC, Balk RA, Fein AM, Perl TM, Wenzel RP, Reines HD, et al. A second large controlled clinical study of E5, a monoclonal antibody to endotoxin: results of a prospective, multicenter, randomized, controlled trial. The E5 Sepsis Study Group. Critical care medicine. 1995;23:994-1006.

[156] McCloskey RV, Straube RC, Sanders C, Smith SM, Smith CR. Treatment of septic shock with human monoclonal antibody HA-1A. A randomized, double-blind, placebo-controlled trial. CHESS Trial Study Group. Annals of internal medicine. 1994;121:1-5.

[157] Peri F, Piazza M. Therapeutic targeting of innate immunity with Toll-like receptor 4 (TLR4) antagonists. Biotechnology advances. 2012;30:251-60.

[158] Sakaue M, Hoffman BB. Glucocorticoids induce transcription and expression of the alpha 1B adrenergic receptor gene in DTT1 MF-2 smooth muscle cells. The Journal of clinical investigation. 1991;88:385-9.

[159] Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. The New England Journal of Medicine. 2003;348:138-50.

[160] Shenker Y, Skatrud JB. Adrenal insufficiency in critically ill patients. American journal of respiratory and critical care medicine. 2001;163:1520-3.

[161] Sprung CL, Annane D, Keh D, Moreno R, Singer M, Freivogel K, et al. Hydrocortisone therapy for patients with septic shock. The New England Journal of Medicine. 2008;358:111-24.

[162] Wang C, Sun J, Zheng J, Guo L, Ma H, Zhang Y, et al. Low-dose hydrocortisone therapy attenuates septic shock in adult patients but does not reduce 28-day mortality: a meta-analysis of randomized controlled trials. Anesthesia and analgesia. 2014;118:346-57.

[163] Patel GP, Balk RA. Systemic steroids in severe sepsis and septic shock. American journal of respiratory and critical care medicine. 2012;185:133-9.

[164] Opal SM, Dellinger RP, Vincent JL, Masur H, Angus DC. The next generation of sepsis clinical trial designs: what is next after the demise of recombinant human activated protein C? Critical care medicine. 2014;42:1714-21.

[165] Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature. 1987;330:662-4.

[166] Vincent JL, Sun Q, Dubois MJ. Clinical trials of immunomodulatory therapies in severe sepsis and septic shock. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2002;34:1084-93.

[167] Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Critical care medicine. 2006;34:1589-96.

[168] Paul M, Shani V, Muchtar E, Kariv G, Robenshtok E, Leibovici L. Systematic review and meta-analysis of the efficacy of appropriate empiric antibiotic therapy for sepsis. Antimicrobial agents and chemotherapy. 2010;54:4851-63.

[169] Lyle NH, Pena OM, Boyd JH, Hancock RE. Barriers to the effective treatment of sepsis: antimicrobial agents, sepsis definitions, and host-directed therapies. Annals of the New York Academy of Sciences. 2014;1323:101-14.

[170] Kaufman D, Haas CE, Edinger R, Hollick G. Antibiotic susceptibility in the surgical intensive care unit compared with the hospital-wide antibiogram. Archives of surgery. 1998;133:1041-5.

[171] Raineri E, Pan A, Mondello P, Acquarolo A, Candiani A, Crema L. Role of the infectious diseases specialist consultant on the appropriateness of antimicrobial therapy prescription in an intensive care unit. American journal of infection control. 2008;36:283-90.

[172] Fowler VG, Jr., Sanders LL, Sexton DJ, Kong L, Marr KA, Gopal AK, et al. Outcome of Staphylococcus aureus bacteremia according to compliance with recommendations of infectious diseases specialists: experience with 244 patients. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1998;27:478-86.

[173] Varpula M, Karlsson S, Parviainen I, Ruokonen E, Pettila V, Finnsepsis Study G. Community-acquired septic shock: early management and outcome in a nationwide study in Finland. Acta anaesthesiologica Scandinavica. 2007;51:1320-6.

[174] Ferrer R, Artigas A, Suarez D, Palencia E, Levy MM, Arenzana A, et al. Effectiveness of treatments for severe sepsis: a prospective, multicenter, observational study. American journal of respiratory and critical care medicine. 2009;180:861-6.

[175] Nguyen HB, Corbett SW, Steele R, Banta J, Clark RT, Hayes SR, et al. Implementation of a bundle of quality indicators for the early management of severe sepsis and septic shock is associated with decreased mortality. Critical care medicine. 2007;35:1105-12.

[176] Garnacho-Montero J, Garcia-Garmendia JL, Barrero-Almodovar A, Jimenez-Jimenez FJ, Perez-Paredes C, Ortiz-Leyba C. Impact of adequate empirical antibiotic therapy on the outcome of patients admitted to the intensive care unit with sepsis. Critical care medicine. 2003;31:2742-51.

[177] Knudsen JD, Frimodt-Moller N, Espersen F. Pharmacodynamics of penicillin are unaffected by bacterial growth phases of Streptococcus pneumoniae in the mouse peritonitis model. The Journal of antimicrobial chemotherapy. 1998;41:451-9.

[178] Lodise TP, Jr., Patel N, Kwa A, Graves J, Furuno JP, Graffunder E, et al. Predictors of 30-day mortality among patients with Pseudomonas aeruginosa bloodstream infections: impact of delayed appropriate antibiotic selection. Antimicrobial agents and chemotherapy. 2007;51:3510-5.

[179] Barochia AV, Cui X, Vitberg D, Suffredini AF, O'Grady NP, Banks SM, et al. Bundled care for septic shock: an analysis of clinical trials. Critical care medicine. 2010;38:668-78.

[180] Roberts JA, Lipman J. Pharmacokinetic issues for antibiotics in the critically ill patient. Critical care medicine. 2009;37:840-51.

[181] Erstad BL. Dosing of medications in morbidly obese patients in the intensive care unit setting. Intensive care medicine. 2004;30:18-32.

[182] McKenzie C. Antibiotic dosing in critical illness. The Journal of antimicrobial chemotherapy. 2011;66 Suppl 2:ii25-31.

[183] Moore RD, Lietman PS, Smith CR. Clinical response to aminoglycoside therapy: importance of the ratio of peak concentration to minimal inhibitory concentration. The Journal of infectious diseases. 1987;155:93-9.

[184] Rhodes NJ, MacVane SH, Kuti JL, Scheetz MH. Impact of loading doses on the time to adequate predicted beta-lactam concentrations in prolonged and continuous infusion dosing schemes. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2014;59:905-7.

[185] Lepper MH, Dowling HF. Treatment of pneumococcic meningitis with penicillin compared with penicillin plus aureomycin; studies including observations on an apparent antagonism between penicillin and aureomycin. AMA archives of internal medicine. 1951;88:489-94.

[186] Yahav D, Lador A, Paul M, Leibovici L. Efficacy and safety of tigecycline: a systematic review and meta-analysis. The Journal of antimicrobial chemotherapy. 2011;66:1963-71.

[187] Prasad P, Sun J, Danner RL, Natanson C. Excess deaths associated with tigecycline after approval based on noninferiority trials. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2012;54:1699-709.

[188] Wilson WR, Karchmer AW, Dajani AS, Taubert KA, Bayer A, Kaye D, et al. Antibiotic treatment of adults with infective endocarditis due to streptococci, enterococci, staphylococci, and HACEK microorganisms. American Heart Association. Jama. 1995;274:1706-13.

[189] Andes DR, Safdar N, Baddley JW, Playford G, Reboli AC, Rex JH, et al. Impact of treatment strategy on outcomes in patients with candidemia and other forms of invasive candidiasis: a patient-level quantitative review of randomized trials. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2012;54:1110-22.

[190] Kumar A, Zarychanski R, Light B, Parrillo J, Maki D, Simon D, et al. Early combination antibiotic therapy yields improved survival compared with monotherapy in septic shock: a propensity-matched analysis. Critical care medicine. 2010;38:1773-85.

[191] Kumar A, Safdar N, Kethireddy S, Chateau D. A survival benefit of combination antibiotic therapy for serious infections associated with sepsis and septic shock is contingent only on the risk of death: a meta-analytic/meta-regression study. Critical care medicine. 2010;38:1651-64.

[192] Sick AC, Tschudin-Sutter S, Turnbull AE, Weissman SJ, Tamma PD. Empiric combination therapy for gram-negative bacteremia. Pediatrics. 2014;133:e1148-55.

[193] Skorup P, Maudsdotter L, Lipcsey M, Castegren M, Larsson A, Jonsson AB, et al. Beneficial antimicrobial effect of the addition of an aminoglycoside to a beta-lactam antibiotic in an E. coli porcine intensive care severe sepsis model. PloS one. 2014;9:e90441.

[194] Al-Hasan MN, Wilson JW, Lahr BD, Thomsen KM, Eckel-Passow JE, Vetter EA, et al. Beta-lactam and fluoroquinolone combination antibiotic therapy for bacteremia caused by gram-negative bacilli. Antimicrobial agents and chemotherapy. 2009;53:1386-94.

[195] Diaz-Martin A, Martinez-Gonzalez ML, Ferrer R, Ortiz-Leyba C, Piacentini E, Lopez-Pueyo MJ, et al. Antibiotic prescription patterns in the empiric therapy of severe sepsis: combination of antimicrobials with different mechanisms of action reduces mortality. Critical care. 2012;16:R223.

[196] Micek ST, Welch EC, Khan J, Pervez M, Doherty JA, Reichley RM, et al. Empiric combination antibiotic therapy is associated with improved outcome against sepsis due to

Gram-negative bacteria: a retrospective analysis. Antimicrobial agents and chemotherapy. 2010;54:1742-8.

[197] Garnacho-Montero J, Gutierrez-Pizarraya A, Escoresca-Ortega A, Corcia-Palomo Y, Fernandez-Delgado E, Herrera-Melero I, et al. De-escalation of empirical therapy is associated with lower mortality in patients with severe sepsis and septic shock. Intensive care medicine. 2014;40:32-40.

[198] Leone M, Bourgoin A, Cambon S, Dubuc M, Albanese J, Martin C. Empirical antimicrobial therapy of septic shock patients: adequacy and impact on the outcome. Critical care medicine. 2003;31:462-7.

[199] Singh H, Thangaraj P, Chakrabarti A. Acinetobacter baumannii: A Brief Account of Mechanisms of Multidrug Resistance and Current and Future Therapeutic Management. Journal of clinical and diagnostic research : JCDR. 2013;7:2602-5.

[200] Saha R, Jain S, Kaur IR. Metallo beta-lactamase producing pseudomonas species--a major cause of concern among hospital associated urinary tract infection. Journal of the Indian Medical Association. 2010;108:344-8.

[201] Levison ME, Levison JH. Pharmacokinetics and pharmacodynamics of antibacterial agents. Infectious disease clinics of North America. 2009;23:791-815.

[202] Craig WA. Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1998;26:1-10; quiz 1-2.

[203] Drusano GL. Antimicrobial pharmacodynamics: critical interactions of 'bug and drug'. Nature reviews Microbiology. 2004;2:289-300.

[204] Kikuchi E, Kikuchi J, Nasuhara Y, Oizumi S, Ishizaka A, Nishimura M. Comparison of the pharmacodynamics of biapenem in bronchial epithelial lining fluid in healthy volunteers given half-hour and three-hour intravenous infusions. Antimicrobial agents and chemotherapy. 2009;53:2799-803.

[205] Adembri C, Fallani S, Cassetta MI, Arrigucci S, Ottaviano A, Pecile P, et al. Linezolid pharmacokinetic/pharmacodynamic profile in critically ill septic patients: intermittent versus continuous infusion. International journal of antimicrobial agents. 2008;31:122-9.

[206] Schentag JJ, Smith IL, Swanson DJ, DeAngelis C, Fracasso JE, Vari A, et al. Role for dual individualization with cefmenoxime. The American journal of medicine. 1984;77:43-50.

[207] McKinnon PS, Paladino JA, Schentag JJ. Evaluation of area under the inhibitory curve (AUIC) and time above the minimum inhibitory concentration (T>MIC) as predictors of outcome for cefepime and ceftazidime in serious bacterial infections. International journal of antimicrobial agents. 2008;31:345-51.

[208] Crandon JL, Bulik CC, Kuti JL, Nicolau DP. Clinical pharmacodynamics of cefepime in patients infected with Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy. 2010;54:1111-6.

[209] Chytra I, Stepan M, Benes J, Pelnar P, Zidkova A, Bergerova T, et al. Clinical and microbiological efficacy of continuous versus intermittent application of meropenem in critically ill patients: a randomized open-label controlled trial. Critical care. 2012;16:R113.

[210] Bliziotis IA, Petrosillo N, Michalopoulos A, Samonis G, Falagas ME. Impact of definitive therapy with beta-lactam monotherapy or combination with an aminoglycoside or a quinolone for Pseudomonas aeruginosa bacteremia. PloS one. 2011;6:e26470.

[211] Vardakas KZ, Tansarli GS, Bliziotis IA, Falagas ME. Beta-Lactam plus aminoglycoside or fluoroquinolone combination versus beta-lactam monotherapy for Pseudomonas aeruginosa infections: A meta-analysis. International journal of antimicrobial agents. 2013;41:301-10.

[212] Kashuba AD, Nafziger AN, Drusano GL, Bertino JS, Jr. Optimizing aminoglycoside therapy for nosocomial pneumonia caused by gram-negative bacteria. Antimicrobial agents and chemotherapy. 1999;43:623-9.

[213] Zelenitsky SA, Harding GK, Sun S, Ubhi K, Ariano RE. Treatment and outcome of Pseudomonas aeruginosa bacteraemia: an antibiotic pharmacodynamic analysis. The Journal of antimicrobial chemotherapy. 2003;52:668-74.

[214] Zelenitsky SA, Ariano RE. Support for higher ciprofloxacin AUC 24/MIC targets in treating Enterobacteriaceae bloodstream infection. The Journal of antimicrobial chemotherapy. 2010;65:1725-32.

[215] van Zanten AR, Polderman KH, van Geijlswijk IM, van der Meer GY, Schouten MA, Girbes AR. Ciprofloxacin pharmacokinetics in critically ill patients: a prospective cohort study. Journal of critical care. 2008;23:422-30.

[216] Sanchez JP, Gogliotti RD, Domagala JM, Gracheck SJ, Huband MD, Sesnie JA, et al. The synthesis, structure-activity, and structure-side effect relationships of a series of 8-alkoxy- and 5-amino-8-alkoxyquinolone antibacterial agents. Journal of medicinal chemistry. 1995;38:4478-87.

[217] Sousa J, Alves G, Fortuna A, Falcao A. Third and fourth generation fluoroquinolone antibacterials: a systematic review of safety and toxicity profiles. Current drug safety. 2014;9:89-105.

[218] Lipsky BA, Baker CA. Fluoroquinolone toxicity profiles: a review focusing on newer agents. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1999;28:352-64.

[219] Zeuli JD, Wilson JW, Estes LL. Effect of combined fluoroquinolone and azole use on QT prolongation in hematology patients. Antimicrobial agents and chemotherapy. 2013;57:1121-7.

[220] Craig WA. Once-daily versus multiple-daily dosing of aminoglycosides. Journal of chemotherapy. 1995;7 Suppl 2:47-52.

[221] Turnidge J. Pharmacodynamics and dosing of aminoglycosides. Infectious disease clinics of North America. 2003;17:503-28, v.

[222] Croes S, Koop AH, van Gils SA, Neef C. Efficacy, nephrotoxicity and ototoxicity of aminoglycosides, mathematically modelled for modelling-supported therapeutic drug monitoring. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2012;45:90-100.

[223] Rougier F, Claude D, Maurin M, Maire P. Aminoglycoside nephrotoxicity. Current drug targets Infectious disorders. 2004;4:153-62.

[224] Wargo KA, Edwards JD. Aminoglycoside-Induced Nephrotoxicity. Journal of pharmacy practice. 2014.

[225] Bitner-Glindzicz M, Rahman S. Ototoxicity caused by aminoglycosides. Bmj. 2007;335:784-5.

[226] Avent ML, Rogers BA, Cheng AC, Paterson DL. Current use of aminoglycosides: indications, pharmacokinetics and monitoring for toxicity. Internal medicine journal. 2011;41:441-9.

[227] McKay GA, Thompson PR, Wright GD. Broad spectrum aminoglycoside phosphotransferase type III from Enterococcus: overexpression, purification, and substrate specificity. Biochemistry. 1994;33:6936-44.

[228] Arya DP. Aminoglycoside antibiotics: from chemical biology to drug discovery: John Wiley & Sons2007.

[229] Wainwright M. Streptomycin: discovery and resultant controversy. History and philosophy of the life sciences. 1991;13:97-124.

[230] Taber HW, Mueller JP, Miller PF, Arrow AS. Bacterial uptake of aminoglycoside antibiotics. Microbiological reviews. 1987;51:439-57.

[231] Fourmy D, Recht MI, Blanchard SC, Puglisi JD. Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science. 1996;274:1367-71.

[232] Shakil S, Khan R, Zarrilli R, Khan AU. Aminoglycosides versus bacteria-a description of the action, resistance mechanism, and nosocomial battleground. Journal of biomedical science. 2008;15:5-14.

[233] Mikkelsen NE, Johansson K, Virtanen A, Kirsebom LA. Aminoglycoside binding displaces a divalent metal ion in a tRNA-neomycin B complex. Nature structural biology. 2001;8:510-4.

[234] McCollister BD, Hoffman M, Husain M, Vazquez-Torres A. Nitric oxide protects bacteria from aminoglycosides by blocking the energy-dependent phases of drug uptake. Antimicrobial agents and chemotherapy. 2011;55:2189-96.

[235] Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. Aminoglycosides: activity and resistance. Antimicrobial agents and chemotherapy. 1999;43:727-37.

[236] Bryan LE, Kwan S. Roles of ribosomal binding, membrane potential, and electron transport in bacterial uptake of streptomycin and gentamicin. Antimicrobial agents and chemotherapy. 1983;23:835-45.

[237] Vakulenko SB, Mobashery S. Versatility of aminoglycosides and prospects for their future. Clinical microbiology reviews. 2003;16:430-50.

[238] Hamada Y, Tamura K, Koyama I, Kuroyama M, Yago K, Sunakawa K. Clinical efficacy of arbekacin for Gram-negative bacteria. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy. 2011;17:876-9.

[239] Hwang JH, Lee JH, Moon MK, Kim JS, Won KS, Lee CS. The usefulness of arbekacin compared to vancomycin. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 2012;31:1663-6.

[240] Hwang JH, Lee JH, Moon MK, Kim JS, Won KS, Lee CS. The efficacy and safety of arbekacin and vancomycin for the treatment in skin and soft tissue MRSA infection: preliminary study. Infection & chemotherapy. 2013;45:62-8.

[241] Reyonolds AV, Hamilton-Miller JM, Brumfitt W. In vitro activity of amikacin and ten other aminoglycoside antibiotics against gentamicin-resistant bacterial strains. The Journal of infectious diseases. 1976;134 SUPPL:S291-6.

[242] Hanberger H, Edlund C, Furebring M, C GG, Melhus A, Nilsson LE, et al. Rational use of aminoglycosides-review and recommendations by the Swedish Reference Group for Antibiotics (SRGA). Scandinavian journal of infectious diseases. 2013;45:161-75.

[243] Goossens H, Ghysels G, Van Laethem Y, De Wit S, Levy J, De Mol P, et al. Predicting gentamicin resistance from annual usage in hospital. Lancet. 1986;2:804-5.

[244] Stanojevic S, Waters V, Mathew JL, Taylor L, Ratjen F. Effectiveness of inhaled tobramycin in eradicating Pseudomonas aeruginosa in children with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2014;13:172-8.

[245] Kapusnik JE, Hackbarth CJ, Chambers HF, Carpenter T, Sande MA. Single, large, daily dosing versus intermittent dosing of tobramycin for treating experimental pseudomonas pneumonia. The Journal of infectious diseases. 1988;158:7-12.

[246] Gilleland LB, Gilleland HE, Gibson JA, Champlin FR. Adaptive resistance to aminoglycoside antibiotics in Pseudomonas aeruginosa. Journal of medical microbiology. 1989;29:41-50.

[247] Xiong YQ, Caillon J, Kergueris MF, Drugeon H, Baron D, Potel G, et al. Adaptive resistance of Pseudomonas aeruginosa induced by aminoglycosides and killing kinetics in a rabbit endocarditis model. Antimicrobial agents and chemotherapy. 1997;41:823-6.

[248] Craig WA, Redington J, Ebert SC. Pharmacodynamics of amikacin in vitro and in mouse thigh and lung infections. The Journal of antimicrobial chemotherapy. 1991;27 Suppl C:29-40.

[249] Gudmundsson S, Einarsson S, Erlendsdottir H, Moffat J, Bayer W, Craig WA. The post-antibiotic effect of antimicrobial combinations in a neutropenic murine thigh infection model. The Journal of antimicrobial chemotherapy. 1993;31 Suppl D:177-91.

[250] Tsuji BT, Rybak MJ. Short-course gentamicin in combination with daptomycin or vancomycin against Staphylococcus aureus in an in vitro pharmacodynamic model with simulated endocardial vegetations. Antimicrob Agents Chemother. 2005;49:2735-45.

[251] Mitra AG, Whitten MK, Laurent SL, Anderson WE. A randomized, prospective study comparing once-daily gentamicin versus thrice-daily gentamicin in the treatment of puerperal infection. American journal of obstetrics and gynecology. 1997;177:786-92.

[252] Raz R, Adawi M, Romano S. Intravenous administration of gentamicin once daily versus thrice daily in adults. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 1995;14:88-91.

[253] Krishnan L, George SA. Gentamicin therapy in preterms: a comparison of two dosage regimens. Indian pediatrics. 1997;34:1075-80.

[254] Nayak-Rao S. Aminoglycoside use in renal failure. Indian journal of nephrology. 2010;20:121-4.

[255] Mueller EW, Boucher BA. The use of extended-interval aminoglycoside dosing strategies for the treatment of moderate-to-severe infections encountered in critically ill surgical patients. Surgical infections. 2009;10:563-70.

[256] Taccone FS, Laterre PF, Spapen H, Dugernier T, Delattre I, Layeux B, et al. Revisiting the loading dose of amikacin for patients with severe sepsis and septic shock. Critical care. 2010;14:R53.

[257] Bennett WM, Plamp CE, Gilbert DN, Parker RA, Porter GA. The influence of dosage regimen on experimental gentamicin nephrotoxicity: dissociation of peak serum levels from renal failure. The Journal of infectious diseases. 1979;140:576-80.

[258] Beauchamp D, Labrecque G. Aminoglycoside nephrotoxicity: do time and frequency of administration matter? Current opinion in critical care. 2001;7:401-8.

[259] Blaser J, Stone BB, Zinner SH. Efficacy of intermittent versus continuous administration of netilmicin in a two-compartment in vitro model. Antimicrobial agents and chemotherapy. 1985;27:343-9.

[260] Nicolau DP, Freeman CD, Belliveau PP, Nightingale CH, Ross JW, Quintiliani R. Experience with a once-daily aminoglycoside program administered to 2,184 adult patients. Antimicrobial agents and chemotherapy. 1995;39:650-5.

[261] Heininger U, Bowing B, Stehr K, Solbach W. [Aminoglycosides in patients with mucoviscidosis and pulmonary exacerbation. Comparison of once or three times daily administration]. Klinische Padiatrie. 1993;205:18-22.

[262] Prins JM, Buller HR, Kuijper EJ, Tange RA, Speelman P. Once versus thrice daily gentamicin in patients with serious infections. Lancet. 1993;341:335-9.

[263] Carapetis JR, Jaquiery AL, Buttery JP, Starr M, Cranswick NE, Kohn S, et al. Randomized, controlled trial comparing once daily and three times daily gentamicin in children with urinary tract infections. Pediatr Infect Dis J. 2001;20:240-6.

[264] Abdel-Bari A, Mokhtar MS, Sabry NA, El-Shafi SA, Bazan NS. Once versus individualized multiple daily dosing of aminoglycosides in critically ill patients. Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society. 2011;19:9-17.

[265] Munckhof WJ, Grayson ML, Turnidge JD. A meta-analysis of studies on the safety and efficacy of aminoglycosides given either once daily or as divided doses. The Journal of antimicrobial chemotherapy. 1996;37:645-63.

[266] Hatala R, Dinh T, Cook DJ. Once-daily aminoglycoside dosing in immunocompetent adults: a meta-analysis. Annals of internal medicine. 1996;124:717-25.

[267] Pea F. Plasma pharmacokinetics of antimicrobial agents in critically ill patients. Current clinical pharmacology. 2013;8:5-12.

[268] Marik PE. Aminoglycoside volume of distribution and illness severity in critically ill septic patients. Anaesthesia and intensive care. 1993;21:172-3.

[269] Rea RS, Capitano B, Bies R, Bigos KL, Smith R, Lee H. Suboptimal aminoglycoside dosing in critically ill patients. Ther Drug Monit. 2008;30:674-81.

[270] Fuhs DW, Mann HJ, Kubajak CA, Cerra FB. Intrapatient variation of aminoglycoside pharmacokinetics in critically ill surgery patients. Clinical pharmacy. 1988;7:207-13.

[271] Dasta JF, Armstrong DK. Variability in aminoglycoside pharmacokinetics in critically ill surgical patients. Critical care medicine. 1988;16:327-30.

[272] Oparaoji EC, Siram S, Shoheiber O, Cornwell EE, 3rd, Mezghebe HM. Appropriateness of a 4 mg/kg gentamicin or tobramycin loading dose in post-operative septic shock patients. Journal of clinical pharmacy and therapeutics. 1998;23:185-90.

[273] Chelluri L, Warren J, Jastremski MS. Pharmacokinetics of a 3 mg/kg body weight loading dose of gentamicin or tobramycin in critically ill patients. Chest. 1989;95:1295-7.

[274] Chelluri L, Jastremski MS. Inadequacy of standard aminoglycoside loading doses in acutely ill patients. Critical care medicine. 1987;15:1143-5.

[275] Dorman T, Swoboda S, Zarfeshenfard F, Trentler B, Lipsett PA. Impact of altered aminoglycoside volume of distribution on the adequacy of a three milligram per kilogram loading dose. . Surgery. 1998;124:73-8.

[276] Henderson-Begg SK, Livermore DM, Hall LM. Effect of subinhibitory concentrations of antibiotics on mutation frequency in Streptococcus pneumoniae. The Journal of antimicrobial chemotherapy. 2006;57:849-54.

[277] Yap FH, Joynt GM, Buckley TA, Wong EL. Association of serum albumin concentration and mortality risk in critically ill patients. Anaesthesia and intensive care. 2002;30:202-7.

[278] Gatta A, Verardo A, Bolognesi M. Hypoalbuminemia. Internal and emergency medicine. 2012;7 Suppl 3:S193-9.

[279] Rosenkranz H, Scheer M, Scholtan W. Binding of aminoglycoside antibiotics to human serum proteins. III. Effect of experimental conditions. Infection. 1978;6:57-64.

[280] Zhanel GG, Ariano RE. Once daily aminoglycoside dosing: maintained efficacy with reduced nephrotoxicity? Renal failure. 1992;14:1-9.

[281] Meyer RD. Risk factors and comparisons of clinical nephrotoxicity of aminoglycosides. The American journal of medicine. 1986;80:119-25.

[282] Matsukawa S, Suh JH, Hashimoto Y, Kato M, Satoh D, Saito S, et al. Neuromuscular blocking actions of the aminoglycoside antibiotics sisomicin and micronomicin in the rabbit. The Tohoku journal of experimental medicine. 1997;181:471-3.

[283] Vital Brazil O, Prado-Franceschi J. The neuromuscular blocking action of gentamicin. Archives internationales de pharmacodynamie et de therapie. 1969;179:65-77.

[284] Matz GJ. Aminoglycoside cochlear ototoxicity. Otolaryngologic clinics of North America. 1993;26:705-12.

[285] Forge A, Wright T. The molecular architecture of the inner ear. British medical bulletin. 2002;63:5-24.

[286] Yang Y, Kong WJ, Hu YJ, Li J, Zhong Y, Zhao XY, et al. Protection of cochlear function from aminoglycosides ototoxicity by manganese superoxide dismutase gene in aging rat. Chinese journal of otorhinolaryngology head and neck surgery. 2009;44:657-63.

[287] Bremer HG, de Groot JC, Versnel H, Klis SF. Combined administration of kanamycin and furosemide does not result in loss of vestibular function in Guinea pigs. Audiology & neuro-otology. 2012;17:25-38.

[288] Bamonte F, Monopoli A, Ongini E, Sabetta F, Ferraresi A, Pettorossi VE. Comparative actions of four aminoglycoside antibiotics on the vestibular function in guinea-pigs. Archives internationales de pharmacodynamie et de therapie. 1986;282:161-76.

[289] Greenwald BD, Gurley JM. Balance and vestibular function. NeuroRehabilitation. 2013;32:433-5.

[290] Lawson BD, Rupert AH, Cho TH. Functional screening for vestibular and balance problems soon after head injury: options in development for the field or aid station. Journal of special operations medicine. 2013;13:42-8.

[291] Dulon D, Hiel H, Aurousseau C, Erre JP, Aran JM. Pharmacokinetics of gentamicin in the sensory hair cells of the organ of Corti: rapid uptake and long term persistence. C R Acad Sci III. 1993;316:682-7.

[292] Wang Q, Steyger PS. Trafficking of systemic fluorescent gentamicin into the cochlea and hair cells. Journal of the Association for Research in Otolaryngology : JARO. 2009;10:205-19.

[293] Dai CF, Steyger PS. A systemic gentamicin pathway across the stria vascularis. Hearing research. 2008;235:114-24.

[294] Forge A, Wright A, Davies SJ. Analysis of structural changes in the stria vascularis following chronic gentamicin treatment. Hearing research. 1987;31:253-65.

[295] Alamo C, Lopez-Munnoz F, Cobeta I, Rivera T. Pathophysiological basis of aminoglycoside-induced ototoxicity. Acta otorrinolaringologica espanola. 1996;47:181-6.

[296] Bareggi R, Narducci P, Grill V, Mallardi F, Zweyer M, Fusaroli P. Localization of an aminoglycoside (streptomycin) in the inner ear after its systemic administration. A histochemical study using fluorescence microscopy. Histochemistry. 1986;84:237-40.

[297] Kitahara T, Li HS, Balaban CD. Changes in transient receptor potential cation channel superfamily V (TRPV) mRNA expression in the mouse inner ear ganglia after kanamycin challenge. Hearing research. 2005;201:132-44.

[298] Guthrie OW. Aminoglycoside induced ototoxicity. Toxicology. 2008;249:91-6.

[299] McFadden SL, Ding D, Salvemini D, Salvi RJ. M40403, a superoxide dismutase mimetic, protects cochlear hair cells from gentamicin, but not cisplatin toxicity. Toxicology and applied pharmacology. 2003;186:46-54.

[300] Lesniak W, Pecoraro VL, Schacht J. Ternary complexes of gentamicin with iron and lipid catalyze formation of reactive oxygen species. Chemical research in toxicology. 2005;18:357-64.

[301] Priuska EM, Schacht J. Formation of free radicals by gentamicin and iron and evidence for an iron/gentamicin complex. Biochemical pharmacology. 1995;50:1749-52.

[302] Schacht J. Antioxidant therapy attenuates aminoglycoside-induced hearing loss. Annals of the New York Academy of Sciences. 1999;884:125-30.

[303] Wrzesniok D, Buszman E, Miernik-Biela E. Amikacin, kanamycin and tobramycin binding to melanin in the presence of Ca(2+) and Mg(2+) ions. Acta poloniae pharmaceutica. 2012;69:1035-41.

[304] Wrzesniok D, Buszman E, Grzegorczyk M, Grzegorczyk A, Hryniewicz T. Impact of metal ions on netilmicin-melanin interaction. Acta poloniae pharmaceutica. 2012;69:41-5.

[305] Baliga R, Ueda N, Walker PD, Shah SV. Oxidant mechanisms in toxic acute renal failure. Drug metabolism reviews. 1999;31:971-97.

[306] Jeong SW, Kim LS, Hur D, Bae WY, Kim JR, Lee JH. Gentamicin-induced spiral ganglion cell death: apoptosis mediated by ROS and the JNK signaling pathway. Acta oto-laryngologica. 2010;130:670-8.

[307] Szczepanik W, Kaczmarek P, Jezowska-Bojczuk M. Oxidative activity of copper(II) complexes with aminoglycoside antibiotics as implication to the toxicity of these drugs. Bioinorganic chemistry and applications. 2004:55-68.

[308] Matsui JI, Gale JE, Warchol ME. Critical signaling events during the aminoglycoside-induced death of sensory hair cells in vitro. Journal of neurobiology. 2004;61:250-66.

[309] Nakagawa T, Yamane H. Cytochrome c redistribution in apoptosis of guinea pig vestibular hair cells. Brain research. 1999;847:357-9.

[310] Al-Malky G, Dawson SJ, Sirimanna T, Bagkeris E, Suri R. High-frequency audiometry reveals high prevalence of aminoglycoside ototoxicity in children with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2014.

[311] Guan MX. Mitochondrial 12S rRNA mutations associated with aminoglycoside ototoxicity. Mitochondrion. 2011;11:237-45.

[312] Gardner JC, Goliath R, Viljoen D, Sellars S, Cortopassi G, Hutchin T, et al. Familial streptomycin ototoxicity in a South African family: a mitochondrial disorder. Journal of medical genetics. 1997;34:904-6.

[313] Guo ZF, Guo WS, Xiao L, Gao GQ, Lan F, Lu XG, et al. Discrimination of A1555G and C1494T point mutations in the mitochondrial 12S rRNA gene by on/off switch. Applied biochemistry and biotechnology. 2012;166:234-42.

[314] Shulman E, Belakhov V, Wei G, Kendall A, Meyron-Holtz EG, Ben-Shachar D, et al. Designer aminoglycosides that selectively inhibit cytoplasmic rather than mitochondrial ribosomes show decreased ototoxicity: a strategy for the treatment of genetic diseases. The Journal of biological chemistry. 2014;289:2318-30.

[315] Lee JE, Nakagawa T, Kim TS, Iguchi F, Endo T, Kita T, et al. Signaling pathway for apoptosis of vestibular hair cells of mice due to aminoglycosides. Acta oto-laryngologica Supplementum. 2004:69-74.

[316] Nakagawa T, Yamane H, Shibata S, Nakai Y. Gentamicin ototoxicity induced apoptosis of the vestibular hair cells of guinea pigs. European archives of otorhinolaryngology. 1997;254:9-14.

[317] Fee WE, Jr. Aminoglycoside ototoxicity in the human. The Laryngoscope. 1980;90:1-19.

[318] Chen KS, Bach A, Shoup A, Winick NJ. Hearing loss and vestibular dysfunction among children with cancer after receiving aminoglycosides. Pediatric blood & cancer. 2013;60:1772-7.

[319] Chong TK, Piraino B, Bernardini J. Vestibular toxicity due to gentamicin in peritoneal dialysis patients. Peritoneal dialysis international : journal of the International Society for Peritoneal Dialysis. 1991;11:152-5.

[320] Scott CS, Retsch-Bogart GZ, Henry MM. Renal failure and vestibular toxicity in an adolescent with cystic fibrosis receiving gentamicin and standard-dose ibuprofen. Pediatric pulmonology. 2001;31:314-6.

[321] Ariano RE, Zelenitsky SA, Kassum DA. Aminoglycoside-induced vestibular injury: maintaining a sense of balance. The Annals of pharmacotherapy. 2008;42:1282-9.

[322] Black FO, Pesznecker S, Stallings V. Permanent gentamicin vestibulotoxicity. Otology & neurotology. 2004;25:559-69.

[323] Nakashima T, Teranishi M, Hibi T, Kobayashi M, Umemura M. Vestibular and cochlear toxicity of aminoglycosides-a review. Acta oto-laryngologica. 2000;120:904-11.

[324] Dhanireddy S, Liles WC, Gates GA. Vestibular toxic effects induced by once-daily aminoglycoside therapy. Archives of otolaryngology-head & neck surgery. 2005;131:46-8.

[325] Selimoglu E, Kalkandelen S, Erdogan F. Comparative vestibulotoxicity of different aminoglycosides in the Guinea pigs. Yonsei medical journal. 2003;44:517-22.

[326] Kitasato I, Yokota M, Inouye S, Igarashi M. Comparative ototoxicity of ribostamycin, dactimicin, dibekacin, kanamycin, amikacin, tobramycin, gentamicin, sisomicin and netilmicin in the inner ear of guinea pigs. Chemotherapy. 1990;36:155-68.

[327] Matz GJ. Aminoglycoside ototoxicity. American journal of otolaryngology. 1986;7:117-9.

[328] Tanyeri H, Lopez I, Honrubia V. Histological evidence for hair cell regeneration after ototoxic cell destruction with local application of gentamicin in the chinchilla crista ampullaris. Hearing research. 1995;89:194-202.

[329] Duncan LJ, Mangiardi DA, Matsui JI, Anderson JK, McLaughlin-Williamson K, Cotanche DA. Differential expression of unconventional myosins in apoptotic and regenerating chick hair cells confirms two regeneration mechanisms. The Journal of comparative neurology. 2006;499:691-701.

[330] Peloquin CA, Berning SE, Nitta AT, Simone PM, Goble M, Huitt GA, et al. Aminoglycoside toxicity: daily versus thrice-weekly dosing for treatment of mycobacterial diseases. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2004;38:1538-44.

[331] Roland PS. New developments in our understanding of ototoxicity. Ear, nose, & throat journal. 2004;83:15-6; discussion 6-7.

[332] Fetoni AR, Sergi B, Ferraresi A, Paludetti G, Troiani D. alpha-Tocopherol protective effects on gentamicin ototoxicity: an experimental study. International journal of audiology. 2004;43:166-71.

[333] Bates DE. Aminoglycoside ototoxicity. Drugs Today (Barc). 2003;39:277-85.

[334] Chu HQ, Xiong H, Zhou XQ, Han F, Wu ZG, Zhang P, et al. Aminoglycoside ototoxicity in three murine strains and effects on NKCC1 of stria vascularis. Chinese medical journal. 2006;119:980-5.

[335] Wu WJ, Sha SH, McLaren JD, Kawamoto K, Raphael Y, Schacht J. Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague-Dawley rat. Hearing research. 2001;158:165-78.

[336] Conlon BJ, Smith DW. Topical aminoglycoside ototoxicity: attempting to protect the cochlea. Acta oto-laryngologica. 2000;120:596-9.

[337] Mazurek B, Lou X, Olze H, Haupt H, Szczepek AJ. In vitro protection of auditory hair cells by salicylate from the gentamicin-induced but not neomycin-induced cell loss. Neuroscience letters. 2012;506:107-10.

[338] Sha SH, Schacht J. Salicylate attenuates gentamicin-induced ototoxicity. Laboratory investigation; a journal of technical methods and pathology. 1999;79:807-13.

[339] Plajer SM, Chin PK, Vella-Brincat JW, Buffery PJ, Begg EJ. Gentamicin and renal function: lessons from 15 years' experience of a pharmacokinetic service for extended interval dosing of gentamicin. Therapeutic drug monitoring. 2015;37:98-103.

[340] Roberts JA, Field J, Visser A, Whitbread R, Tallot M, Lipman J, et al. Using population pharmacokinetics to determine gentamicin dosing during extended daily diafiltration in critically ill patients with acute kidney injury. Antimicrobial agents and chemotherapy. 2010;54:3635-40.

[341] Elhanan K, Siplovich L, Raz R. Gentamicin once-daily versus thrice-daily in children. The Journal of antimicrobial chemotherapy. 1995;35:327-32.

[342] Galloe AM, Graudal N, Christensen HR, Kampmann JP. Aminoglycosides: single or multiple daily dosing? A meta-analysis on efficacy and safety. European journal of clinical pharmacology. 1995;48:39-43.

[343] Rasmussen HH, Ibels LS. Acute renal failure. Multivariate analysis of causes and risk factors. The American journal of medicine. 1982;73:211-8.

[344] Bertino JS, Jr., Booker LA, Franck PA, Jenkins PL, Franck KR, Nafziger AN. Incidence of and significant risk factors for aminoglycoside-associated nephrotoxicity in patients dosed by using individualized pharmacokinetic monitoring. The Journal of infectious diseases. 1993;167:173-9.

[345] Moore RD, Smith CR, Lipsky JJ, Mellits ED, Lietman PS. Risk factors for nephrotoxicity in patients treated with aminoglycosides. Annals of internal medicine. 1984;100:352-7.

[346] Sawyers CL, Moore RD, Lerner SA, Smith CR. A model for predicting nephrotoxicity in patients treated with aminoglycosides. The Journal of infectious diseases. 1986;153:1062-8.

[347] Oliveira JF, Silva CA, Barbieri CD, Oliveira GM, Zanetta DM, Burdmann EA. Prevalence and risk factors for aminoglycoside nephrotoxicity in intensive care units. Antimicrobial agents and chemotherapy. 2009;53:2887-91.

[348] Prins JM, Buller HR, Kuijper EJ, Tange RA, Speelman P. Once-daily gentamicin versus once-daily netilmicin in patients with serious infections-a randomized clinical trial. The Journal of antimicrobial chemotherapy. 1994;33:823-35.

[349] Pancorbo S, Compty C, Heissler J. Comparison of gentamicin and tobramycin nephrotoxicity in patients receiving individualized-pharmacokinetic dosing regimens. Biopharmaceutics & drug disposition. 1982;3:83-8.

[350] Sweileh WM. A prospective comparative study of gentamicin- and amikacin-induced nephrotoxicity in patients with normal baseline renal function. Fundamental & clinical pharmacology. 2009;23:515-20.

[351] Pannu N, Nadim MK. An overview of drug-induced acute kidney injury. Critical care medicine. 2008;36:S216-23.

[352] Luft FC, Yum MN, Walker PD, Kleit SA. Gentamicin gradient patterns and morphological changes in human kidneys. Nephron. 1977;18:167-74.

[353] Ali BH, Al Za'abi M, Blunden G, Nemmar A. Experimental gentamicin nephrotoxicity and agents that modify it: a mini-review of recent research. Basic & clinical pharmacology & toxicology. 2011;109:225-32.

[354] El Mouedden M, Laurent G, Mingeot-Leclercq MP, Taper HS, Cumps J, Tulkens PM. Apoptosis in renal proximal tubules of rats treated with low doses of aminoglycosides. Antimicrobial agents and chemotherapy. 2000;44:665-75.

[355] Choi KH, Kim TI, Chong DL, Lee HY, Han DS. Gentamicin induced apoptosis of renal tubular epithelial (LLC-PK1) cells. The Korean journal of internal medicine. 2000;15:218-23.

[356] Avasthi PS, Evan AP, Huser JW, Luft FC. Effect of gentamicin on glomerular ultrastructure. The Journal of laboratory and clinical medicine. 1981;98:444-54.

[357] Baylis C. The mechanism of the decline in glomerular filtration rate in gentamicin-induced acute renal failure in the rat. The Journal of antimicrobial chemotherapy. 1980;6:381-8.

[358] Mene P, Simonson MS, Dunn MJ. Physiology of the mesangial cell. Physiological reviews. 1989;69:1347-424.

[359] Pfeilschifter J. Cross-talk between transmembrane signalling systems: a prerequisite for the delicate regulation of glomerular haemodynamics by mesangial cells. European journal of clinical investigation. 1989;19:347-61.

[360] Martinez-Salgado C, Eleno N, Morales AI, Perez-Barriocanal F, Arevalo M, Lopez-Novoa JM. Gentamicin treatment induces simultaneous mesangial proliferation and apoptosis in rats. Kidney international. 2004;65:2161-71.

[361] Cui S, Verroust PJ, Moestrup SK, Christensen EI. Megalin/gp330 mediates uptake of albumin in renal proximal tubule. The American journal of physiology. 1996;271:F900-7.

[362] Nagai J, Katsube T, Murakami T, Takano M. Effect of gentamicin on pharmacokinetics of lysozyme in rats: interaction between megalin substrates in the kidney. The Journal of pharmacy and pharmacology. 2002;54:1491-6.

[363] Quiros Y, Vicente-Vicente L, Morales AI, Lopez-Novoa JM, Lopez-Hernandez FJ. An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin. Toxicological sciences : an official journal of the Society of Toxicology. 2011;119:245-56.

[364] Nagai J, Takano M. Entry of aminoglycosides into renal tubular epithelial cells via endocytosis-dependent and endocytosis-independent pathways. Biochemical pharmacology. 2014;90:331-7.

[365] Nagai J, Takano M. Molecular-targeted approaches to reduce renal accumulation of nephrotoxic drugs. Expert opinion on drug metabolism & toxicology. 2010;6:1125-38.

[366] Decorti G, Malusa N, Furlan G, Candussio L, Klugmann FB. Endocytosis of gentamicin in a proximal tubular renal cell line. Life sciences. 1999;65:1115-24.

[367] Nagai J, Tanaka H, Nakanishi N, Murakami T, Takano M. Role of megalin in renal handling of aminoglycosides. American journal of physiology Renal physiology. 2001;281:F337-44.

[368] Hostetler KY, Hall LB. Inhibition of kidney lysosomal phospholipases A and C by aminoglycoside antibiotics: possible mechanism of aminoglycoside toxicity. Proceedings of the National Academy of Sciences of the United States of America. 1982;79:1663-7.

[369] Lopez-Novoa JM, Quiros Y, Vicente L, Morales AI, Lopez-Hernandez FJ. New insights into the mechanism of aminoglycoside nephrotoxicity: an integrative point of view. Kidney international. 2011;79:33-45.

[370] Nonclercq D, Wrona S, Toubeau G, Zanen J, Heuson-Stiennon JA, Schaudies RP, et al. Tubular injury and regeneration in the rat kidney following acute exposure to gentamicin: a time-course study. Renal failure. 1992;14:507-21.

[371] De Broe ME, Paulus GJ, Verpooten GA, Roels F, Buyssens N, Wedeen R, et al. Early effects of gentamicin, tobramycin, and amikacin on the human kidney. Kidney international. 1984;25:643-52.

[372] Chwieralski CE, Welte T, Buhling F. Cathepsin-regulated apoptosis. Apoptosis : an international journal on programmed cell death. 2006;11:143-9.

[373] Schnellmann RG, Williams SW. Proteases in renal cell death: calpains mediate cell death produced by diverse toxicants. Renal failure. 1998;20:679-86.

[374] Ward DT, Maldonado-Perez D, Hollins L, Riccardi D. Aminoglycosides induce acute cell signaling and chronic cell death in renal cells that express the calcium-sensing receptor. Journal of the American Society of Nephrology : JASN. 2005;16:1236-44.

[375] Brown EM, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. Physiological reviews. 2001;81:239-97.

[376] Gibbons CE, Maldonado-Perez D, Shah AN, Riccardi D, Ward DT. Calcium-sensing receptor antagonism or lithium treatment ameliorates aminoglycoside-induced cell death in renal epithelial cells. Biochimica et biophysica acta. 2008;1782:188-95.

[377] Weinberg JM, Simmons F, Jr., Humes HD. Alterations of mitochondrial respiration induced by aminoglycoside antibiotics. Research communications in chemical pathology and pharmacology. 1980;27:521-31.

[378] Servais H, Jossin Y, Van Bambeke F, Tulkens PM, Mingeot-Leclercq MP. Gentamicin causes apoptosis at low concentrations in renal LLC-PK1 cells subjected to electroporation. Antimicrob Agents Chemother. 2006;50:1213-21.

[379] Morales AI, Detaille D, Prieto M, Puente A, Briones E, Arevalo M, et al. Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway. Kidney international. 2010;77:861-9.

[380] Walker PD, Shah SV. Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. The Journal of clinical investigation. 1988;81:334-41.

[381] Cuzzocrea S, Mazzon E, Dugo L, Serraino I, Di Paola R, Britti D, et al. A role for superoxide in gentamicin-mediated nephropathy in rats. European journal of pharmacology. 2002;450:67-76.

[382] Aramaki Y, Takahashi M, Inaba A, Ishii Y, Tsuchiya S. Uptake of aminoglycoside antibiotics into brush-border membrane vesicles and inhibition of (Na+ + K+)-ATPase activity of basolateral membrane. Biochimica et biophysica acta. 1986;862:111-8.

[383] Kerbiriou M, Teng L, Benz N, Trouve P, Ferec C. The calpain, caspase 12, caspase 3 cascade leading to apoptosis is altered in F508del-CFTR expressing cells. PloS one. 2009;4:e8436.

[384] Zhang F, Hamanaka RB, Bobrovnikova-Marjon E, Gordan JD, Dai MS, Lu H, et al. Ribosomal stress couples the unfolded protein response to p53-dependent cell cycle arrest. The Journal of biological chemistry. 2006;281:30036-45.

[385] Sens MA, Hazen-Martin DJ, Blackburn JG, Hennigar GR, Sens DA. Growth characteristics of cultured human proximal tubule cells exposed to aminoglycoside antibiotics. Annals of clinical and laboratory science. 1989;19:266-79.

[386] Koyner JL, Sher Ali R, Murray PT. Antioxidants. Do they have a place in the prevention or therapy of acute kidney injury? Nephron Experimental nephrology. 2008;109:e109-17.

[387] Morales AI, Buitrago JM, Santiago JM, Fernandez-Tagarro M, Lopez-Novoa JM, Perez-Barriocanal F. Protective effect of trans-resveratrol on gentamicin-induced nephrotoxicity. Antioxidants & redox signaling. 2002;4:893-8.

[388] Saleem U, Ahmad B, Rehman K, Mahmood S, Alam M, Erum A. Nephro-protective effect of vitamin C and Nigella sativa oil on gentamicin associated nephrotoxicity in rabbits. Pakistan journal of pharmaceutical sciences. 2012;25:727-30.

[389] Said MM. The protective effect of eugenol against gentamicin-induced nephrotoxicity and oxidative damage in rat kidney. Fundamental & clinical pharmacology. 2011;25:708-16.

[390] Abdel-Raheem IT, El-Sherbiny GA, Taye A. Green tea ameliorates renal oxidative damage induced by gentamicin in rats. Pakistan journal of pharmaceutical sciences. 2010;23:21-8.

[391] Giuliano RA, Verpooten GA, Verbist L, Wedeen RP, De Broe ME. In vivo uptake kinetics of aminoglycosides in the kidney cortex of rats. The Journal of pharmacology and experimental therapeutics. 1986;236:470-5.

[392] Verpooten GA, Giuliano RA, Verbist L, Eestermans G, De Broe ME. Once-daily dosing decreases renal accumulation of gentamicin and netilmicin. Clinical pharmacology and therapeutics. 1989;45:22-7.

[393] ter Braak EW, de Vries PJ, Bouter KP, van der Vegt SG, Dorrestein GC, Nortier JW, et al. Once-daily dosing regimen for aminoglycoside plus beta-lactam combination therapy of serious bacterial infections: comparative trial with netilmicin plus ceftriaxone. The American journal of medicine. 1990;89:58-66.

[394] Tange RA, Dreschler WA, Prins JM, Buller HR, Kuijper EJ, Speelman P. Ototoxicity and nephrotoxicity of gentamicin vs netilmicin in patients with serious infections. A randomized clinical trial. Clinical otolaryngology and allied sciences. 1995;20:118-23.

[395] Downes KJ, Rao MB, Kahill L, Nguyen H, Clancy JP, Goldstein SL. Daily serum creatinine monitoring promotes earlier detection of acute kidney injury in children and adolescents with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2014;13:435-41.

[396] Malvino E, Goldenberg D, Giniger R, Casabella A, Jorge M. Gentamicin dosage regimen based on serum creatinine concentration. Chemotherapy. 1987;33:316-21.

[397] Gatell JM, SanMiguel JG, Zamora L, Araujo V, Castells C, Moreno A, et al. Tobramycin and amikacin nephrotoxicity. Value of serum creatinine versus urinary concentration of beta-2-microglobulin. Nephron. 1985;41:337-43.

[398] Rybak MJ, Abate BJ, Kang SL, Ruffing MJ, Lerner SA, Drusano GL. Prospective evaluation of the effect of an aminoglycoside dosing regimen on rates of observed nephrotoxicity and ototoxicity. Antimicrobial agents and chemotherapy. 1999;43:1549-55.

[399] Sha SH, Schacht J. Antioxidants attenuate gentamicin-induced free radical formation in vitro and ototoxicity in vivo: D-methionine is a potential protectant. Hearing research. 2000;142:34-40.

[400] Talbot PA. Potentiation of aminoglycoside-induced neuromuscular blockade by protons in vitro and in vivo. The Journal of pharmacology and experimental therapeutics. 1987;241:686-94.

[401] Warner WA, Sanders E. Neuromuscular blockade associated with gentamicin therapy. Jama. 1971;215:1153-4.

[402] Fiekers JF. Effects of the aminoglycoside antibiotics, streptomycin and neomycin, on neuromuscular transmission. II. Postsynaptic considerations. The Journal of pharmacology and experimental therapeutics. 1983;225:496-502.

[403] Singh YN, Harvey AL, Marshall IG. Antibiotic-induced paralysis of the mouse phrenic nerve-hemidiaphragm preparation, and reversibility by calcium and by neostigmine. Anesthesiology. 1978;48:418-24.

[404] Udumula V, Ham YW, Fosso MY, Chan KY, Rai R, Zhang J, et al. Investigation of antibacterial mode of action for traditional and amphiphilic aminoglycosides. Bioorganic & medicinal chemistry letters. 2013;23:1671-5.

[405] Martin GS. Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes. Expert review of anti-infective therapy. 2012;10:701-6.

[406] Cross AS, Zollinger W, Mandrell R, Gemski P, Sadoff J. Evaluation of immunotherapeutic approaches for the potential treatment of infections caused by K1-positive Escherichia coli. The Journal of infectious diseases. 1983;147:68-76.

[407] Johnson JR, Moseley SL, Roberts PL, Stamm WE. Aerobactin and other virulence factor genes among strains of Escherichia coli causing urosepsis: association with patient characteristics. Infection and immunity. 1988;56:405-12.

[408] Moulin-Schouleur M, Schouler C, Tailliez P, Kao MR, Bree A, Germon P, et al. Common virulence factors and genetic relationships between O18:K1:H7 Escherichia coli isolates of human and avian origin. Journal of clinical microbiology. 2006;44:3484-92.

[409] Studena S, Martinkova J, Slizova D, Krs O, Senkerik M, Springer D, et al. A rat model of early sepsis: relationships between gentamicin pharmacokinetics and systemic and renal effects of bacterial lipopolysaccharide combined with interleukin-2. Biol Pharm Bull. 2012;35:1703-10.

[410] Yoburn BC, Morales R, Inturrisi CE. Chronic vascular catheterization in the rat: comparison of three techniques. Physiology & behavior. 1984;33:89-94.

[411] Sardana A, Kalra S, Khanna D, Balakumar P. Nephroprotective effect of catechin on gentamicin-induced experimental nephrotoxicity. Clinical and experimental nephrology. 2014:10.1007/s10157-014-0980-3.

[412] Darzynkiewicz Z, Galkowski D, Zhao H. Analysis of apoptosis by cytometry using TUNEL assay. Methods. 2008;44:250-4.

[413] Gao Z, Liu G, Hu Z, Li X, Yang X, Jiang B, et al. Grape seed proanthocyanidin extract protects from cisplatin-induced nephrotoxicity by inhibiting endoplasmic reticulum stress-induced apoptosis. Molecular medicine reports. 2014;9:801-7.

[414] Ohira Y, Nomura T, Kawano F, Sato Y, Ishihara A, Nonaka I. Effects of nine weeks of unloading on neuromuscular activities in adult rats. Journal of gravitational physiology : a journal of the International Society for Gravitational Physiology. 2002;9:49-59.

[415] Kay IS, Davies WE. The effect of nimodipine on salicylate ototoxicity in the rat as revealed by the auditory evoked brain-stem response. European archives of otorhinolaryngology. 1993;250:51-4.

[416] Le Prell CG, Ojano-Dirain C, Rudnick EW, Nelson MA, Deremer SJ, Prieskorn DM, et al. Assessment of nutrient supplement to reduce gentamicin-induced ototoxicity. Journal of the Association for Research in Otolaryngology : JARO. 2014;15:375-93.

[417] Ye LF, Tao ZZ, Hua QQ, Xiao BK, Zhou XH, Li J, et al. Protective effect of melatonin against gentamicin ototoxicity. The Journal of laryngology and otology. 2009;123:598-602.

[418] Murillo-Cuesta S, Contreras J, Cediel R, Varela-Nieto I. Comparison of different aminoglycoside antibiotic treatments to refine ototoxicity studies in adult mice. Laboratory animals. 2010;44:124-31.

[419] Llorens J, Dememes D, Sans A. The behavioral syndrome caused by 3,3'-iminodipropionitrile and related nitriles in the rat is associated with degeneration of the vestibular sensory hair cells. Toxicology and applied pharmacology. 1993;123:199-210.

[420] Llorens J, Rodriguez-Farre E. Comparison of behavioral, vestibular, and axonal effects of subchronic IDPN in the rat. Neurotoxicology and teratology. 1997;19:117-27.

[421] Llorens J, Aguillo A, Rodriguez-Farre E. Behavioral disturbances and vestibular pathology following crotonitrile exposure in rats. Journal of the peripheral nervous system : JPNS. 1998;3:189-96.

[422] Boadas-Vaello P, Jover E, Saldana-Ruiz S, Soler-Martin C, Chabbert C, Bayona JM, et al. Allylnitrile metabolism by CYP2E1 and other CYPs leads to distinct lethal and vestibulotoxic effects in the mouse. Toxicological sciences : an official journal of the Society of Toxicology. 2009;107:461-72.

[423] Al Deeb S, Al Moutaery K, Khan HA, Tariq M. Exacerbation of iminodipropionitrile-induced behavioral toxicity, oxidative stress, and vestibular hair cell degeneration by gentamicin in rats. Neurotoxicology and teratology. 2000;22:213-20.

[424] Soler-Martin C, Diez-Padrisa N, Boadas-Vaello P, Llorens J. Behavioral disturbances and hair cell loss in the inner ear following nitrile exposure in mice, guinea pigs, and frogs. Toxicological sciences : an official journal of the Society of Toxicology. 2007;96:123-32.

[425] Ossenkopp KP, Prkacin A, Hargreaves EL. Sodium arsanilate-induced vestibular dysfunction in rats: effects on open-field behavior and spontaneous activity in the automated digiscan monitoring system. Pharmacology, biochemistry, and behavior. 1990;36:875-81.

[426] Petterino C, Argentino-Storino A. Clinical chemistry and haematology historical data in control Sprague-Dawley rats from pre-clinical toxicity studies. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie. 2006;57:213-9.

[427] Bourke JB, Balfour TW, Elliott J, MacShane L. Cefotaxime plus metronidazole appears more effective than piperacillin in the prevention of postappendicectomy wound sepsis. Preliminary results of a comparative trial. Drugs. 1988;35 Suppl 2:106-10.

[428] Boxenbaum H. Interspecies variation in liver weight, hepatic blood flow, and antipyrine intrinsic clearance: extrapolation of data to benzodiazepines and phenytoin. Journal of pharmacokinetics and biopharmaceutics. 1980;8:165-76.

[429] Kaye B, Brearley CJ, Cussans NJ, Herron M, Humphrey MJ, Mollatt AR. Formation and pharmacokinetics of the active drug candoxatrilat in mouse, rat, rabbit, dog and man following administration of the prodrug candoxatril. Xenobiotica; the fate of foreign compounds in biological systems. 1997;27:1091-102.

[430] Ramachandran G, Tulapurkar ME, Harris KM, Arad G, Shirvan A, Shemesh R, et al. A peptide antagonist of CD28 signaling attenuates toxic shock and necrotizing soft-tissue infection induced by Streptococcus pyogenes. The Journal of infectious diseases. 2013;207:1869-77.

[431] Bourget P, Delouis JM. [Review of a technic for the estimation of area under the concentration curve in pharmacokinetic analysis]. Therapie. 1993;48:1-5.

[432] Manley HJ, Bailie GR, McClaran ML, Bender WL. Gentamicin pharmacokinetics during slow daily home hemodialysis. Kidney Int. 2003;63:1072-8.

[433] Zhanel GG, Voth D, Nichol K, Karlowsky JA, Noreddin AM, Hoban DJ. Pharmacodynamic activity of ceftobiprole compared with vancomycin versus methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-intermediate Staphylococcus aureus (VISA) and vancomycin-resistant Staphylococcus aureus (VRSA) using an in vitro model. The Journal of antimicrobial chemotherapy. 2009;64:364-9.

[434] Renna G, Siro-Brigiani G, Cuomo V. Comparative evaluation of the neuromuscular blocking activity of three new aminoglycoside antibiotics in rats. Toxicology letters. 1981;9:107-12.

[435] Dell RB, Holleran S, Ramakrishnan R. Sample size determination. ILAR journal / National Research Council, Institute of Laboratory Animal Resources. 2002;43:207-13.

[436] Schacht J, Talaska AE, Rybak LP. Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention. Anat Rec (Hoboken). 2012;295:1837-50.

[437] Muhammad FS, Goode AK, Kock ND, Arifin EA, Cline JM, Adams MR, et al. Effects of 4-vinylcyclohexene diepoxide on peripubertal and adult Sprague-Dawley rats: ovarian, clinical, and pathologic outcomes. Comparative medicine. 2009;59:46-59.

[438] Fabre J, Rudhardt M, Blanchard P, Regamey C. Persistence of sisomicin and gentamicin in renal cortex and medulla compared with other organs and serum of rats. Kidney international. 1976;10:444-9.

[439] Shum HP, Chan KC, Kwan MC, Yeung AW, Cheung EW, Yan WW. Timing for initiation of continuous renal replacement therapy in patients with septic shock and acute kidney injury. Therapeutic apheresis and dialysis : official peer-reviewed journal of the International Society for Apheresis, the Japanese Society for Apheresis, the Japanese Society for Dialysis Therapy. 2013;17:305-10.

[440] Taccone FS, Hites M, Beumier M, Scolletta S, Jacobs F. Appropriate antibiotic dosage levels in the treatment of severe sepsis and septic shock. Current infectious disease reports. 2011;13:406-15.

[441] Paterson DL, Rice LB. Empirical antibiotic choice for the seriously ill patient: are minimization of selection of resistant organisms and maximization of individual outcome mutually exclusive? Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2003;36:1006-12.

[442] Kollef MH, Sherman G, Ward S, Fraser VJ. Inadequate antimicrobial treatment of infections: a risk factor for hospital mortality among critically ill patients. Chest. 1999;115:462-74.

[443] Rasigade JP, Raulin O, Picaud JC, Tellini C, Bes M, Grando J, et al. Methicillin-resistant Staphylococcus capitis with reduced vancomycin susceptibility causes late-onset sepsis in intensive care neonates. PloS one. 2012;7:e31548.

[444] Zeni F, Freeman B, Natanson C. Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment. Critical care medicine. 1997;25:1095-100.

[445] Angus DC. The search for effective therapy for sepsis: back to the drawing board? Jama. 2011;306:2614-5.

[446] Eichacker PQ, Parent C, Kalil A, Esposito C, Cui X, Banks SM, et al. Risk and the efficacy of antiinflammatory agents: retrospective and confirmatory studies of sepsis. American journal of respiratory and critical care medicine. 2002;166:1197-205.

[447] Abraham E, Wunderink R, Silverman H, Perl TM, Nasraway S, Levy H, et al. Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group. Jama. 1995;273:934-41.

[448] Truwit JD, Bernard GR, Steingrub J, Matthay MA, Liu KD, Albertson TE, et al. Rosuvastatin for sepsis-associated acute respiratory distress syndrome. The New England Journal of Medicine. 2014;370:2191-200.

[449] Christaki E, Opal SM. Is the mortality rate for septic shock really decreasing? Current opinion in critical care. 2008;14:580-6.

[450] Kumar A, Haery C, Paladugu B, Symeoneides S, Taiberg L, Osman J, et al. The duration of hypotension before the initiation of antibiotic treatment is a critical determinant of survival in a murine model of Escherichia coli septic shock: association with serum lactate and inflammatory cytokine levels. The Journal of infectious diseases. 2006;193:251-8.

[451] Dulhunty JM, Roberts JA, Davis JS, Webb SA, Bellomo R, Gomersall C, et al. Continuous infusion of beta-lactam antibiotics in severe sepsis: a multicenter double-blind, randomized controlled trial. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2013;56:236-44.

[452] Triginer C, Izquierdo I, Fernandez R, Rello J, Torrent J, Benito S, et al. Gentamicin volume of distribution in critically ill septic patients. Intensive care medicine. 1990;16:303-6.

[453] Mann HJ, Fuhs DW, Awang R, Ndemo FA, Cerra FB. Altered aminoglycoside pharmacokinetics in critically ill patients with sepsis. Clinical pharmacy. 1987;6:148-53.

[454] Galvez R, Luengo C, Cornejo R, Kosche J, Romero C, Tobar E, et al. Higher than recommended amikacin loading doses achieve pharmacokinetic targets without associated toxicity. International journal of antimicrobial agents. 2011;38:146-51.

[455] de Montmollin E, Bouadma L, Gault N, Mourvillier B, Mariotte E, Chemam S, et al. Predictors of insufficient amikacin peak concentration in critically ill patients receiving a 25 mg/kg total body weight regimen. Intensive care medicine. 2014;40:998-1005.

[456] Ulldemolins M, Vaquer S, Llaurado-Serra M, Pontes C, Calvo G, Soy D, et al. Beta-lactam dosing in critically ill patients with septic shock and continuous renal replacement therapy. Critical care. 2014;18:227.

[457] Taccone FS, Cotton F, Roisin S, Vincent JL, Jacobs F. Optimal meropenem concentrations to treat multidrug-resistant Pseudomonas aeruginosa septic shock. Antimicrobial agents and chemotherapy. 2012;56:2129-31.

[458] DeGrado JR, Cios D, Greenwood BC, Kubiak DW, Szumita PM. Pharmacodynamic target attainment with high-dose extended-interval tobramycin therapy in patients with cystic fibrosis. Journal of chemotherapy. 2014;26:101-4.

[459] Touw DJ, Knox AJ, Smyth A. Population pharmacokinetics of tobramycin administered thrice daily and once daily in children and adults with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2007;6:327-33.

[460] Fink MP. Animal models of sepsis. Virulence. 2014;5:143-53.

[461] Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proceedings of the National Academy of Sciences of the United States of America. 2013;110:3507-12.

[462] Saslaw S, Carlisle HN, Moheimani M. Comparison of colistin-carbenicillin, colistin, and carbenicillin in Pseudomonas sepsis in monkeys. Antimicrobial agents and chemotherapy. 1973;3:118-24.

[463] Morgan RA, Manning PB, Coran AG, Drongowski RA, Till GO, Ward PD, et al. Oxygen free radical activity during live E. coli septic shock in the dog. Circulatory shock. 1988;25:319-23.

[464] Matyal R, Vin Y, Delude RL, Lee C, Creasey AA, Fink MP. Extremely low doses of tissue factor pathway inhibitor decrease mortality in a rabbit model of septic shock. Intensive care medicine. 2001;27:1274-80.

[465] Carr C, Bild GS, Chang AC, Peer GT, Palmier MO, Frazier RB, et al. Recombinant E. coli-derived tissue factor pathway inhibitor reduces coagulopathic and lethal effects in the baboon gram-negative model of septic shock. Circulatory shock. 1994;44:126-37.

[466] Coletta C, Modis K, Olah G, Brunyanszki A, Herzig DS, Sherwood ER, et al. Endothelial dysfunction is a potential contributor to multiple organ failure and mortality in aged mice subjected to septic shock: preclinical studies in a murine model of cecal ligation and puncture. Critical care. 2014;18:511.

[467] Buras JA, Holzmann B, Sitkovsky M. Animal models of sepsis: setting the stage. Nature reviews Drug discovery. 2005;4:854-65.

[468] Deitch EA. Animal models of sepsis and shock: a review and lessons learned. Shock. 1998;9:1-11.

[469] Matute-Bello G, Frevert CW, Kajikawa O, Skerrett SJ, Goodman RB, Park DR, et al. Septic shock and acute lung injury in rabbits with peritonitis: failure of the neutrophil response to localized infection. American journal of respiratory and critical care medicine. 2001;163:234-43.

[470] Shih MF, Chen LY, Tsai PJ, Cherng JY. In vitro and in vivo therapeutics of beta-thujaplicin on LPS-induced inflammation in macrophages and septic shock in mice. International journal of immunopathology and pharmacology. 2012;25:39-48.

[471] Sakaue Y, Nezu Y, Komori S, Hara Y, Tagawa M, Ogawa R. Evaluation of hepatosplanchnic circulation and intestinal oxygenation in dogs with a condition that mimicked septic shock induced by continuous infusion of a low dose of lipopolysaccharide. American journal of veterinary research. 2004;65:1347-54.

[472] Jansen PM, Eisele B, de Jong IW, Chang A, Delvos U, Taylor FB, Jr., et al. Effect of C1 inhibitor on inflammatory and physiologic response patterns in primates suffering from lethal septic shock. J Immunol. 1998;160:475-84.

[473] Sun Q, Dimopoulos G, Nguyen DN, Tu Z, Nagy N, Hoang AD, et al. Low-dose vasopressin in the treatment of septic shock in sheep. American journal of respiratory and critical care medicine. 2003;168:481-6.

[474] Wu JY, Tsou MY, Chen TH, Chen SJ, Tsao CM, Wu CC. Therapeutic effects of melatonin on peritonitis-induced septic shock with multiple organ dysfunction syndrome in rats. Journal of pineal research. 2008;45:106-16.

[475] Kumar A MJ, Zelenitsky S, Ariano R, Parrillo JE. Effect of antibiotic sequence on blood bacterial counts in a rat model of E. coli peritonitis/septic shock. ICAAC Proceedings. 2004:page 26; A-1296.

[476] Cross AS, Opal SM, Sadoff JC, Gemski P. Choice of bacteria in animal models of sepsis. Infection and immunity. 1993;61:2741-7.

[477] Hamzaoui O, Georger JF, Monnet X, Ksouri H, Maizel J, Richard C, et al. Early administration of norepinephrine increases cardiac preload and cardiac output in septic patients with life-threatening hypotension. Critical care. 2010;14:R142.

[478] Gomes RN, Teixeira-Cunha MG, Figueiredo RT, Almeida PE, Alves SC, Bozza PT, et al. Bacterial clearance in septic mice is modulated by MCP-1/CCL2 and nitric oxide. Shock. 2013;39:63-9.

[479] Sassi N, Paul C, Martin A, Bettaieb A, Jeannin JF. Lipid A-induced responses in vivo. Advances in experimental medicine and biology. 2010;667:69-80.

[480] Barnett A, Ackermann E. Neuromuscular blocking activity of gentamicin in cats and mice. Archives internationales de pharmacodynamie et de therapie. 1969;181:109-17.

[481] Finland M. The symposium on gentamicin. The Journal of infectious diseases. 1969;119:537-40.

[482] Crassaris LG, Mironidou M, Salpigides G, Giala M, Papadakis E, Paradelis AG. Interaction of aminoglycoside antibiotics with calcium channel blockers at the neuromuscular junctions. Journal of chemotherapy. 1989;1:619-20.

[483] Paradelis AG, Triantaphyllidis CJ, Mironidou M, Crassaris LG, Karachalios DN, Giala MM. Interaction of aminoglycoside antibiotics and calcium channel blockers at the neuromuscular junctions. Methods and findings in experimental and clinical pharmacology. 1988;10:687-90.

[484] Del Pozo E, Baeyens JM. Effects of calcium channel blockers on neuromuscular blockade induced by aminoglycoside antibiotics. European journal of pharmacology. 1986;128:49-54.

[485] Albiero L, Bamonte F, Ongini E, Parravicini L. Comparison of neuromuscular effects and acute toxicity of some aminoglycoside antibiotics. Archives internationales de pharmacodynamie et de therapie. 1978;233:343-50.

[486] Liu M, Kato M, Hashimoto Y. Neuromuscular blocking effects of the aminoglycoside antibiotics arbekacin, astromicin, isepamicin and netilmicin on the diaphragm and limb muscles in the rabbit. Pharmacology. 2001;63:142-6.

[487] Paradelis AG, Triantaphyllidis C, Fidani V, Logaras G. The action of the aminoglycosidic antibiotic gentamicin of the isolated rat diaphragm. Potentiation of the neuromuscular blocking activity of gentamicin during combined application with muscle relaxant agents and magnesium. Arzneimittel-Forschung. 1974;24:1774-9.

[488] Paradelis AG, Triantaphyllidis C, Giala MM. Neuromuscular blocking activity of aminoglycoside antibiotics. Methods and findings in experimental and clinical pharmacology. 1980;2:45-51.

[489] Murray MJ, Cowen J, DeBlock H, Erstad B, Gray AW, Jr., Tescher AN, et al. Clinical practice guidelines for sustained neuromuscular blockade in the adult critically ill patient. Critical care medicine. 2002;30:142-56.

[490] Dahlgren JG, Anderson ET, Hewitt WL. Gentamicin blood levels: a guide to nephrotoxicity. Antimicrobial agents and chemotherapy. 1975;8:58-62.

[491] Fuller BM, Mohr NM, Dettmer M, Kennedy S, Cullison K, Bavolek R, et al. Mechanical ventilation and acute lung injury in emergency department patients with severe sepsis and septic shock: an observational study. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine. 2013;20:659-69.

[492] Adams HR. Cardiovascular depressant effects of neomycin and gentamicin in rhesus monkeys. British journal of pharmacology. 1975;54:453-61.

[493] Adams HR. Cardiovascular depressant effects of the neomycin-streptomycin group of antibiotics. American journal of veterinary research. 1975;36:103-8.

[494] Sobek V. The effect of calcium, neostigmine and 4-aminopyridine upon respiratory arrest and depression of cardiovascular functions after aminoglycosidic antibiotics. Arzneimittel-Forschung. 1982;32:222-4.

[495] Makris K, Markou N, Evodia E, Dimopoulou E, Drakopoulos I, Ntetsika K, et al. Urinary neutrophil gelatinase-associated lipocalin (NGAL) as an early marker of acute kidney injury in critically ill multiple trauma patients. Clinical chemistry and laboratory medicine : CCLM / FESCC. 2009;47:79-82.

[496] Devarajan P. Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomarkers in medicine. 2010;4:265-80.

[497] Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. Journal of the American Society of Nephrology : JASN. 2003;14:2534-43.

[498] McCullough PA, Williams FJ, Stivers DN, Cannon L, Dixon S, Alexander P, et al. Neutrophil gelatinase-associated lipocalin: a novel marker of contrast nephropathy risk. American journal of nephrology. 2012;35:509-14.

[499] Han M, Li Y, Liu M, Li Y, Cong B. Renal neutrophil gelatinase associated lipocalin expression in lipopolysaccharide-induced acute kidney injury in the rat. BMC nephrology. 2012;13:25.

[500] Bao G, Clifton M, Hoette TM, Mori K, Deng SX, Qiu A, et al. Iron traffics in circulation bound to a siderocalin (Ngal)-catechol complex. Nature chemical biology. 2010;6:602-9.

[501] Ferguson MA, Vaidya VS, Bonventre JV. Biomarkers of nephrotoxic acute kidney injury. Toxicology. 2008;245:182-93.

[502] Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, Saito Y, et al. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. Kidney international. 2009;75:285-94.

[503] Zhou X, Ma B, Lin Z, Qu Z, Huo Y, Wang J, et al. Evaluation of the usefulness of novel biomarkers for drug-induced acute kidney injury in beagle dogs. Toxicology and applied pharmacology. 2014;280:30-5.

[504] Bennett M, Dent CL, Ma Q, Dastrala S, Grenier F, Workman R, et al. Urine NGAL predicts severity of acute kidney injury after cardiac surgery: a prospective study. Clinical journal of the American Society of Nephrology : CJASN. 2008;3:665-73.

[505] Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. Lancet. 2005;365:1231-8.

[506] Provoost AP, Adejuyigbe O, Wolff ED. Nephrotoxicity of aminoglycosides in young and adult rats. Pediatric research. 1985;19:1191-6.

[507] Moreira MA, Nascimento MA, Bozzo TA, Cintra A, da Silva SM, Dalboni MA, et al. Ascorbic acid reduces gentamicin-induced nephrotoxicity in rats through the control of reactive oxygen species. Clin Nutr. 2014;33:296-301.

[508] Sahu BD, Tatireddy S, Koneru M, Borkar RM, Kumar JM, Kuncha M, et al. Naringin ameliorates gentamicin-induced nephrotoxicity and associated mitochondrial dysfunction, apoptosis and inflammation in rats: possible mechanism of nephroprotection. Toxicology and applied pharmacology. 2014;277:8-20.

[509] Foidart JB, Mahieu P. Glomerular mesangial cell contractility in vitro is controlled by an angiotensin-prostaglandin balance. Molecular and cellular endocrinology. 1986;47:163-73.

[510] Fandrey J, Jelkmann W. Prostaglandin E2 and atriopeptin III oppose the contractile effect of angiotensin II in rat kidney mesangial cell cultures. Prostaglandins. 1988;36:249-57.

[511] Polony G, Humli V, Ando R, Aller M, Horvath T, Harnos A, et al. Protective effect of rasagiline in aminoglycoside ototoxicity. Neuroscience. 2014;265:263-73.

[512] Ojano-Dirain CP, Antonelli PJ, Le Prell CG. Mitochondria-targeted antioxidant MitoQ reduces gentamicin-induced ototoxicity. Otology & neurotology. 2014;35:533-9.

[513] Fetoni AR, Sergi B, Scarano E, Paludetti G, Ferraresi A, Troiani D. Protective effects of alpha-tocopherol against gentamicin-induced Oto-vestibulo toxicity: an experimental study. Acta oto-laryngologica. 2003;123:192-7.

[514] Angunsri N, Taura A, Nakagawa T, Hayashi Y, Kitajiri S, Omi E, et al. Insulin-like growth factor 1 protects vestibular hair cells from aminoglycosides. Neuroreport. 2011;22:38-43.

[515] Ylikoski J, Xing-Qun L, Virkkala J, Pirvola U. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. Hearing research. 2002;166:33-43.

[516] Matsui JI, Haque A, Huss D, Messana EP, Alosi JA, Roberson DW, et al. Caspase inhibitors promote vestibular hair cell survival and function after aminoglycoside treatment in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2003;23:6111-22.

[517] Humphrey MJ, Jevons S, Tarbit MH. Pharmacokinetic evaluation of UK-49,858, a metabolically stable triazole antifungal drug, in animals and humans. Antimicrobial agents and chemotherapy. 1985;28:648-53.

[518] Rodrigues WF, Miguel CB, Napimoga MH, Oliveira CJ, Lazo-Chica JE. Establishing standards for studying renal function in mice through measurements of body size-adjusted creatinine and urea levels. BioMed research international. 2014;2014:872827.

[519] Katz S, Jimenez MA, Lehmkuhler WE, Grosfeld JL. Liver bacterial clearance following hepatic artery ligation and portacaval shunt. The Journal of surgical research. 1991;51:267-70.

[520] Gordon SB, Read RC. Macrophage defences against respiratory tract infections. British medical bulletin. 2002;61:45-61.

[521] Gordon S, Keshav S, Chung LP. Mononuclear phagocytes: tissue distribution and functional heterogeneity. Current opinion in immunology. 1988;1:26-35.

[522] Bonventre PF, Imhoff JG. Uptake of h-dihydrostreptomycin by macrophages in culture. Infect Immun. 1970;2:89-95.

[523] Tulkens P, Trouet A. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. Biochem Pharmacol. 1978;27:415-24.

[524] Mackaness GB. The action of drugs on intracellular tubercle bacilli. J Pathol Bacteriol. 1952;64:429-46.

[525] Coopersmith CM, Amiot DM, 2nd, Stromberg PE, Dunne WM, Davis CG, Osborne DF, et al. Antibiotics improve survival and alter the inflammatory profile in a murine model of sepsis from Pseudomonas aeruginosa pneumonia. Shock. 2003;19:408-14.

[526] Fjell CD, Thair S, Hsu JL, Walley KR, Russell JA, Boyd J. Cytokines and signaling molecules predict clinical outcomes in sepsis. PloS one. 2013;8:e79207.

[527] Vianna RC, Gomes RN, Bozza FA, Amancio RT, Bozza PT, David CM, et al. Antibiotic treatment in a murine model of sepsis: impact on cytokines and endotoxin release. Shock. 2004;21:115-20.

[528] Silverstein R, Wood JG, Xue Q, Norimatsu M, Horn DL, Morrison DC. Differential host inflammatory responses to viable versus antibiotic-killed bacteria in experimental microbial sepsis. Infection and immunity. 2000;68:2301-8.

[529] Yousef AA, Suliman GA. The predictive prognostic values of serum TNF-alpha in comparison to SOFA score monitoring in critically ill patients. BioMed research international. 2013;2013:258029.

[530] Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. Annals of internal medicine. 1993;119:771-8.

[531] Martin C, Saux P, Mege JL, Perrin G, Papazian L, Gouin F. Prognostic values of serum cytokines in septic shock. Intensive care medicine. 1994;20:272-7.

[532] Hack CE, De Groot ER, Felt-Bersma RJ, Nuijens JH, Strack Van Schijndel RJ, Eerenberg-Belmer AJ, et al. Increased plasma levels of interleukin-6 in sepsis. Blood. 1989;74:1704-10.

[533] Blackwell TS, Christman JW. Sepsis and cytokines: current status. British journal of anaesthesia. 1996;77:110-7.

[534] Borden EC, Chin P. Interleukin-6: a cytokine with potential diagnostic and therapeutic roles. The Journal of laboratory and clinical medicine. 1994;123:824-9.

[535] Creasey AA, Stevens P, Kenney J, Allison AC, Warren K, Catlett R, et al. Endotoxin and cytokine profile in plasma of baboons challenged with lethal and sublethal Escherichia coli. Circulatory shock. 1991;33:84-91.

[536] Aderka D, Le JM, Vilcek J. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. J Immunol. 1989;143:3517-23.

[537] Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA. Correlations 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. 1990;75:40-7.

[538] Libert C, Vink A, Coulie P, Brouckaert P, Everaerdt B, Van Snick J, et al. Limited involvement of interleukin-6 in the pathogenesis of lethal septic shock as revealed by the effect of monoclonal antibodies against interleukin-6 or its receptor in various murine models. European journal of immunology. 1992;22:2625-30.

[539] Goscinski G, Lipcsey M, Eriksson M, Larsson A, Tano E, Sjolin J. Endotoxin neutralization and anti-inflammatory effects of tobramycin and ceftazidime in porcine endotoxin shock. Critical care. 2004;8:R35-41.

[540] Marecaux G, Pinsky MR, Dupont E, Kahn RJ, Vincent JL. Blood lactate levels are better prognostic indicators than TNF and IL-6 levels in patients with septic shock. Intensive care medicine. 1996;22:404-8.

[541] Hermans MA, Leffers P, Jansen LM, Keulemans YC, Stassen PM. The value of the Mortality in Emergency Department Sepsis (MEDS) score, C reactive protein and lactate in predicting 28-day mortality of sepsis in a Dutch emergency department. Emergency medicine journal : EMJ. 2012;29:295-300.

[542] Jones AE, Shapiro NI, Trzeciak S, Arnold RC, Claremont HA, Kline JA, et al. Lactate clearance vs central venous oxygen saturation as goals of early sepsis therapy: a randomized clinical trial. Jama. 2010;303:739-46.

[543] Chvojka J, Sykora R, Krouzecky A, Radej J, Varnerova V, Karvunidis T, et al. Renal haemodynamic, microcirculatory, metabolic and histopathological responses to peritonitis-induced septic shock in pigs. Critical care. 2008;12:R164.

[544] Savva A, Roger T. Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. Frontiers in immunology. 2013;4:387.

[545] Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449:819-26.

[546] Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical microbiology reviews. 2009;22:240-73, Table of Contents.

[547] Russell JA. Management of sepsis. The New England Journal of Medicine. 2006;355:1699-713.

[548] Kruszewski M, Iwanenko T. Labile iron pool correlates with iron content in the nucleus and the formation of oxidative DNA damage in mouse lymphoma L5178Y cell lines. Acta biochimica Polonica. 2003;50:211-5.

CHAPTER 8

8. List of Tables

Table 1. Clinical manifestations of sepsis

	Physiological	**Clinical**
Vascular system	Increased vascular permeability, Decrease in systemic vascular resistance	Decreased intravascular volume, Hypotension, Hypo-perfusion of end organs Decreased cardiac output (pre fluid-resuscitation) Increased cardiac output (post-fluid resuscitation)
Heart	Decreased myocardial contractility	Hypotension
Coagulation	Activation of coagulation pathways	Disseminated intravascular coagulopathy, Thrombocytopenia
Lungs	Increase in intravascular coagulopathy	Acute respiratory distress syndrome
Liver	Elevated liver enzymes, Increase bilirubin	Liver dysfunction
Kidneys	Decrease glomerular filtration rate	Acute tubular necrosis and renal dysfunction

Adapted from Manifestations of Sepsis. *Arch Intern Med.* 1987;

147(11):1895-1906. **License number:** 3567920158615

Table 2.   Toxins from some bacterial strains

Organism	Toxins	Function	Target
S. aureus	Enterotoxins (A – E)	Super antigen	TCR/MHC-II
	Toxic shock syndrome toxin (TSST-1)	Super antigen	TCR/MHC-II
	α-hemolysin	Pore forming	Plasma membrane
S. pyogenes	Pyrogenic endotoxin A and C	Super antigen	TCR/MHC-I
	Streptolysin - O	Pore forming	Cholesterol
	Heat stable enterotoxin	Guanylate cyclase	G-proteins
E. coli	Hemolysin	Pore forming	Plasma membrane
L. monocytogenes	Listeriolysin O	Pore forming	Plasma membrane
C. perfringens	Perfringolysin O	Pore forming	Cholesterol

Adapted from Clare K. Schmitt, Karen C. Meysick, and Alison D. O'Brien. Bacterial Toxins:

Friends or Foes? Emerg Infect Dis [Volume 5, Number 2]. April 1999. Available from

http://wwwnc.cdc.gov/eid/article/5/2/99-0206_article

Table 3. TLRs and corresponding PAMP/ ligands

TLR	PAMPS/Ligands	Origin
TLR1	Triacyl lipopeptides Soluble factors	Bacteria, *Mycobacteria* spp, *Neisseria meningitidis*
TLR2	Lipoproteins, lipopeptides, Lipoteichoic acid, Peptidoglycan, Lipoarabinomannan, Phenol-soluble modulin, Porins	Gram-positive bacteria *Mycobacteria* spp *S. epidermidis* *Neisseria* spp
TLR3	Double-stranded RNA	Viruses
TLR4	LPS O-linked mannan Fusion and envelope protein	Gram –ve bacteria Fungi Respiratory syncytial virus, Mouse mammary tumor virus
TLR5	Flagellin	Flagellated bacteria
TLR6	Diacyl lipopeptides, lipoteichoic acid, b-glucan	Mycoplasma, Gram-positive bacteria, fungi
TLR7	Single-stranded RNA, Imidazoquinoline, loxoribine, bropirimine	Viruses, Bacteria Synthetic compounds
TLR8	Single-stranded RNA, Imidazoquinoline	Viruses, Bacteria Synthetic compounds
TLR9	CpG-containing DNA Homozoin	Bacteria, viruses, fungi *Plasmodium falciparum*
TLR10	Lipopeptides (prediction)	
TLR11	Flagellin	Flagellated bacteria
TLR12	Profilin	Apicomplexan parasites
TLR13	23S RNA	Bacteria

Reprinted with permission from Frontiers in Immunology. Savva A, Roger T., Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. Front Immunol (2013)4:387 [544].

Table 4. Inflammatory mediators of sepsis and septic shock

Pro-inflammatory mediators	Anti-inflammatory mediators
Tumor Necrosis Factor (TNF- α)	Interleukin -1 receptor antagonist
Interleukins (1, 6, 8, 12, 18, 22)	Soluble IL – 1 receptor
Interferon-γ	Transforming growth factor (TGF) - β
Complement system	Interleukins (4,10, 11, 13)
Nitric oxide (NO)	Soluble TNF-α receptors
Granulocyte macrophage colony stimulating factor (GM-CSF)	LPS binding protein
Macrophage migration inhibitory factor	Leukotriene B4-receptor antagonism
High mobility group box 1 (HMGB-1)	Soluble thrombomodulin
Histamine	Apoptotic cells
Thrombin	
Factor X	
Tissue Factor (TF)	
Necrotic cells	

Adapted from 'Cytokine modulation in sepsis and septic shock' *Expert Opinion on Investigational Drugs*, published by Informa Healthcare © 2002, Informa UK Limited[545].

Table 5.   Antimicrobial determinants of pathogen clearance in septic shock

1) Early antimicrobial therapy
a. Initiate microbially-appropriate therapy
b. Ensure maximally rapid initiation (avoid delays)
c. Utilize a loading dose when possible
2) Antimicrobial potency
a. Ensure antimicrobial cidality
b. Optimize pharmacokinetic indices
i. Time-dependent agents
ii. Concentration-dependent agents
c. Utilize combination therapy with antimicrobials possessing different mechanisms of action
3) Supplemental therapies
a. Source control

Reproduced with permission from Kumar A, *Virulence,* 5(1), 80–97 © 2014, published by Landes Bioscience.

Table 6. Aminoglycoside antibiotics and clinical use

Aminoglycoside antibiotic	Clinical application
Streptomycin	Tularemia, Tuberculosis, Plague
Gentamicin and Amikacin	Sepsis, meningitis and pneumonia
Neomycin	Burn infections, wound infections, ulcers etc.
Spectinomycin	Gonorrhea
Tobramycin	Cystic fibrosis

Original table with general information regarding aminoglycoside antibiotics and their use in the clinics.

Table 7. **Cytokine assay upper and lower limits of detection**

Cytokine Assay	rIL-1a (pg/ml)	rIL-1b (pg/ml)	rIL-2 (pg/ml)	rIL-4 (pg/ml)	rIL-6 (pg/ml)	rIL-10 (pg/ml)	rIL-12 (pg/ml)	rIFNy (pg/ml)	rTNFa (pg/ml)
Upper Limit of Quantification	1895.7	19839.3	25994.1	646.36	53829.1	2836.3	57110.2	19469.7	3385.0
Lower Limit of Quantification	693.19	93.68	326.83	256.94	5731.89	9.89	235.2	19469.7	371.28
Limit of Detection	459.68	6.22	22.48	5.46	437.79	2.76	14.53	167.3	254.13

Manual of ELISA kit from QUANSYS (Rat cytokine - inflammation (9-plex) – 110451RT)

Table 8. *In vitro* **study: Determination of Minimum inhibitory concentration by broth micro dilution method**

Organism	Minimum inhibitory concentration (MIC) in µg / mL		
	Gentamicin	Tobramycin	Cefotaxime
E. coli (Bort) ATCC 700973	1	1	0.125

Table 9. Time duration of neuromuscular paralysis and ventilator dependency

9a. Neuromuscular paralysis and ventilator dependency with individual antibiotic:

N = 3

Antibiotic	Dose	Time duration of neuromuscular paralysis (minutes)	Statistics
Gentamicin	80 mg/kg	29.3 ± 10.06	No statistically significant difference.
Tobramycin	80 mg/kg	35 ± 8.88	p-value – 0.505
Gentamicin	160 mg/kg	45.3 ± 3.78	No statistically significant difference.
Tobramycin	160 mg/kg	49 ± 2.64	p-value –0.241

*- statistics calculated using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

t-tt was used to determine the significance.

Value ± Standard deviation

9b. Neuromuscular paralysis and ventilator dependency of gentamicin and tobramycin combined at each dose

N = 6 (3 animals/ gentamicin + 3 animals/ tobramycin)

Aminoglycoside Dose	Time duration of neuromuscular paralysis (minutes)
80 mg/kg	32.16 ± 9.04 minutes
160 mg/kg	47.10 ± 3.54 minutes

Aminoglycoside 80 vs 160 mg/kg dose duration of paralysis, p=0.0361, Students t-test

Value ± Standard deviation

Table 10. Invasive study - Aminoglycosides 80 mg/kg dose by intra venous route

Hemodynamic parameters using Heart Pressure Analyzer: (3 animals - Gentamicin 80

mg/kg + 3 animals - Tobramycin 80 mg/kg) (N = 6)

Time point	HR (bpm)	Max-P (mmHg)	Min-P (mmHg)	Mean (mmHg)
Base Line	383.92 ±27.6	92.33 ± 6	57.87 ± 12	73.42 ± 8.7
10 min	380.8 ± 70.1	86.42 ± 8.1	52.70 ± 9.4	70.48 ± 10.2
20 min	378.6 ± 76.8	79.12 ± 6.42	51.47 ± 15.7	65.18 ± 6.7
0 h	356.9 ± 74.9	88.77 ± 10.5	58.85 ± 10.2	69.85 ± 7.3
0.75 h	375.6 ± 32.8	93.18 ± 9.22	57.57 ± 13.5	72.68 ± 13
1.5 h	381.7 ± 26.0	96.58 ± 9.22	63.65 ± 12.3	80.52 ± 6.3
3 h	357.5 ± 45.2	81.27 ± 4.7	49.07 ± 12.8	62.65 ± 7.0
6 h	366.5 ± 49.9	71.93 ± 12.32	43.53 ± 19.8	56.9 ± 15.1
9 h	430.9 ± 40.3	69.33 ± 11.56	36.52 ± 4.25	49.15 ± 7.9

Parameters – Value ± Standard deviation

HR   -   Heart Rate

Max-P -   Maximum Pressure (Systolic)

Min-P -   Minimum Pressure (Diastolic)

Mean -   Mean Pressure

Table 11. Invasive study - Aminoglycosides 160 mg/kg dose by intra venous route:

Hemodynamic parameters using Heart Pressure Analyzer: (3 animals - Gentamicin 160 mg/kg + 3 animals - Tobramycin 160 mg/kg) (N = 6)

Time Point	HR (bpm)	Max-P (mmHg)	Min-P (mmHg)	Mean (mmHg)
Base Line	327.05 ± 60.9	93.50 ± 5.71	59.52 ± 6.1	72.98 ± 6.5
10 min	296.55 ± 74.4	67.32 ± 4.7	33.60 ± 4.4	45.97 ± 4.2
20 min	305.68 ± 61.1	65.52 ± 4.8	35.32 ± 3.3	46.28 ± 3.8
0 h	310.15 ± 54.8	71.68 ± 2.9	41.97 ± 4.6	53.20 ± 4.7
0.75 h	321.90 ± 49.3	89.42 ± 4.2	58.33 ± 7.2	70.73 ± 6.7
1.5 h	333.08 ± 56.9	88.67 ± 3.9	60.73 ± 6.7	71.88 ± 6.1
3 h	309.98 ± 60.4	81.42 ± 7.7	53.07 ± 7.9	64.48 ± 7.9
6 h	381.37 ± 47.2	66.73 ± 5.9	46.90 ± 7.9	54.50 ± 6.4
9 h	326.67 ± 53.2	61.63 ± 6.9	42.77 ± 7.0	54.83 ± 4.7

Parameters – Value ± Standard deviation

HR   -   Heart Rate

Max-P -   Maximum Pressure (Systolic)

Min-P -   Minimum Pressure (Diastolic)

Mean -   Mean Pressure

Table 12. Gentamicin pharmacokinetics after ultra-high doses by intravenous route in normal SD rats: N = 3

Gentamicin	AUC tot (mg*h/L)	Clearance (L/kg/h)	Elimination rate (h-1)	Half-life (h)	Vd (area)(L/kg)	C_{max} (ug/mL)
80 mg/kg	426.2 ± 58.5	0.20 ± 0.02	0.74 ± 0.20	0.98 ± 0.27	0.27 ± 0.04	423.6 ± 21
160 mg/kg	725.5 ± 50.5	0.22 ± 0.01	0.74 ± 0.20	0.98 ± 0.27	0.31 ± 0.06	616 ± 36.6

Value ± Standard deviation

Table 13. Vestibular toxicity(behavioral studies): (N=4, 2 gentamicin+2 tobramycin)

Antibiotic Dose	Tail hanging Reflex	Air righting reflex	Contact inhibition of Righting	Final Score	Vestibular Toxicity
Aminoglycosides 80 mg/kg	0.67	0	0	0.67	NO
Aminoglycosides 160 mg/kg	1	0	0	1	NO
Control	0	0	0	0	NO

Total Score 0 – 2: Normal

Total Score 3 – 4: Moderate vestibular toxicity

Total Score 5 – 6: Severe vestibular toxicity

No vestibular toxicity was observed with both the ultra-doses of aminoglycosides. A further study with a larger group of animals is required to confirm these results.

Table 14. Standardization of septic shock model: N = 3/group

a. **Group 1: Single capsule implantation: Bacterial counts from blood**

Time Point	Group 1
	Mean $\log_{10}$ CFU/mL
0 h	< LD
6 h	3.95 ± 1.25
12 h	4.47 ± 0.8
18 h	5.47 ± 0.65
24 h	6.30 ± 1.1
36 h	7.00 ± 0.35
	Euthanized after 36th hour

< LD – Less than limit of detection

Value ± Standard deviation

b. Group 2: Two capsule implantation: Bacterial counts from blood

Time Point	Group 2
	Mean $\log_{10}$ CFU/mL
0 h	< LD
6 h	4.30 ± 0.68
9 h	5.7 ± 0.9
12 h	6.30 ± 0.33
16 h	6.84 ± 0.64
20 h	7.47 ± 0.45
	Euthanized after 20th hour

c. Group 3: Four capsule implantation: Bacterial counts from blood

Time Point	Group 3
	Mean $\log_{10}$ CFU/mL
0 h	< LD
6 h	5.90 ± 0.35
12 h	8.30 ± 0.4
	Euthanized after 12th hour

< LD – Less than limit of detection   Value ± Standard deviation

Table 15. Cefotaxime pK from infection and treated animals by bioassay: N = 6

Cefotaxime	AUC tot (mg*h/L)	Clearance (L/kg/h)	Elimination rate (h-1)	Half-life (h) Median (25% - 75%)	Vd (area) (L/kg)	C_{max} (ug/mL)
15 mg/kg	55.7 ± 2.48	0.27 ± 0.01	0.74 ± 0.20	0.99 (0.87 - 1.1)	0.40 ± 0.12	67.9 ± 2.32
60 mg/kg	212.8 ± 6.29	0.28 ± 0.01	0.60 ± 0.05	1.15 (1.05 - 1.3)	0.47 ± 0.02	265.6 ± 1.90

Value ± Standard deviation

Table 16. Gentamicin pK values from septic animals: N = 6

Gentamicin	AUC tot (mg*h/L)	Clearance (L/kg/h)	Elimination rate (h-1)	Half-life (h) Median (25% - 75 %)	Vd (area)(L/kg)	C_{max} (ug/mL)
5 mg/kg	14.0 ± 3.02	0.37± 0.08	2.25 ± 0.30	0.32 (0.30-0.34)	0.16 ± 0.02	24.1 ± 3.2
10 mg/kg	26.16 ± 6.0	0.40± 0.08	1.38 ± 0.47	0.62 (0.44 - 0.7)	0.36 ± 0.10	41.1 ± 15.6
20 mg/kg	54.0 ± 16.3	0.41± 0.15	0.70 ± 0.15	1.03 (0.84 - 1.2)	0.51 ± 0.07	51.4 ± 12.5
40 mg/kg	201.7±40.6	0.21± 0.05	0.60 ± 0.12	1.19 (1.03 - 1.4)	0.35 ± 0.09	221.8±30.8
80 mg/kg	408.8±51.6	0.20 ±0.02	0.52 ± 0.08	1.33 (1.21 - 1.5)	0.39 ± 0.05	328.7±14.9
160 mg/kg	707 ±120.0	0.23± 0.04	0.48 ± 0.11	1.43 (1.24 – 1.7)	0.50 ± 0.06	536.4 ± 70

Value ± Standard deviation

Table 17. Septic shock model: Hemodynamic parameters

a. Heart rate (bpm): N = 6

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Base Line	357.4 ± 70.4	382.4 ± 76.6	347.1 ± 24.3	380.7 ± 51.1	339.8± 6.8	322.4 ± 22.2	380.8 ± 35.6	345 ± 67.6	384.2 ± 51.7	357.3 ± 42.0
0 h	378.9 ± 89.1	392.6 ± 84	336.9 ± 27.3	410.1 ± 57.6	337.5 ± 7.5	321.9 ± 16.7	362. ± 32.4	360.1 ± 42.9	407.1 ± 37.9	343.9 ± 23.5
0.75 h	417.7± 103.7	432.6 ± 132	350.5 ± 55.2	425.1 ± 55.8	353.3 ± 34	326.3 ± 34.2	396.6 ± 96.8	340.4 ± 35.9	421.9 ± 49.9	351.6 ± 15.7
1.5 h	435.6 ± 143.0	447.0 ± 174	353.0 ± 86	404 ± 26.4	356.3 ± 25.4	343.4 ± 12	395.5 ± 80.5	331.4 ± 24.7	392.1 ± 62.9	323 ± 33.6
3 h	436.0 ± 124.6	428.3 ± 131.1	354.7 ± 57.6	396.5 ± 38.7	381.9 ± 27.4	357.7 ± 37.4	406.6 ± 57.6	320.9 ± 16.8	354.6 ± 44.2	309.7 ± 23.6
6 h	380.5 ± 49.2	444.3 ± 181.4	389.9 ± 144.7	408 ± 27.03	346.5 ± 15	368.6 ± 41.4	377.6 ± 99.7	334.3 ± 57.2	422.5 ± 79.9	324 ± 23.4
8*/9 h	290.2 ± 61.1*	349.6 ± 84.9	379.7 ± 99.1	403.5 ± 31.42	353.3 ± 14.1	352.8 ± 29.2	413.4 ± 60.5	330.6 ± 50.9*	394.3 ± 25.1*	329.5± 43.7

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

b. Max-Pressure (mmHg): N = 6

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Base Line	96.9 ± 28.4	99.6 ± 17.3	112.9±14.2	108.3±17.6	109.5±27.2	93.6 ±15.3	111.7±26.2	110.4 ±38.8	128.8 ±12.2	108.7 ±10.9
0 h	110.0±11.8	104.8±24.9	110.1±22.4	117.4±19.2	78.2 ±17.7	93.5 ±25.7	95.8 ±33.1	112.6 ± 7.9	132.7 ±9.4	102.1 ±3.2
0.75 h	110.7±21.7	100.5±23.5	107.5±25.3	98.5 ±7.6	94.9 ±30.6	107.3±20.9	82.6± 18.6	112.4 ±13.9	130.1 ±15.2	107.32 ±10.7
1.5 h	104.3±20.8	92.3 ±23.8	108.4±20.2	101.1 ±17	91.5 ±33.9	104.8±15.9	98.42 ±40.9	112.0 ±23.3	122.7 ±21.6	110.27 ±11.1
3 h	85.1 ± 15.1	91.0 ±18.2	103.7±24.8	100.2 ± 9.7	84.3 ±15.9	105.2±23.4	78.78 ±19.2	108.4 ±18.2	98.3 ± 21	91.4 ±11.3
6 h	53.7 ± 10.3	90.9 ±19.3	81.1 ±23.6	97.1 ±13.3	64.7 ± 12.2	80.2 ± 33.8	74.83 ±17.3	81.5 ± 13.6	95.4 ± 27.5	89.3 ±3.37
8*/9 h	25.4±9.25*	74.2 ±34.8	61.7 ±27.1	76.7 ±24.1	48.9 ± 20.6	61.3 ±12.3	75.85 ±13.8	70.4 ± 6.5*	74.3 ± 23.3*	71.4 ±27.5

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

c. Min-Pressure (mmHg): N = 6

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Base Line	69.7 ±19.1	65.2 ±16.6	81.2 ± 6.9	71.0 ± 18.7	79.2 ± 22.4	72.5 ±7.8	72.8 ±21.6	70.4 ± 39.5	88.6 ± 9.5	78.6 ± 14.1
0 h	77.8 ±20.0	64.1 ±20.7	76.6 ± 11.0	72.1 ± 15.7	46.0 ± 18.4	69.4 ±19	57.9 ±29.0	76.0±4.4	92.2 ± 11.9	73.2 ±4.6
0.75 h	75.2 ±19.9	64.5 ±20.1	76.2 ± 14.5	51.9 ± 11.1	60.5 ± 30.6	87.1 ± 18.2	46.3 ±13.2	75.1±16.4	92.2 ±17.2	68.9 ± 13.3
1.5 h	72.3 ±18.9	56.0 ±21.3	78.3 ± 13.9	60.5 ±8.5	68.6 ± 29.1	85.5 ±10.8	57.5 ± 21.9	75.7±19.9	88.6 ±25.7	88.6 ±9.2
3 h	53.5± 15.1	56.9 ±22.7	73.8 ± 20.8	55.3 ± 9.2	59.5 ± 15.5	78.3 ± 14.8	47.6 ± 15.2	72.2±17.2	59.9 ± 21.9	59.9 ± 8.8
6 h	18.0 ±12.2	49.1 ±13.2	49.7 ±12.8	43.3 ± 14.0	31.8 ± 9.6	49.4 ± 15.6	41.4 ±10.5	44.5±16.4	52.7 ±27.7	52.7 ± 8.25
8*/9 h	2.3 ± 2.6*	36.8 ±21.1	26.2 ± 15.8	35.1 ±18.4	21.6 ± 16.4	32.6 ± 23.3	43.7 ±9.23	33.2 ±8.8*	30.5±10.9*	30.5 ± 4.8

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

d. Mean (mmHg): N = 6

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Base Line	83.0 ±23.7	80.6 ±17.1	97.4 ±9.2	88.9 ±15.5	94.6 ± 23.2	84.2 ±10.7	90.6 ±22.4	87.4 ±41.3	109.3 ±9.9	93.6 ±12.6
0 h	93.4 ±15.9	81.7 ±22.9	94.2 ±16.2	92.5 ±20.2	62.5 ± 19.6	82.6 ±22.4	74.5 ± 32.8	95.4 ± 7.1	112.5 ±10.3	88.6 ±2.9
0.75 h	92.5 ±20.9	80.6 ±22.0	91.6 ±18.6	70.5 ±10.5	79.5 ± 32.2	98.2 ±20	61.75 ±15.6	94.2 ± 15	110.8 ±16.3	86.9 ±13.6
1.5 h	88.1 ±19.5	70.9 ±20.5	92.9 ±16.3	77.8 ±11.9	80.9 ± 31.2	96.7 ±13.6	80.58 ±36.8	95.0 ±21.1	106.1 ±25.2	91.0 ±8.7
3 h	67.2 ±14.7	70.5 ±20.9	88.0 ±21.7	73.3 ±9.3	72.5 ± 15	93.4 ±18.6	63.42 ±16.1	91.1 ±16.9	77.9 ±21.4	71.7 ± 8.2
6 h	31.6 ±14.2	55.5 ±14.6	59.3 ±15.6	63.4 ±16.8	64.23 ± 9.0	62.8 ±20.8	66.05 ±15.9	60.7 ±17.9	69.8 ±30.3	69.1 ±8.4
8*/9 h	10.2 ±3.7*	54.2 ±25.1	42.1 ±19.9	45.0 ±22.0	45.4 ± 16.4	43.9 ±22.3	51.4 ±18.6	49.2 ±8.8*	46.4 ±16.0*	47.6 ±25.0

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

Table 18. Pulse oximeter readings in all the groups of animals in septic shock model:

a. % O$_2$ Saturation: N = 6

| Time point | UTC | Gentamicin | | | | | | Cefotaxime | | Sham |
		5 mg/kg	10 mg/kg	20mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15mg/kg	60mg/kg	
Baseline	98.9 ±0.82	99.5 ±0.20	99.3 ±0.12	99.3 ±0.25	99.2 ±0.36	99.2 ±0.35	99.3 ±0.25	99.5 ±0.2	99.5 ±0.2	99.3 ±0.23
0 h	99.1 ±0.37	99.2 ±0.34	99.2 ±0.16	99.1 ±0.50	88.9 ±23.1	99.0 ±0.68	98.8 ±0.44	99.4 ±0.1	99.4 ±0.3	99.1 ±0.57
0.75 h	99.2 ±0.40	99.5 ±0.1	99.1 ±0.45	98.9 ±0.43	99.3 ±0.35	99.4 ±0.12	99.0 ±0.32	99.4 ±0.2	97.0 ±1.8	99.3 ±0.04
1.5 h	99.4 ±0.12	99.2 ±0.4	98.9 ±0.73	97.4 ±3.46	98.3 ±0.72	99.1 ±0.65	99.1±0.40	99.4 ±0.1	98.8 ±1.0	99.3 ±0.23
3 h	97.1 ±4.4	99.2 ±0.40	99.1 ±0.48	98.7 ±0.94	98.9 ±0.86	99.1 ±0.30	99.2 ±0.26	99.5 ±0.3	98.4 ±1.7	99.3 ±0.13
6 h	96.7 ±2.2	99.2 ±0.35	98.9 ±0.23	99.0 ±0.40	97.7 ±3.6	95.8 ±5.2	96.9 ±3.2	99.4 ±0.2	97.3 ±3.1	99.1 ±0.75
8*/9 h	*93.5 ±7.9	98.6 ±1.3	98.9 ±0.17	98.9 ±0.67	92.2 ±33.1	90.4 ±18.2	91.6 ±6.9	*99.6 ±0.2	* 95.8 ±5.8	96.7 ±3.8

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

b. Ventilatory/breath rate (brpm): N = 6

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Baseline	35.2 ± 4.2	41.5 ±10.7	48.7± 13.9	50.2± 17.1	41.4 ±5.2	35.8 ±6.2	32.7 ± 12.4	35.3 ± 3.1	39.2 ± 8.2	41± 18
0 h	44.7 ± 4.94	38.6 ± 2.6	41 ±5.2	49.2± 19.0	34.4 ±5.7	40.8± 6.7	66.6 ± 30	41.7 ± 12.4	45.7 ± 8.8	64.8 ±22.8
0.75 h	44.2 ± 4.7	43.6 ± 2.5	60.2 ±14.8	46.6 ±19.2	36.4 ± 4.3	57.6 ±20.6	71.6 ± 19.3	48.3 ± 0.6	106.7 ±131	54 ± 24.3
1.5 h	52.7± 6.9	48.2 ±17.6	55.5 ±10.7	53 ± 7.3	38.8 ±10.0	55±14.5	54.2 ±17.9	48.7 ±10.8	100.5 ±137	57.8 ±20.6
3 h	50.0 ± 0.71	54.4 ± 15.3	49.5 ±10.5	43.6 ±3.7	48.6± 21.7	50 ± 19.8	48.6 ± 15.7	45.0 ± 7.8	47.3± 4.3	48 ± 19.6
6 h	53.8 ± 6.8	45.8 ±17.9	50 ± 8.7	41.4 ± 8.5	48.8 ± 18.2	40.2± 6.8	47 ± 18.3	41.7 ± 9.5	56.7 ± 15.2	56.8 ± 22.7
8*/9 h	*38.7 ± 2.2	49.8 ±22.7	64.7 ±34.8	52.8 ± 9.6	49.2 ± 17.4	48.8 ±15.8	55.2 ±10.9	* 44.0 ± 8.2	* 47.5 ± 18	47.8± 15.O

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

c. **Heart rate (bpm): N = 6**

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Baseline	395.21 ± 63.4	325.75 ± 60.9	350.25 ± 18.6	370.60 ± 21.5	391.20 ± 66.5	328.0 ± 42.8	349.05 ± 36.6	336.67 ± 47.3	373.33 ± 50.5	319.60 ± 20.7
0 h	407.82 ± 74.7	370.00 ± 47.2	353.25 ± 22.1	373.00 ± 45.3	393.80 ± 105.2	353.40 ± 68.8	324.49 ± 20.3	342.67 ± 26.7	407.17 ± 29.0	320.20 ± 15.0
0.75 h	406.65 ± 46.2	367.80 ± 38.7	374.00 ± 23.5	355.60 ± 26.9	359.00 ± 31.9	324.40 ± 45.2	317.00 ± 32.3	331.00 ± 27.2	362.67 ± 27.4	328.60 ± 33.3
1.5 h	411.62 ± 64.8	356.20 ± 41.1	377.75 ± 41.24	348.60 ± 24.4	370.80 ± 37.4	343.80 ± 15.1	338.00 ± 26.5	317.00 ± 9.5	425.67 ± 110.9	334.80 ± 33.8
3 h	380.98 ± 73.2	358.60 ± 64.9	372.75 ± 43.3	343.40 ± 26.4	354.00 ± 27.6	365.00 ± 54.3	346.20 ± 48.1	322.67 ± 28.7	375.33 ± 96.3	333.00 ± 35.3
6 h	332.16 ± 26.4	332.80 ± 79.4	380.00 ± 30.4	390.20 ± 72.3	368.00 ± 34.2	357.00 ± 41.1	352.60 ± 76.9	335.00 ± 63.9	376.33 ± 47.3	343.80 ± 35.6
8*/9 h	* 290.50 ± 19.4	349.60 ± 59.5	356.25 ± 31.26	366.80 ± 25.3	363.20 ± 43.9	360.40 ± 39.01	364.00 ± 56.6	* 327.00 ± 54.7	* 333.2 ± 70.3	339.80 ± 16.8

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

d. Pulse Distention (um): N = 6

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Baseline	19.80 ± 1.6	20.0 ± 2.7	22 ± 1.8	24.4 ± 3.64	21.2 ±11.1	25.9 ± 5.9	20.7 ± 1.38	54.3 ± 5.6	23.8 ± 5.33	27.2 ± 3.7
0 h	12.60 ± 6.7	14.5 ± 1.5	20.7 ± 4.5	25.2 ± 3.80	22.4 ± 9.4	25.1 ± 6.8	10 ± 0.09	48.5 ± 7.7	16.3 ± 3.50	30.7 ± 8.06
0.75 h	21.6 ± 7.2	21.72 ± 3.5	19.5 ± 3.3	21 ± 3.76	26.5 ± 2.7	22.34 ± 7	65.7 ± 0.17	23.2 ± 8.5	10.9 ± 1.97	35.0 ± 5.32
1.5 h	11.3 ± 8.4	20.02 ± 2.4	17.9 ± 2.4	15.2 ± 3.96	27.7 ± 10.1	15 ± 3.67	33.48 ± 3.4	25.4 ± 8.8	8.3 ± 1.58	29.3 ± 6.68
3 h	7.73 ± 3.1	24.5 ± 4.6	11.8 ± 1.9	20.2 ± 3.42	24.6 ± 6.9	15.3 ± 3.9	30.96 ± 1.9	30.8 ± 17.6	9.1 ± 2.23	19.7 ± 5.46
6 h	6.38 ± 1.4	23.8 ± 4.5	16.3 ± 2.0	11.8 ± 3.17	17.5 ± 4.0	15.4 ± 3.4	19.4 ± 1.13	18.9 ± 4.9	11.2 ± 3.88	13.3 ± 2.79
8*/9 h	11.50 ± 7.8	15.38 ± 2.6	14.25 ± 1.4	6.18 ± 2.37	6.8 ± 4.5	7.08 ± 2.6	8.5 ± 1.97	* 18.3 ± 5.8	* 5.4 ± 1.59	15.1 ± 13.6

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

e. **Ventilatory distention (um): N = 6**

Time point	UTC	Gentamicin						Cefotaxime		Sham
		5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	160 mg/kg	15 mg/kg	60 mg/kg	
Baseline	31.4 ± 18	13.9 ± 8.6	21.6 ± 9.3	20.2 ± 17.1	13.6 ± 6.06	14.8 ± 3.5	29.5 ± 1.4	46.9 ± 38.1	19.4 ± 7.2	18.4 ± 14.6
0 h	10.8 ± 12.7	16.1 ± 8.2	18.6 ± 10.2	25.5 ± 17.8	18.7 ± 8.7	16.9 ± 8.7	24.3 ± 1.9	27.5 ± 13.2	17.9 ± 8.8	13.5 ± 9.0
0.75 h	16.8 ± 10.24	20.4 ± 10.3	13.8 ± 6.8	17.2 ± 9.9	18.5 ± 9.5	6.9 ± 4.7	19.5 ± 0.9	17.3 ± 9.4	8.9 ± 5.4	13.2 ± 13
1.5 h	17.3 ± 7.09	16.9 ± 7.0	15.9 ± 8.0	14.3 ± 8.3	16.8 ± 68.9	8.0 ± 6.1	25.9 ± 2.8	15.3 ± 9.8	9.4 ± 4.5	16.1 ± 10.2
3 h	11.9 ± 1.09	17.4 ± 10.0	9.1 ± 2.6	17.2 ± 4.6	17.5 ± 9.9	9.4 ± 5.1	25.8 ± 1.9	16.7 ± 15.6	10.6 ± 5.7	8.4 ± 3.2
6 h	14.1 ± 3.6	17.1 ± 9.4	22.1 ± 6.4	18.6 ± 5.9	15.2 ± 4.3	7.7 ± 3.2	22.6 ± 1.1	9.6 ± 6.0	9.6 ± 8.6	6.8 ± 1.1
8*/ 9 h	* 17.1 ± 1.5	9.5 ± 4.9	15.8 ± 4.1	16.1 ± 3.3	9.3 ± 7.4	9.7 ± 6.5	16.5 ± 1.3	* 10.3 ± 7.9	* 8.1 ± 5.6	5.1 ± 1.8

*- Animals from UTC and cefotaxime groups died by this time point   Value ± Standard deviation

9. Figures:

Figure 1.   Sepsis, severe sepsis and septic shock:
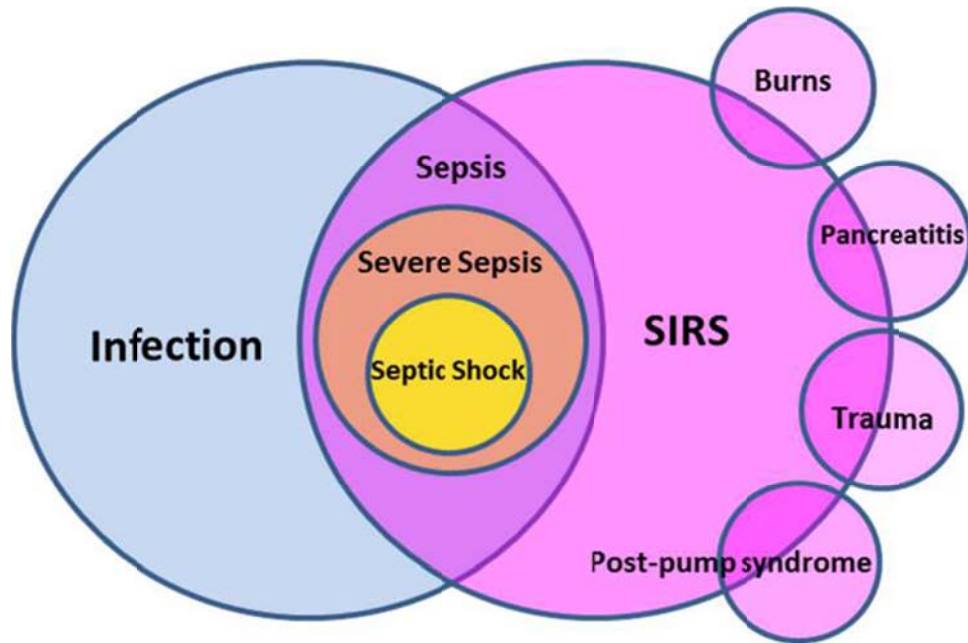
SIRS - systemic inflammatory response.

This Venn diagram depicts a conceptual framework for sepsis. Sepsis exists when SIRS is initiated by the presence of infection. Severe sepsis (sepsis with organ failure) is a subset of septic shock and septic shock (where cardiovascular failure is present) is a subset of severe sepsis. Various non-infectious inflammatory conditions like burns or pancreatitis can independently initiate SIRS. These are not considered to represent sepsis.

Adapted from Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest,*101(6), 1644-1655. [13]

Adapted with permission from Chest 101(6), 1644-1655 © 1992.

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Figure 2. Host responses following infection with microbial pathogens:
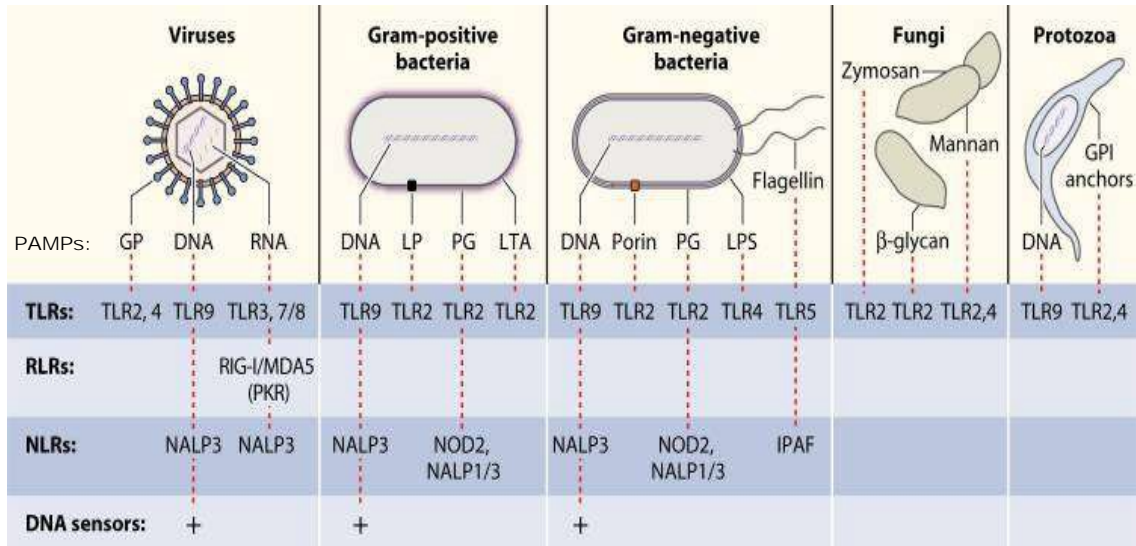
Recognition of PAMPs from different classes of microbial pathogens. Viruses, bacteria, fungi, and protozoa display several different PAMPs, some of which are shared between different classes of pathogens. Major PAMPs are nucleic acids, including DNA, dsRNA, ssRNA, and 5′-triphosphate RNA, as well as surface glycoproteins (GP), lipoproteins (LP), and membrane components (peptidoglycans [PG], lipoteichoic acid [LTA], LPS, and GPI anchors). These PAMPs are recognized by different families of PRRs [546].

PAMPs: pathogen associated molecular patterns,TLRs: Toll-like receptors

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Figure 3. Toll-like Receptors and signaling:
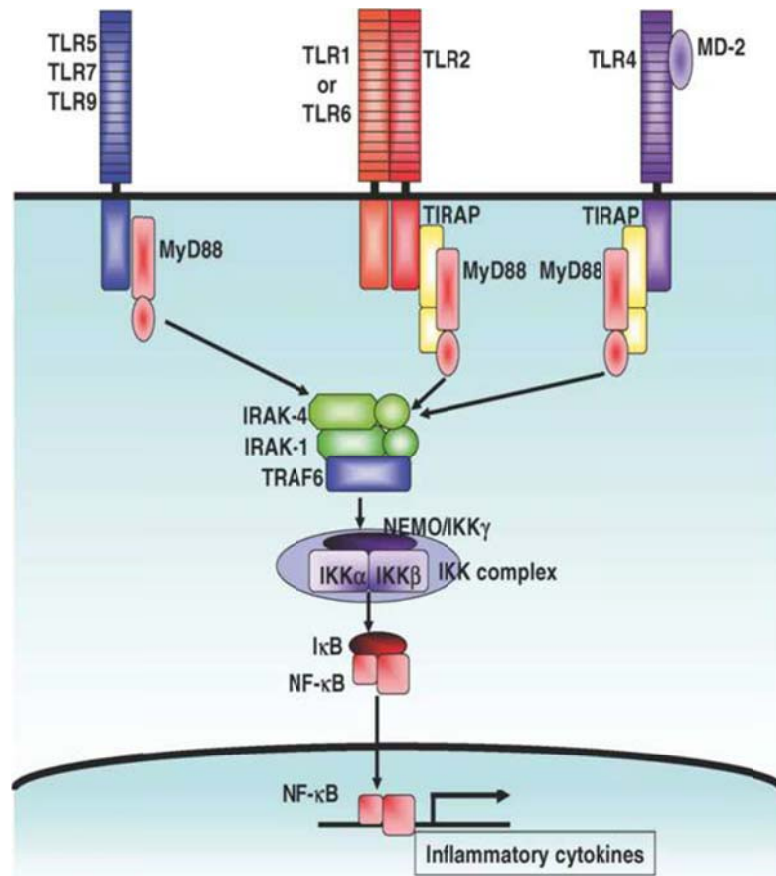
MyD88-dependent signaling pathway. A TIR (Toll interleukin receptor) domain-containing adaptor molecule, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK (Interleukin-1 receptor associated kinase) to the receptor upon ligand binding. The remaining pathway of inflammatory cytokine activation is presented in the figure [69].

Takeda, K., & Akira, S. (2004). Microbial recognition by toll-like receptors. *J Dermatol Sci, 34*(2), 73-82

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Liscence number: 3431650236696

Figure 4. Inflammatory responses to sepsis:
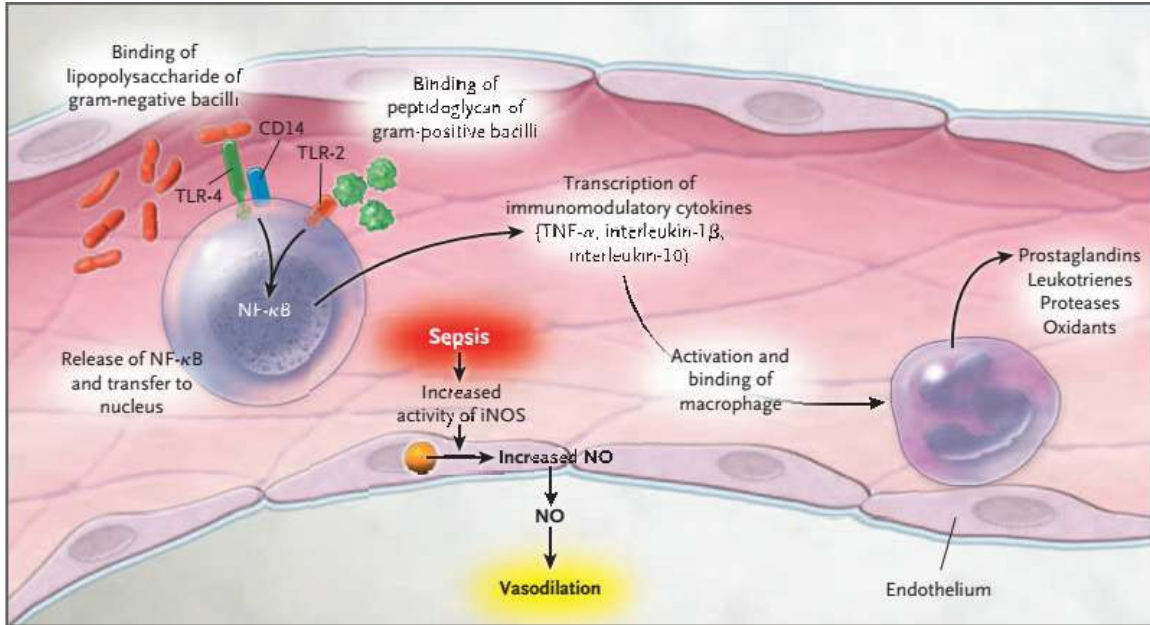
Sepsis initiates a brisk inflammatory response that directly and indirectly causes widespread tissue injury. Shown here are key components of this process and their interactions at the level of the microvasculature of a representative vital organ. Gram-positive and gram-negative bacteria, viruses, and fungi have unique cell-wall molecules called pathogen-associated molecular patterns that bind to pattern-recognition receptors (toll-like receptors [TLRs]) on the surface of immune cells. The lipopolysaccharide of gram-negative bacilli binds to lipopolysaccharide-binding protein, CD14 complex. The peptidoglycan of gram-positive bacteria and the lipopolysaccharide of gram-negative bacteria bind to TLR-2 and TLR-4, respectively. Binding of TLR-2 and TLR-4 activates intracellular signal-transduction pathways that lead to the activation of cytosolic nuclear factor κB (NF-κB). Activated NF-κB moves from the cytoplasm to the nucleus, binds to transcription initiation sites, and increases the transcription of cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β, and interleukin-10. TNF-α and interleukin-1β are pro-inflammatory cytokines that activate the adaptive immune response but also cause both direct and indirect host injury. Interleukin-10 is an anti-inflammatory cytokine that inactivates macrophages and has other anti-inflammatory effects. Sepsis increases the activity of inducible nitric oxide synthase (iNOS), which increases the synthesis of nitric oxide (NO), a potent vasodilator. Cytokines activate endothelial cells by up-regulating adhesion receptors and injure endothelial cells by inducing neutrophils, monocytes, macrophages, and platelets to bind to endothelial cells. These effector cells release mediators such as proteases, oxidants, prostaglandins, and leukotrienes. Key functions of the endothelium are selective permeability, vasoregulation, and provision of an anticoagulant surface. Proteases, oxidants, prostaglandins, and leukotrienes injure endothelial cells, leading to increased permeability, further vasodilation, and alteration of the procoagulant–anticoagulant balance. Cytokines also activate the coagulation cascade [547].
Russell, J. A. (2006). Management of sepsis. *N Engl J Med, 355*(16), 1699-1713
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Figure 5.   Pro-coagulant response in sepsis:
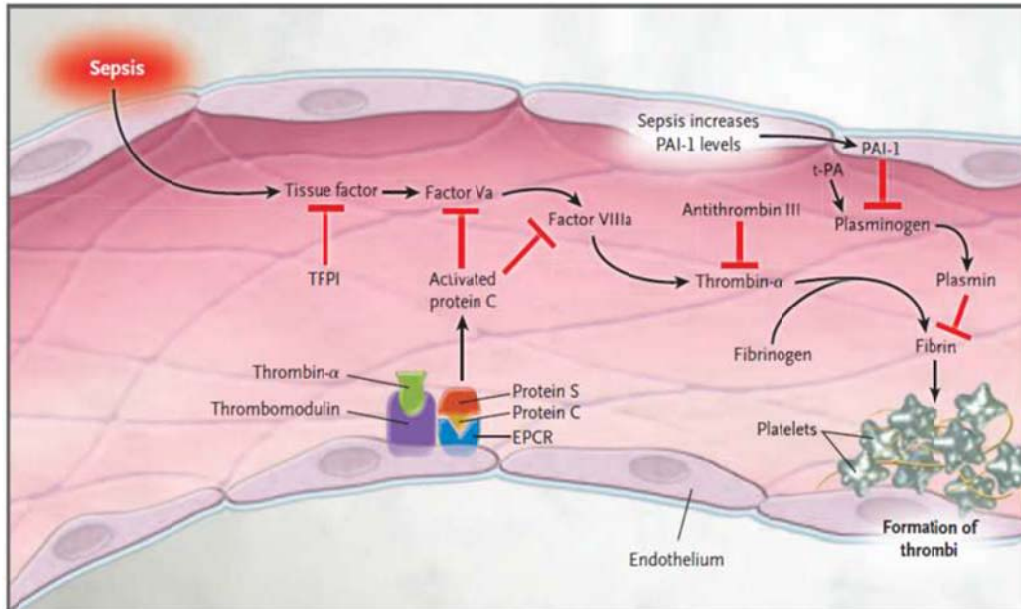
Sepsis initiates coagulation by activating endothelium to increase the expression of tissue factor. Activation of the coagulation cascade, and especially factors Va and VIIIa, leads to the formation of thrombin-α, which converts fibrinogen to fibrin. Fibrin binds to platelets, which in turn adhere to endothelial cells, forming microvascular thrombi. Microvascular thrombi amplify injury through the release of mediators and by microvascular obstruction, which causes distal ischemia and tissue hypoxia. Normally, natural anticoagulants (protein C and protein S), antithrombin III, and tissue factor–pathway inhibitor (TFPI) dampen coagulation, enhance fibrinolysis, and remove microthrombi. Thrombin-α binds to thrombomodulin on endothelial cells, which dramatically increases activation of protein C to activated protein C. Protein C forms a complex with its cofactor protein S. Activated protein C proteolytically inactivates factors Va and VIIIa and decreases the synthesis of plasminogen-activator inhibitor 1 (PAI-1). In contrast, sepsis increases the synthesis of PAI-1. Sepsis also decreases the levels of protein C, protein S, antithrombin III, and TFPI. Lipopolysaccharide and tumor necrosis factor α (TNF-α) decrease the synthesis of thrombomodulin and endothelial protein C receptor (EPCR), thus decreasing the activation of protein C. Sepsis further disrupts the protein C pathway because sepsis also decreases the expression of EPCR, which amplifies the deleterious effects of the sepsis-induced decrease in levels of protein C. Lipopolysaccharide and TNF-α also increase PAI-1 levels so that fibrinolysis is inhibited. The clinical consequences of the changes in coagulation caused by sepsis are increased levels of markers of disseminated intravascular coagulation and widespread organ dysfunction. t-PA denotes tissue plasminogen activator [547].
Russell, J. A. (2006). Management of sepsis. *N Engl J Med, 355*(16), 1699-1713.
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Figure 6.   Immune dysfunction in sepsis:
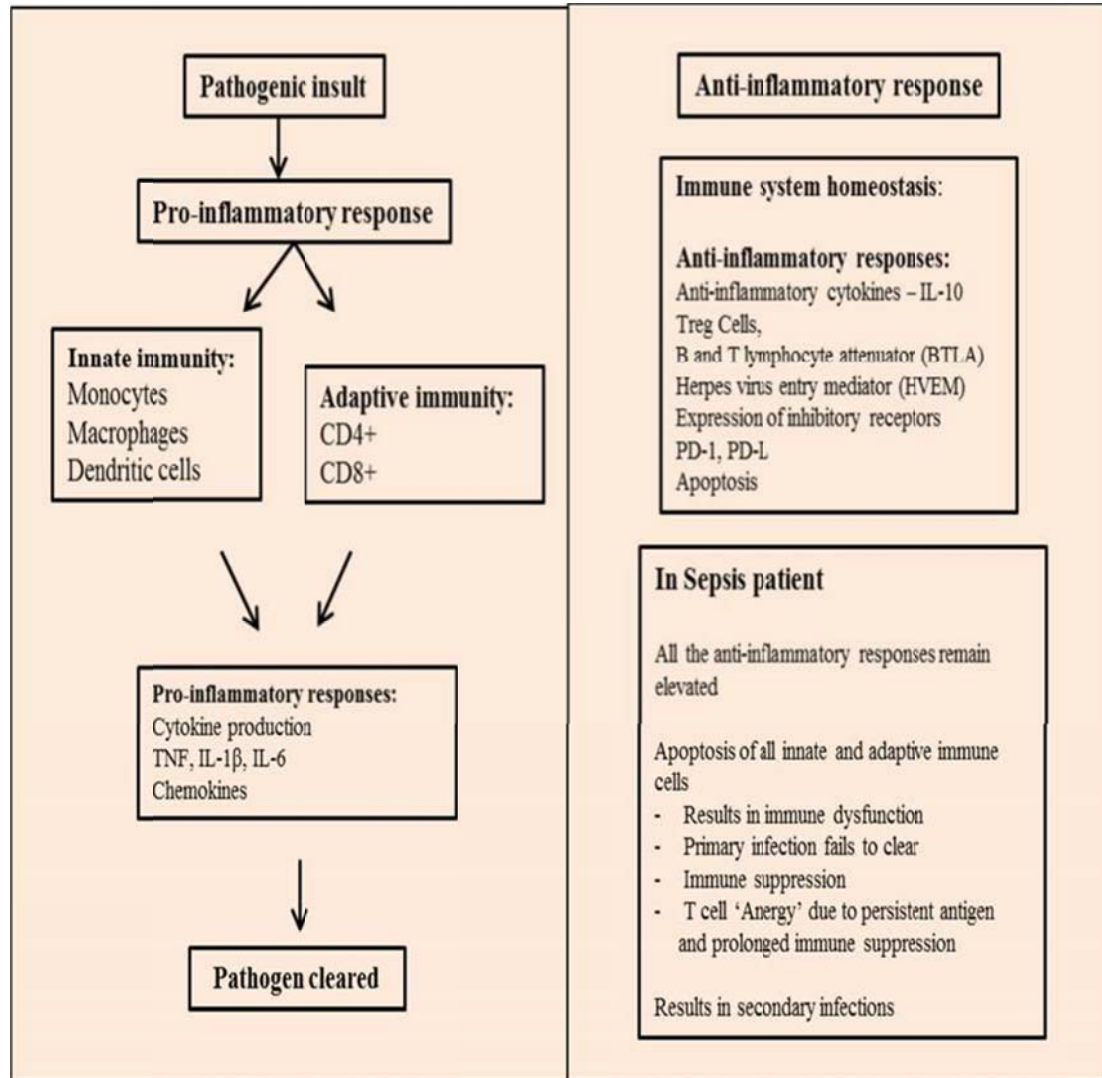
Figure 7. Immunologic model (Current paradigm):
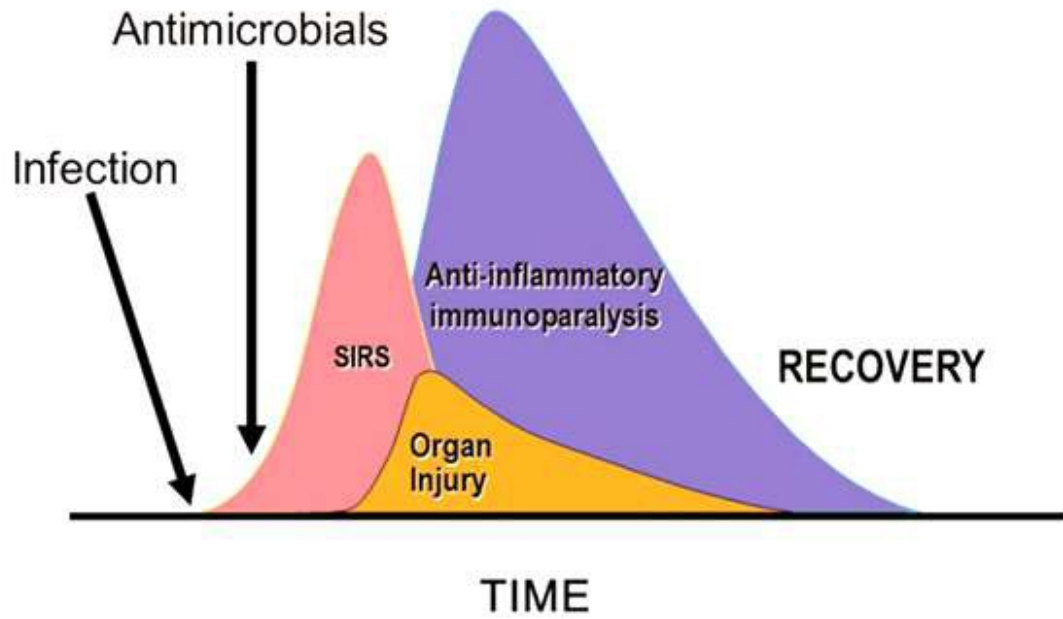
Figure 8. The Classic Paradigm: Microbiologic model:
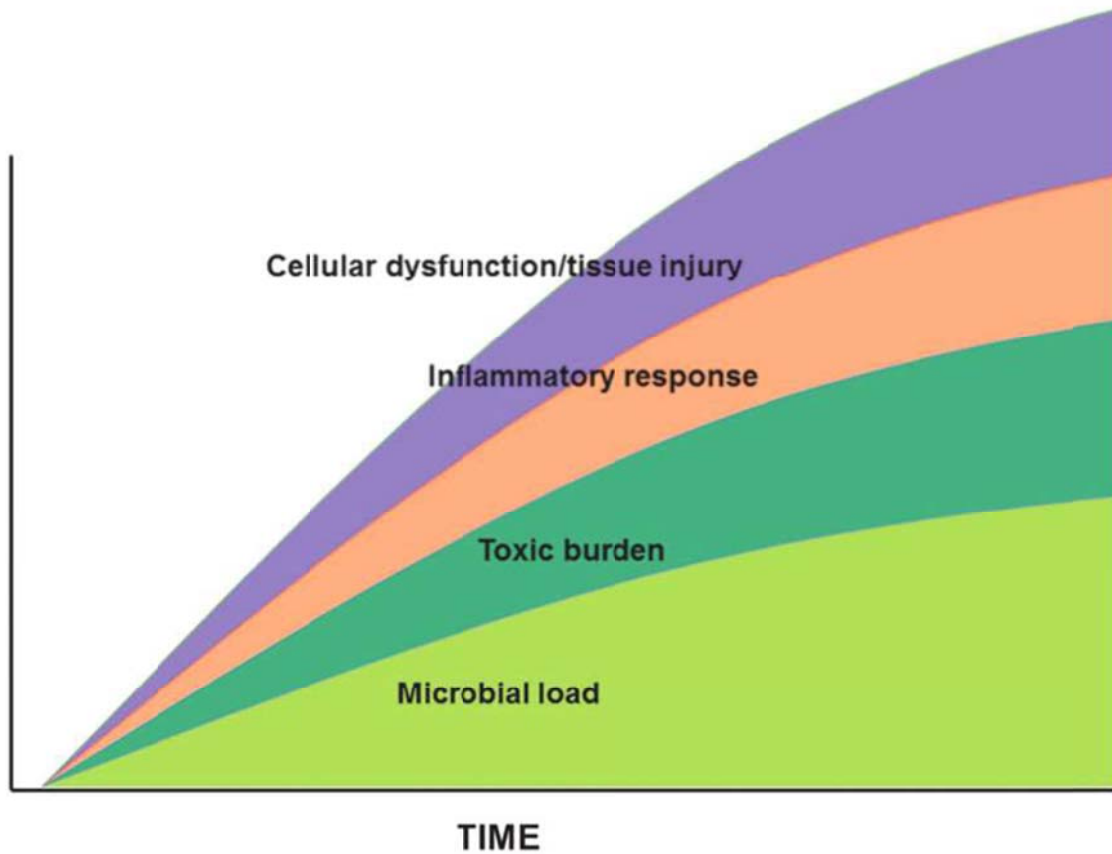
Microbiologic view of sepsis and septic shock

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Figure 9. Integrating shock: Composite view of sepsis and septic shock:
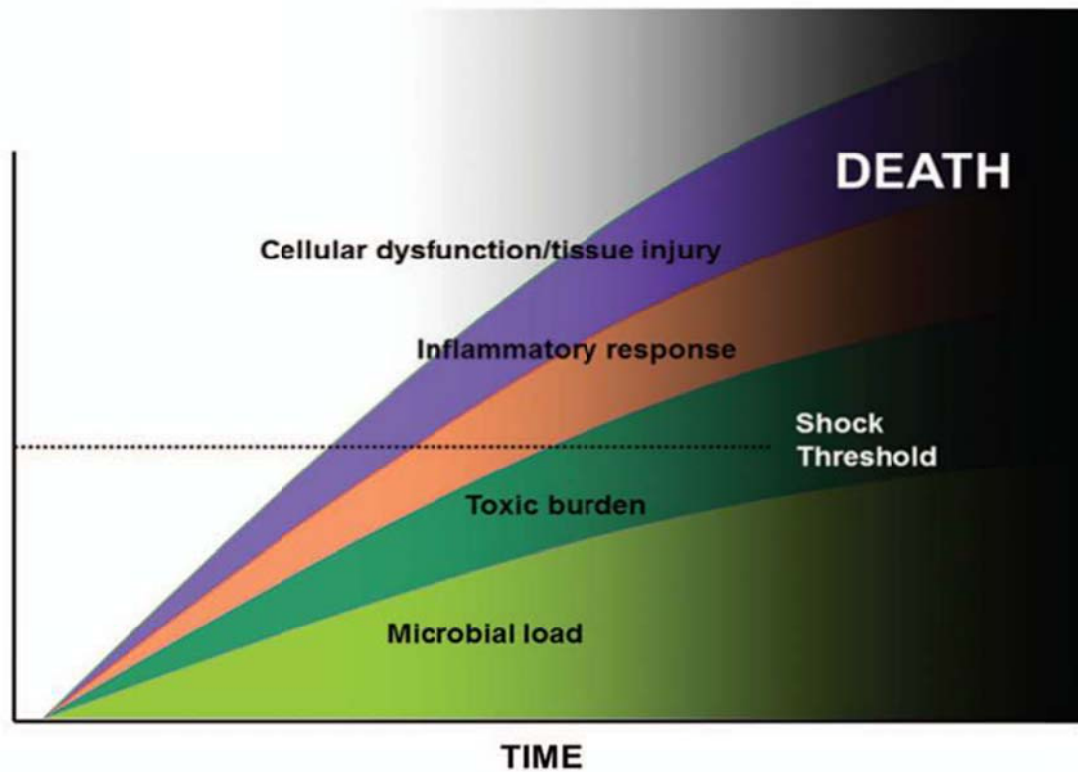
This model is similar to the microbiologic model. The shock threshold may be highly variable between individuals based on their age, cardiovascular reserve and genetic factors. However, all septic patients are on a similar pathway.

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Figure 10. Appropriate antimicrobial therapy:
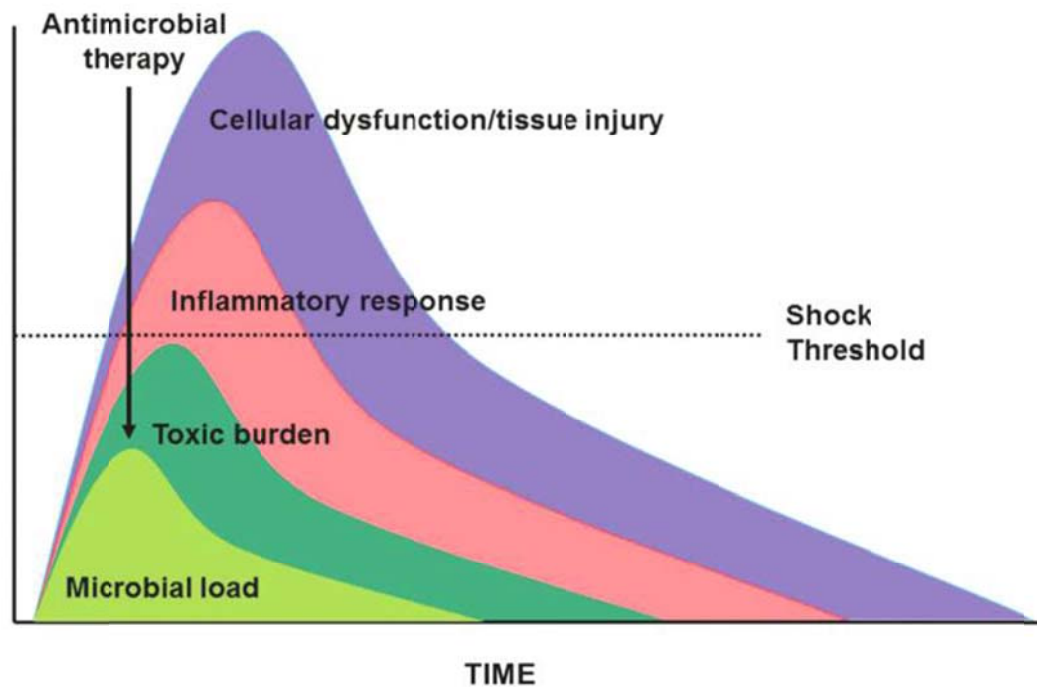
Impact of appropriate antimicrobial therapy in sepsis and septic shock

Reproduced with permission from Kumar A, *Virulence*; 2014, 5(1), 80–97.

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Figure 11. Impact of early antimicrobial therapy in sepsis and septic shock:
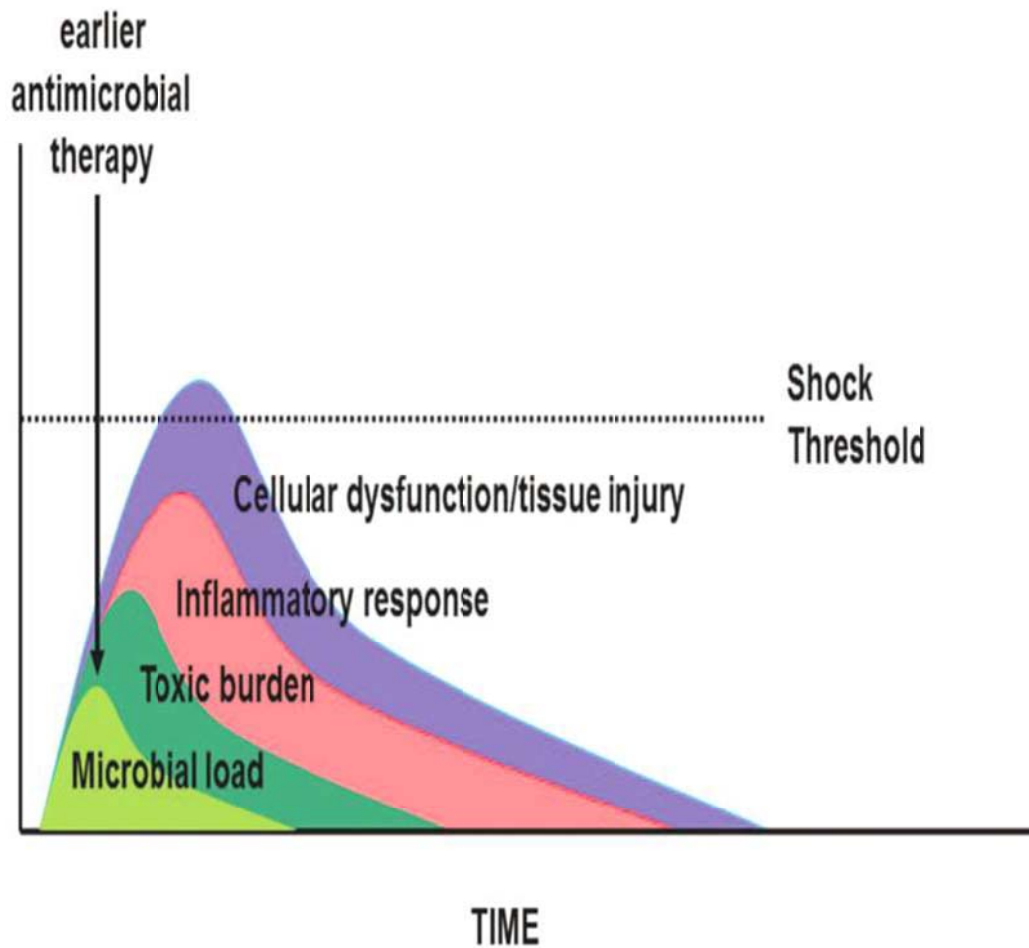
Early initiation of antimicrobial therapy helps to clear the microbial load and allows the patient to remain below the shock threshold at a faster rate.

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Figure 12. Cumulative effective antimicrobial initiation following onset of septic

Shock-associated hypotension and associated survival:
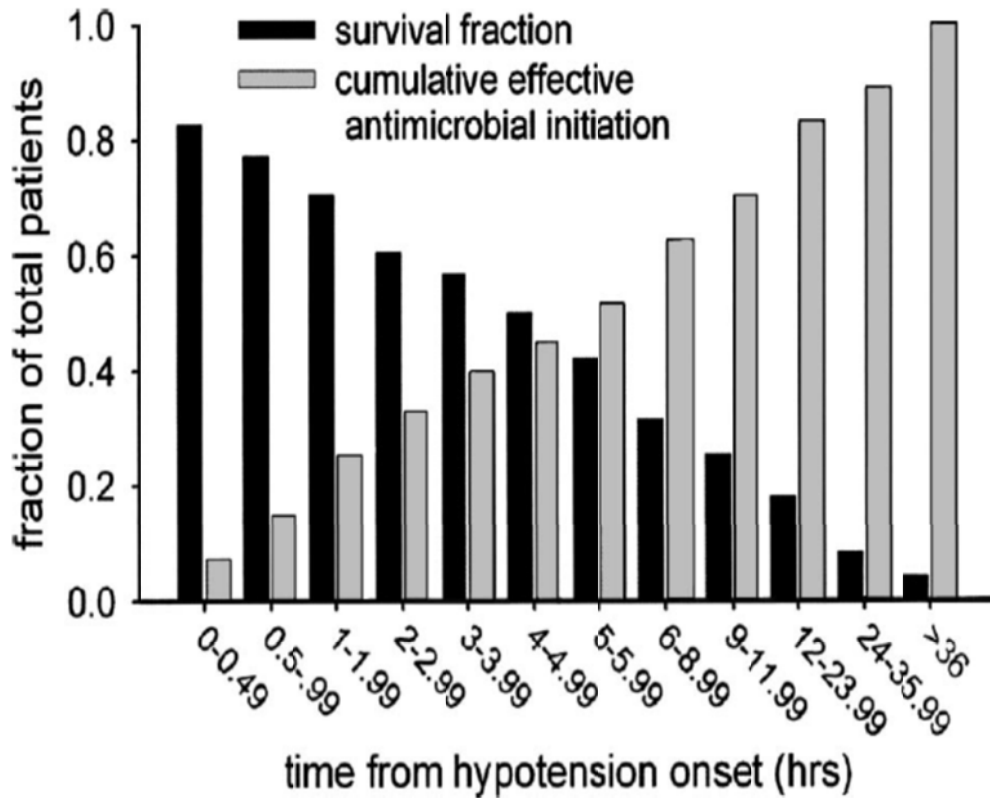
The x-axis represents time (hrs) following first documentation of septic shock-associated hypotension. *Black bars* represent the fraction of patients surviving to hospital discharge for effective therapy initiated within the given time interval. The *gray bars* represent the cumulative fraction of patients having received effective antimicrobials at any given time point.

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Figure 13. Impact of more potent antimicrobial therapy in sepsis and septic shock:
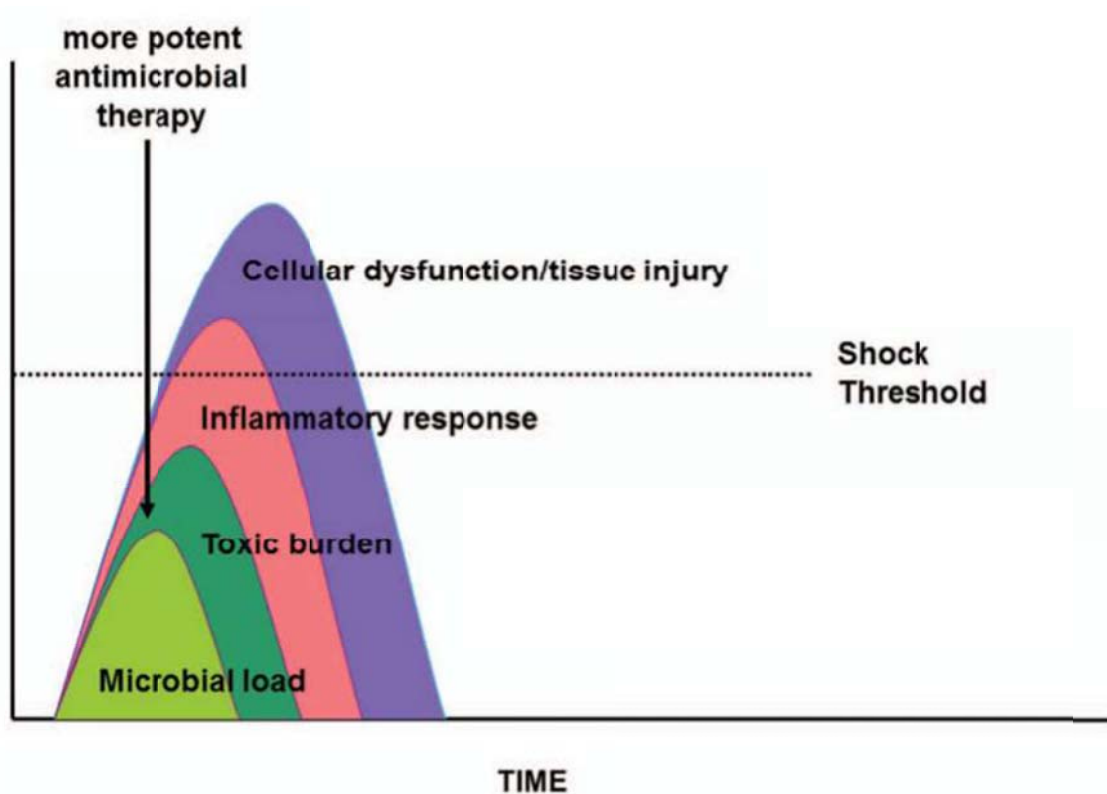
More potent antibiotics will eliminate bacteria at a faster rate. This leads to decreased toxic burden, inflammatory responses and cellular dysfunction with time and allows the individual to come below shock threshold in less time and helps to save the life of the patient.

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Figure 14.   Pathophysiological changes causing alteration in pharmacokinetics:
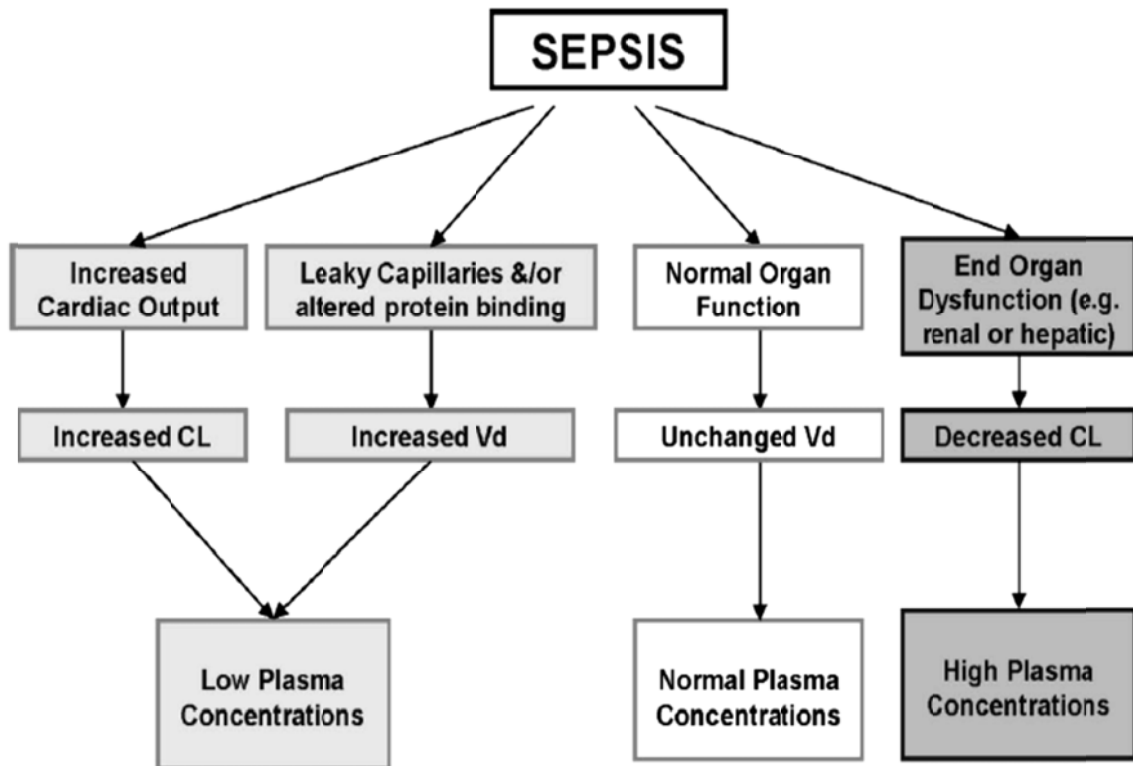
Schematic representation of the basic pathophysiological changes that can occur during sepsis and subscequent pharmacokinetic effects. CL - clearance, Vd – volume of distribution. Roberts, J. A., & Lipman, J. (2009). *Crit Care Med, 37*(3), 840-851**.**

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Figure 15. Aminoglycosides form free radicals by interacting with heme (iron):

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}_{\cdot}OH + OH^-$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + {}^{\cdot}OH + OH^-$$
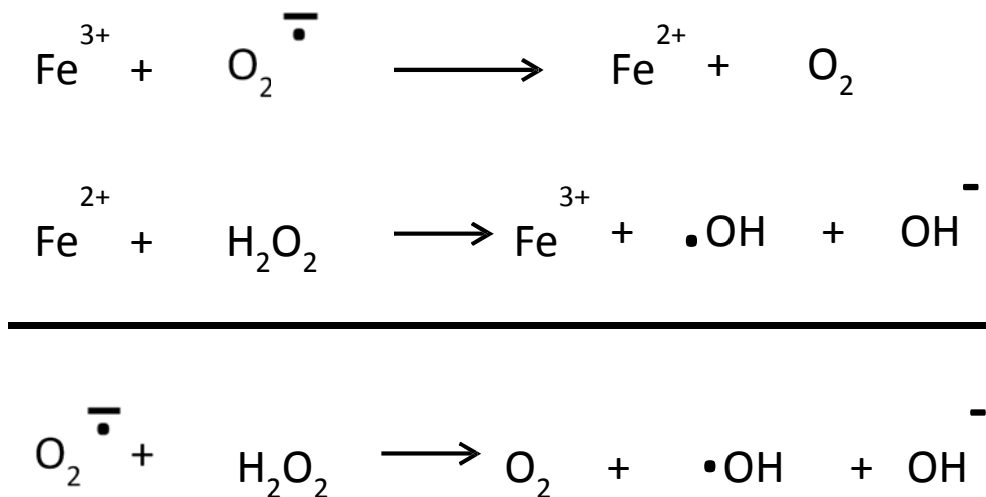
Reactive oxygen species generated cause severe damage by peroxidation of the proteins and lipids and cause oxidative damage to the DNA [548].

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Figure 16.   Reduction in glomerular filtration rate after gentamicin dosing:
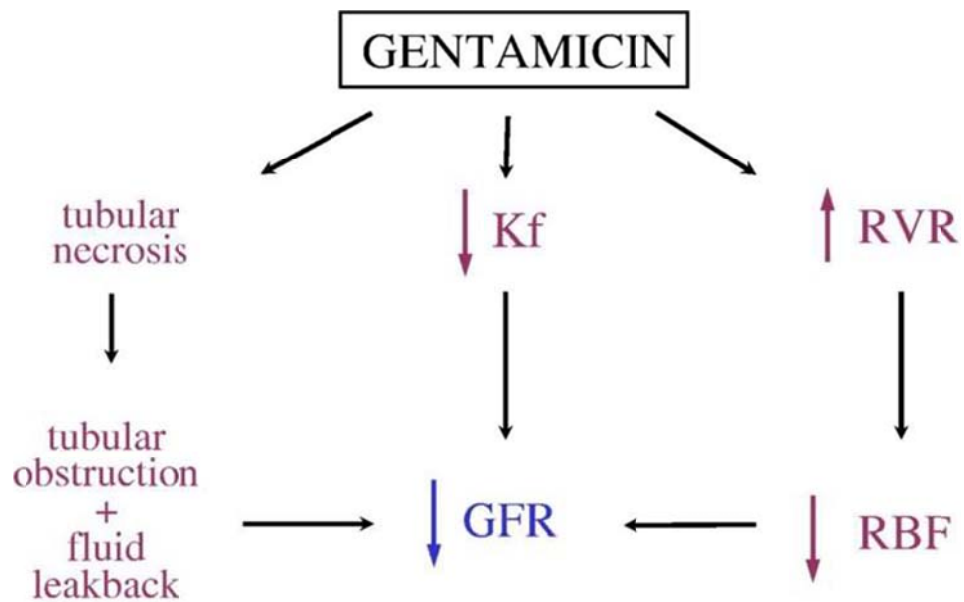
GFR: glomerular filtration rate; Kf: ultrafiltration coefficient; RBF: renal blood flow;

RVR: renal vascular resistance.

Carlos Martínez-Salgado, Francisco J. López-Hernández, José M. López-Novoa, Glomerular nephrotoxicity of aminoglycosides. *Toxicology and Applied Pharmacology*, 223 (1), 2007, 86 - 98

Figure 17. Mechanisms and cell signaling pathways underlying the cytotoxic effect of gentamicin in kidneys:
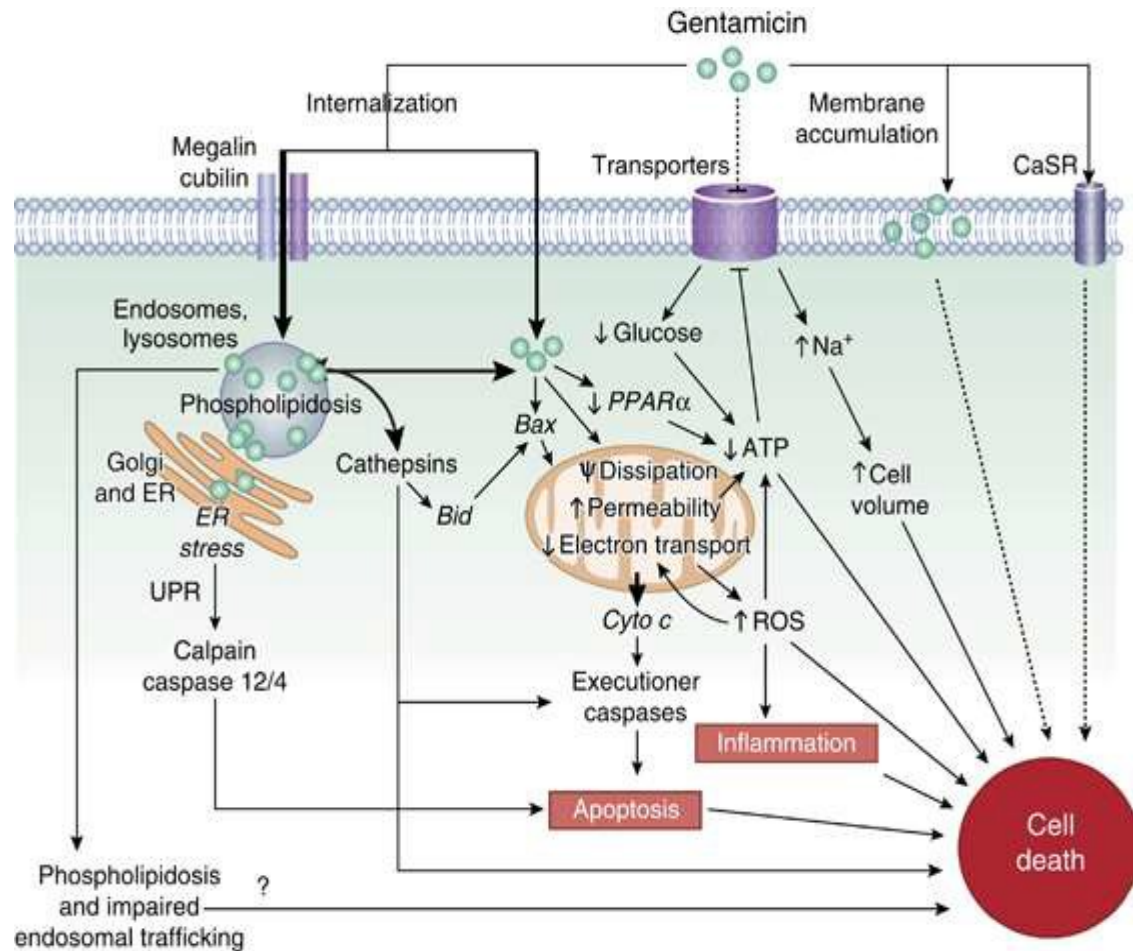
ATP, adenosine triphosphate; CaSR, extracellular calcium-sensing receptor; *Cyto c*, Cytochrome *c*; ER, endoplasmic reticulum; *PPARα*, peroxisome proliferator-activated. Receptor-α; ROS, reactive oxygen species; UPR, unfolded protein response; The contribution of these mechanisms to cell death is not completely known. Lopez Novoa, J. M., Quiros, Y., Vicente, L., Morales, A. I., & Lopez Hernandez, F. J. (2011). New insights into the mechanism of aminoglycoside nephrotoxicity: an integrative point of view. *Kidney Int, 79*(1), 33-45.

Figure 18. Schematic presentation of invasive study:

- Blood and urine samples at time points
- Serum creatinine
- Aminoglycoside concentrations
- Duration of neuromuscular paralysis
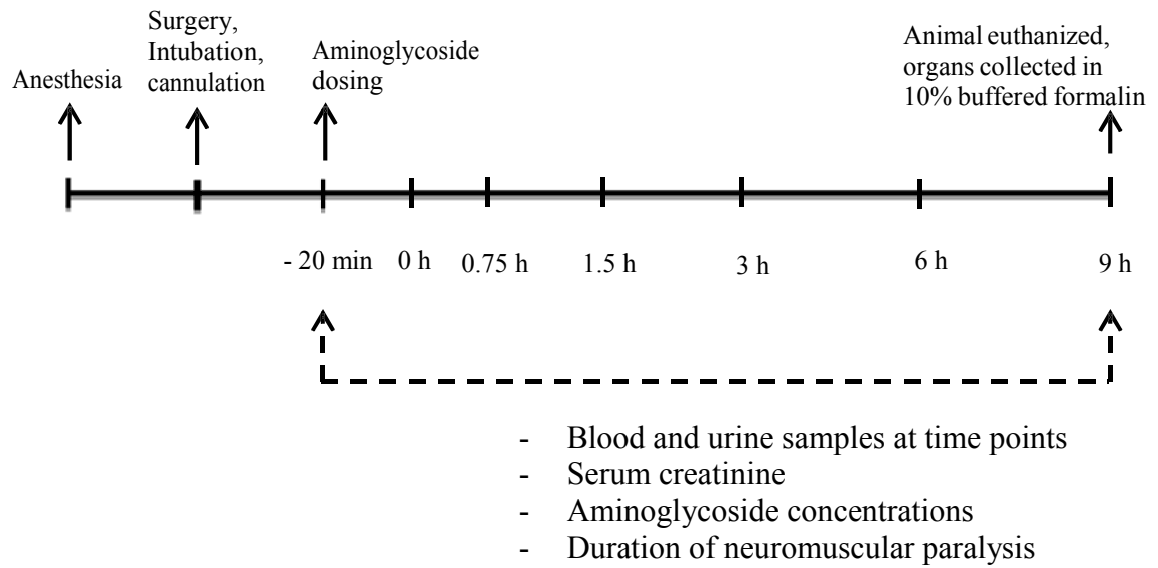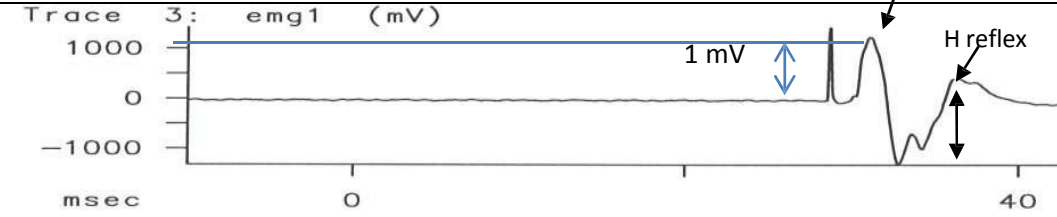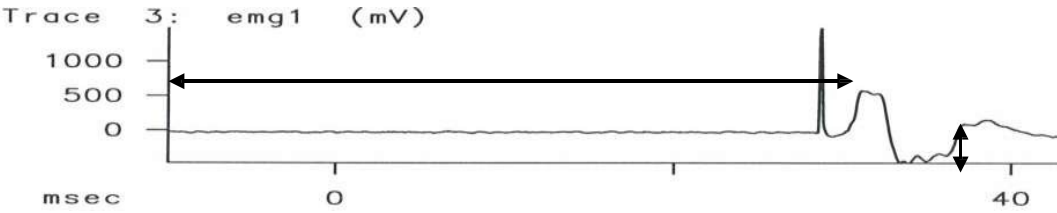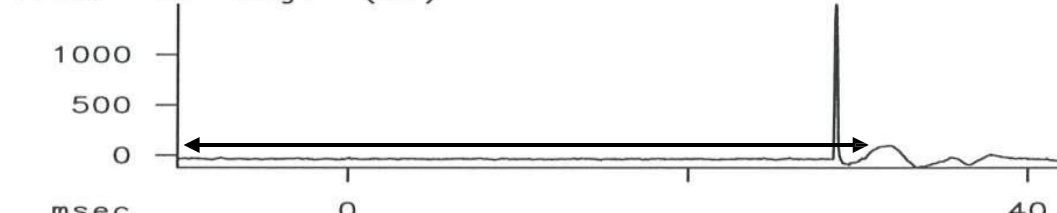
Figure 19. Schematic presentation of septic shock model:
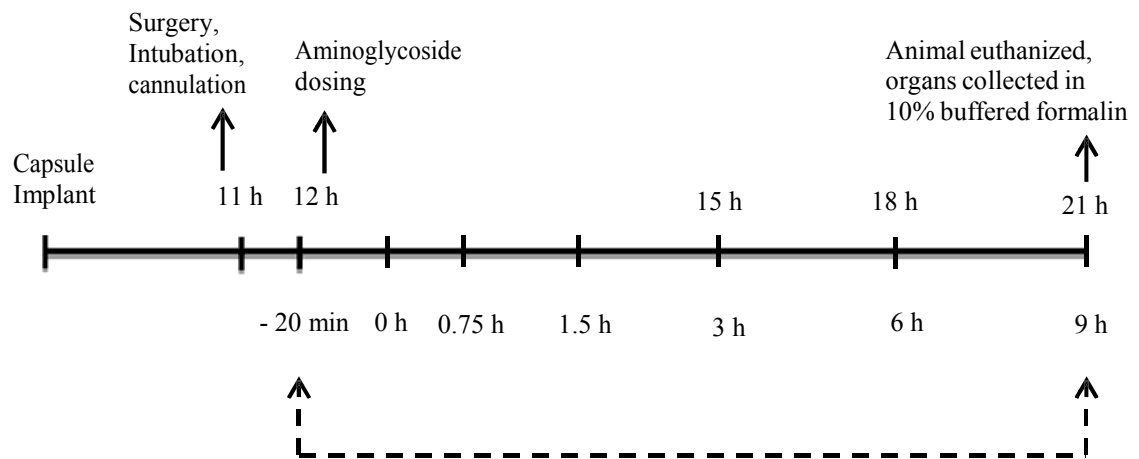
- Blood and urine samples at specific time points;
- Blood bacterial count,
   - Serum creatinine,
- Aminoglycoside concentrations,
   - Serum lactate

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Figure 20. a) Gentamicin 80 mg/kg intravenous dose (IV) and EMG wave recording during infusion for 20 minutes:

Time Point	EMG Wave		Heart rate (bpm)	Blood pressure (mmHg)
Baseline	M Wave / 1 mV / H reflex		284	104
			Normal EMG wave	
Post 40 mg/kg infusion			249	102
			Slight decrease in EMG wave	
Complete 80 mg/kg dose			265	85
			Complete paralysis EMG wave disappeared	

b) Gentamicin 80 mg/kg (IV) and EMG wave for 30 minutes after completing dosing
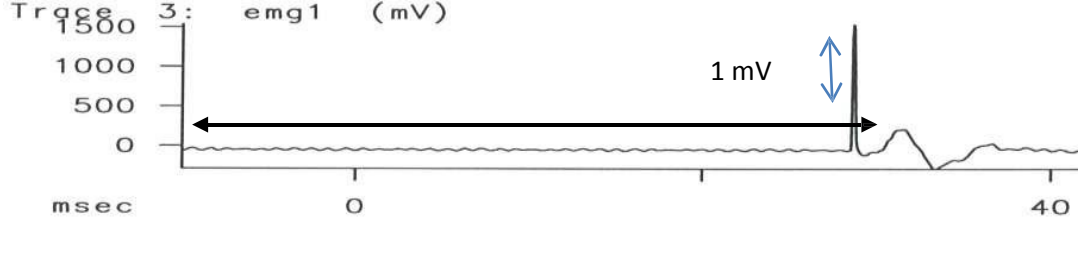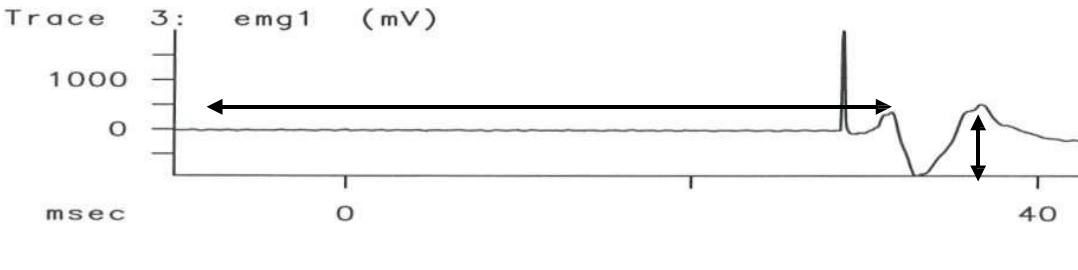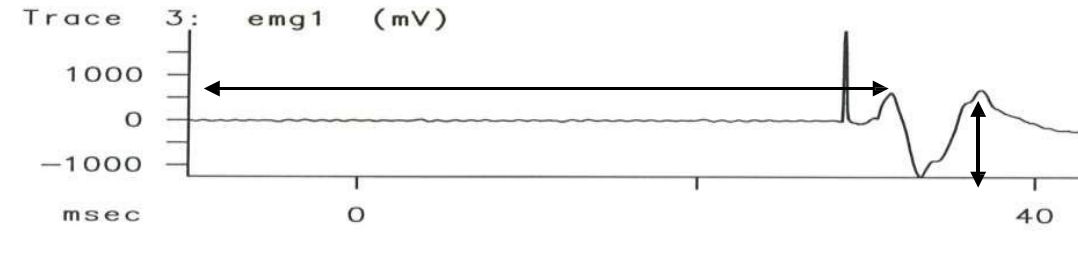
Time Point Post dosing	EMG Wave	Heart rate (bpm)	Blood pressure (mmHg)
10 minutes post dosing	Trace 3: emg1 (mV) 1500 1000 500 0 1 mV msec 0 40	268 No EMG wave, still the animal under paralysis	105
20 minutes post dosing	Trace 3: emg1 (mV) 1000 0 msec 0 40	266 Reappearance of EMG wave	85
30 minutes post dosing	Trace 3: emg1 (mV) 1000 0 -1000 msec 0 40	268 Return of EMG wave and normal functioning	98

Figure 21. Invasive study (9 h) serum creatinine levels:
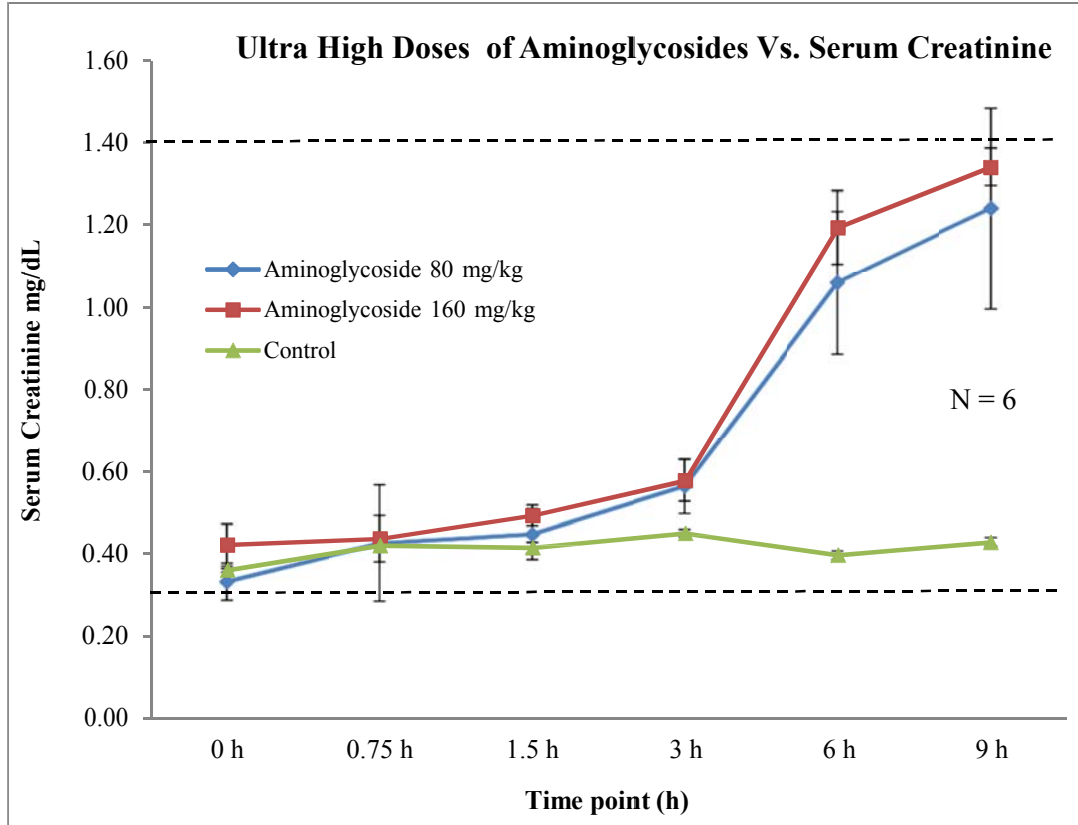
There was no statistically significant difference between single, ultra-high aminoglycoside dose of 80 mg/kg compared to control. There was a statistically significant difference between 160 mg/kg compared to blank control serum with a p-value of < 0.01. Statistics were calculated by repeated measures ANOVA followed by Dunnett's test.

Bars represent standard error.

Figure 22. Invasive study: Hemodynamic parameters using pulse oximeter:
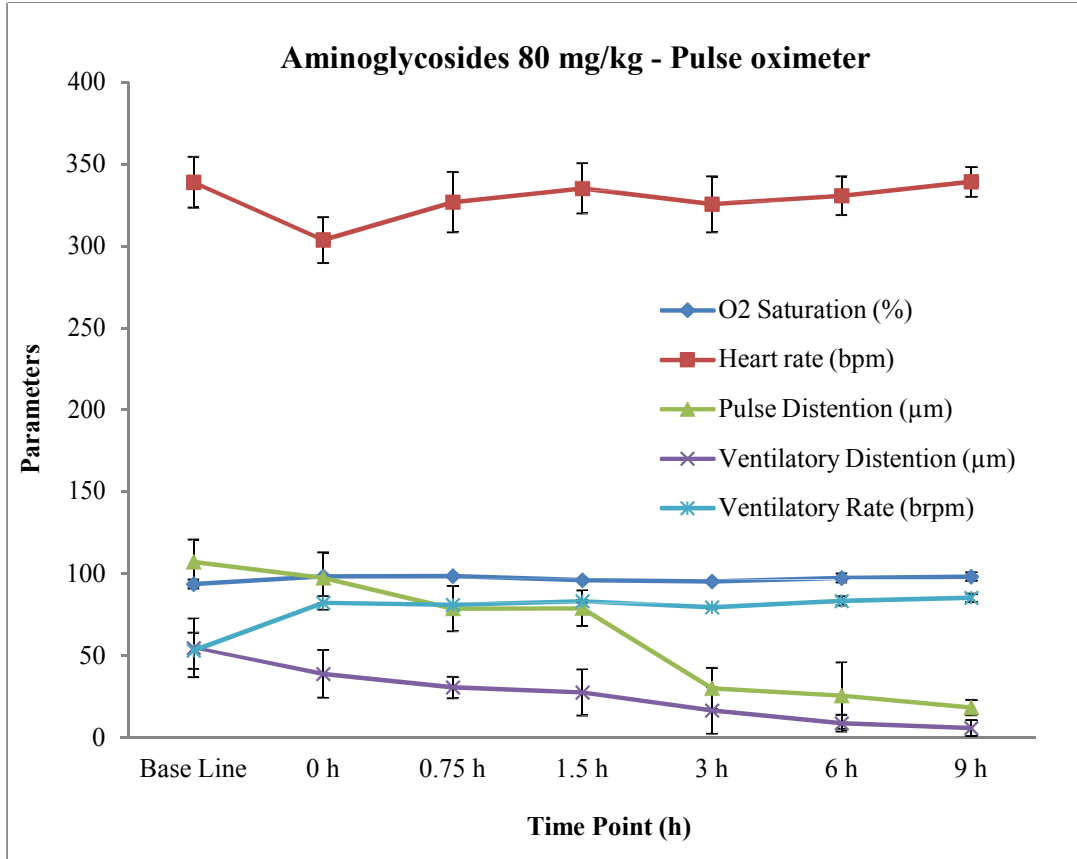
Steady drop in heart rate after administering aminoglycoside antibiotics at 80 mg/kg dose. Heart rate returned to baseline after 0.75 h of dosing.

Bars represent standard error.

Figure 23. Invasive study: Hemodynamic parameters using pulse oximeter:
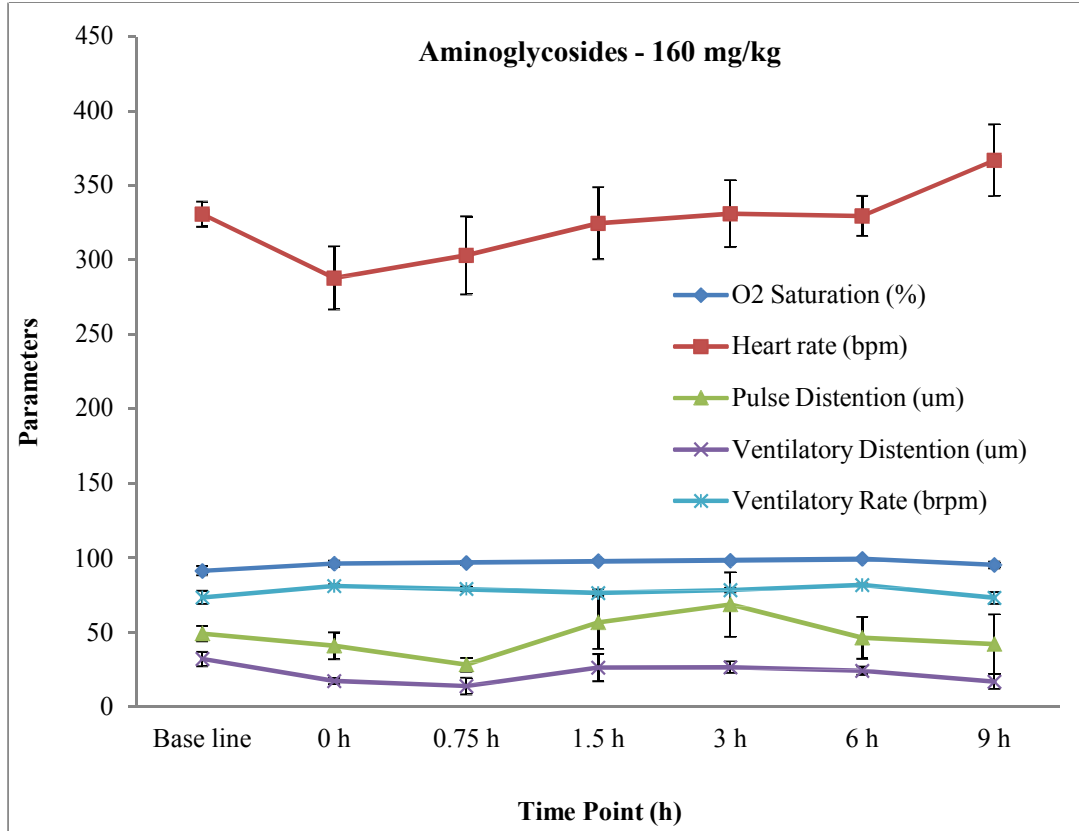
Steady drop in heart rate after administering aminoglycoside antibiotics at 160 mg/kg dose. Heart rate returned to baseline after 1.5 h of dosing.

Bars represent standard error.

Figure 24. Invasive study:

a) Pharmacokinetics of gentamicin at ultra – high doses in uninfected animals
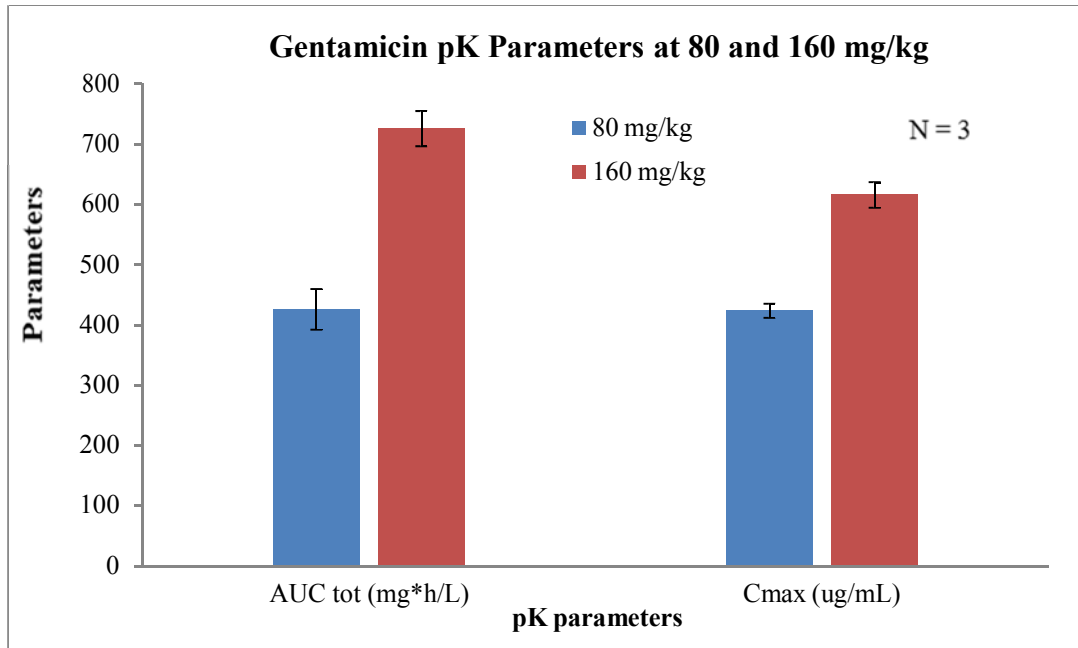
b)
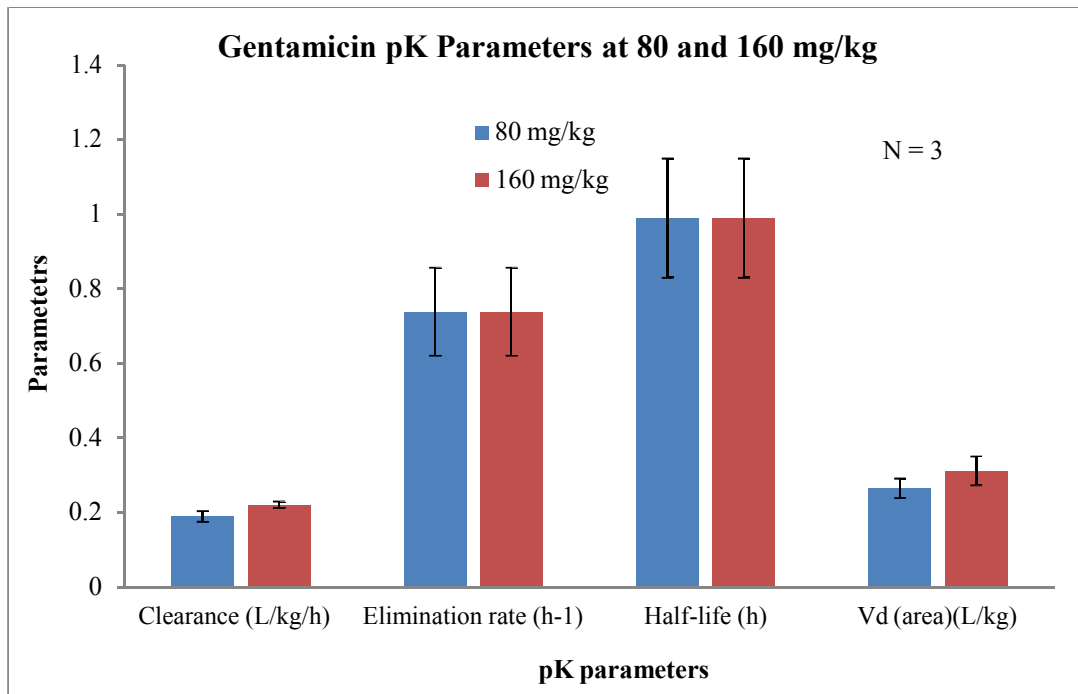
Bars represent standard error.

Figure 25.   Invasive study: Histology of kidneys:

a.   Gentamicin 80 mg/kg - 20 x magnification
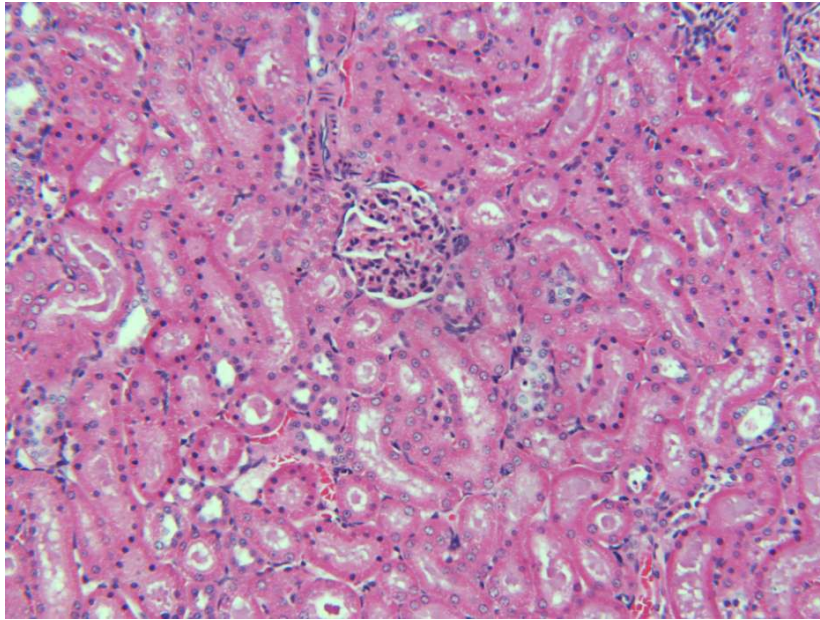
Glomerulus with clear Bowmen's capsule. No toxicity

b.   Gentamicin 160 mg/kg – 20 x magnification
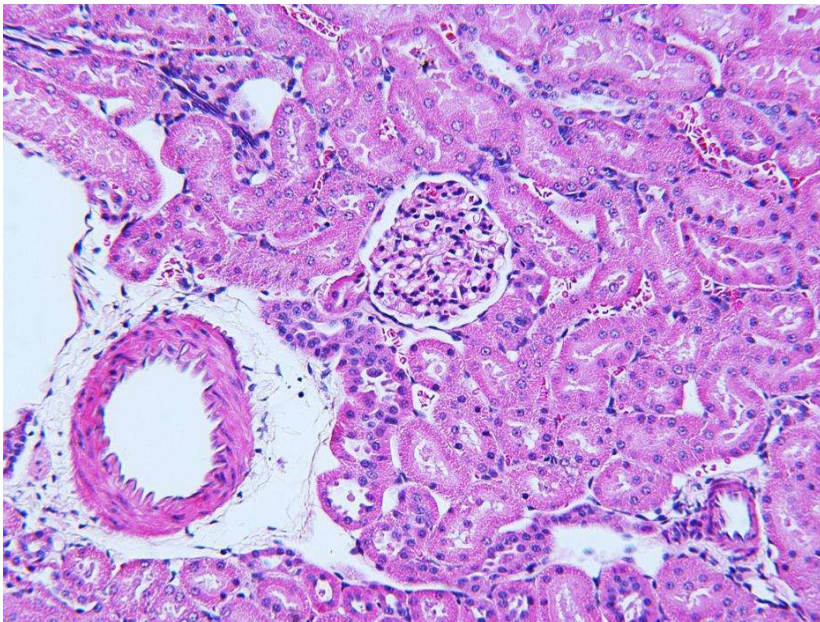
Glomerulus with clear Bowmen's capsule. No toxicity

c) Tobramycin 80 mg/kg: 20 x magnification
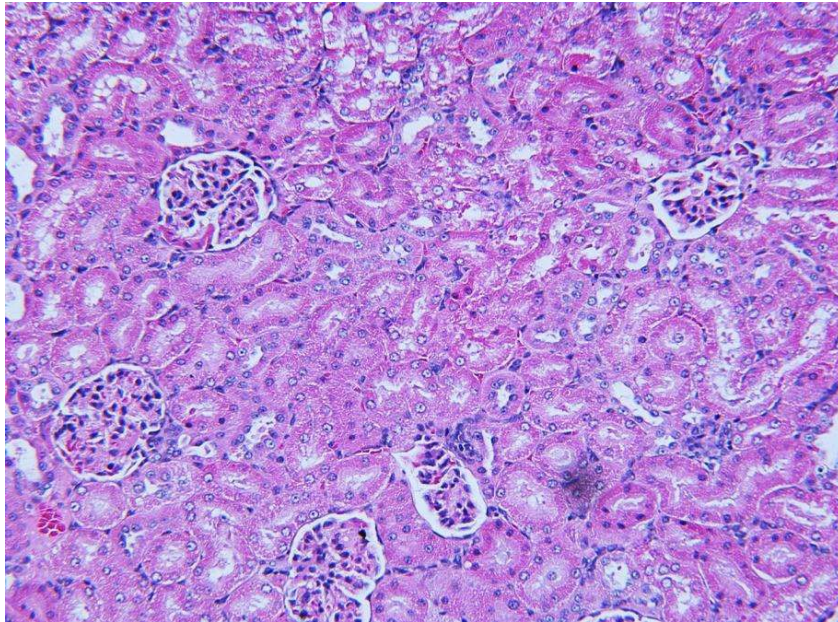
Glomerulus with clear Bowmen's capsule. No toxicity

d) Tobramycin 160 mg/kg: 20 x magnification
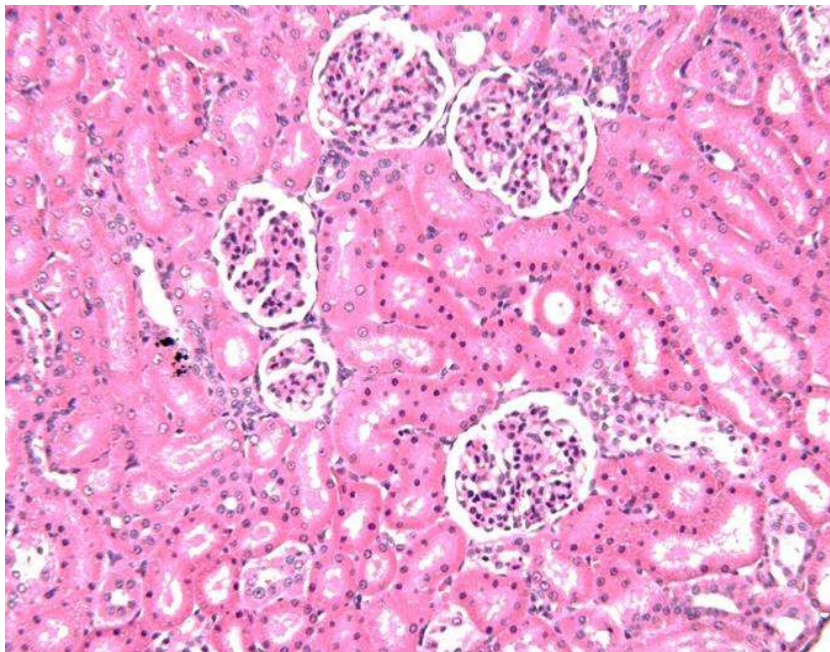
Glomerulus with clear Bowmen's capsule. No toxicity

e) Control : 20 x magnification (Normal animal)
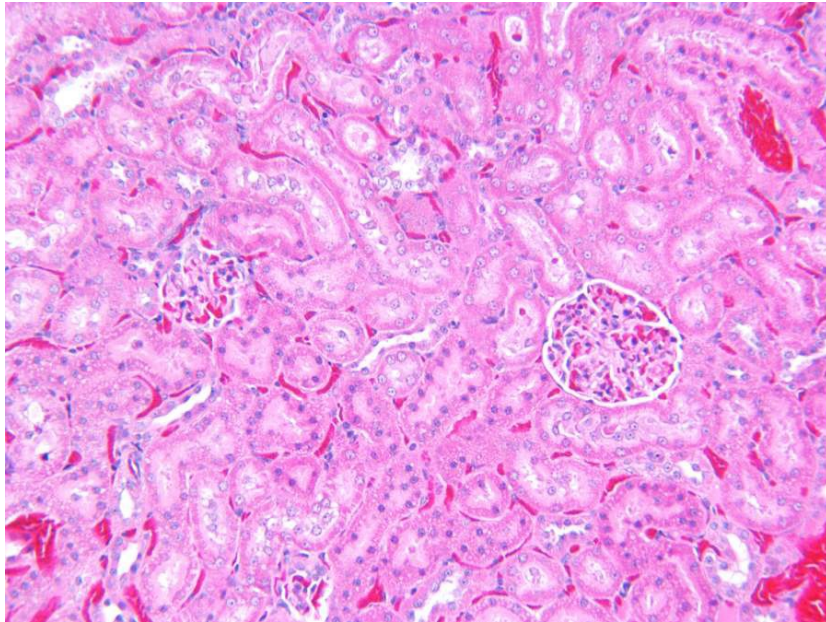
Slides a – e; are the histology slides of kidneys from invasive study (9 hours post ultra-high doses of gentamicin, tobramycin and saline treated control). All histology slides were stained using H & E staining. Usually, when there is kidney injury/ toxicity; hyaline casts, necrosis of tubular epithelial cells, hypertrophy of glomeruli etc. are observed.

None of these toxic symptoms were observed in the ultra-high aminoglycoside dosed animals and the kidneys appeared similar to that of the saline treated control animals indicating no toxicity.

Figure 26. Auditory brain stem response (ABR) study:

a) **Control animals: (N = 2)**
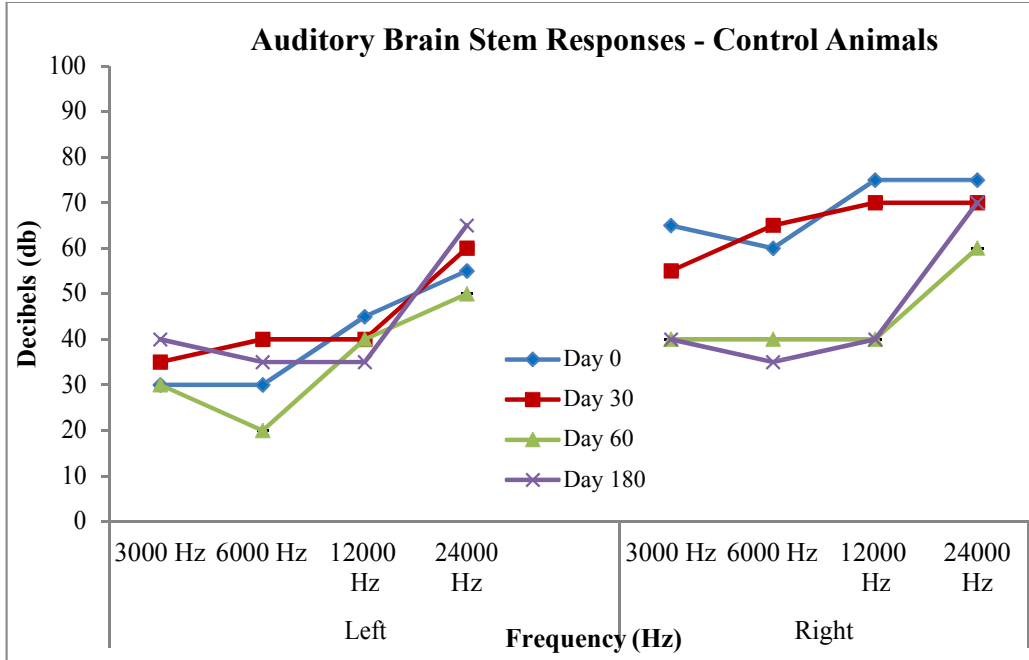
Auditory Brain Stem Responses - Control Animals

Data on Auditory brain stem responses were analyzed by the particular frequency level being tested (3000 Hz, 6000 Hz, 12000 Hz, 24000 Hz). Hearing ability of the animals at each specific frequency was recorded.

b) Aminoglycoside 80 mg/kg dose: (N = 4, 2 gentamicin + 2 tobramycin)
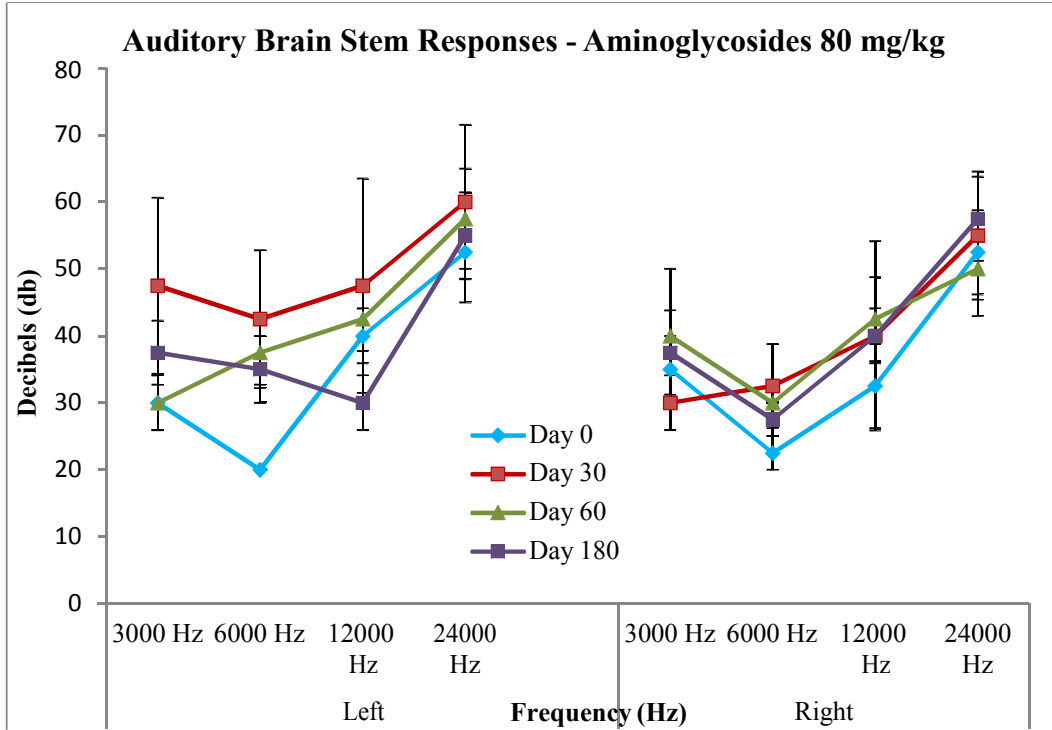
Data on Auditory brain stem responses were analyzed by the particular frequency level being tested (3000 Hz, 6000 Hz, 12000 Hz, 24000 Hz). Generalized estimating equations (GEE) with an identity link and normal distribution were run on the data with saline administered controls, 80 mg/kg and 160 mg/kg of aminoglycoside doses as a between subjects factor and day (0,180) as a repeated measure, as well as the interaction between dose and day. The P - value of ≤ 0.01 was considered significant difference. No statistically significant difference was observed at ultra-high doses of aminoglycosides at all the frequencies tested. Except at 6000 Hz, where, there was a significant decrease in the hearing ability, but this effect was not observed at higher frequencies. Hence this effect is considered because of type I error. Because of small number of animals per dose being used in this experiment, further study in a large group of animals per dose is required to confirm these results. Bars represent standard error.

c) Aminoglycoside 160 mg/kg dose: (N = 4, 2 gentamicin + 2 tobramycin)
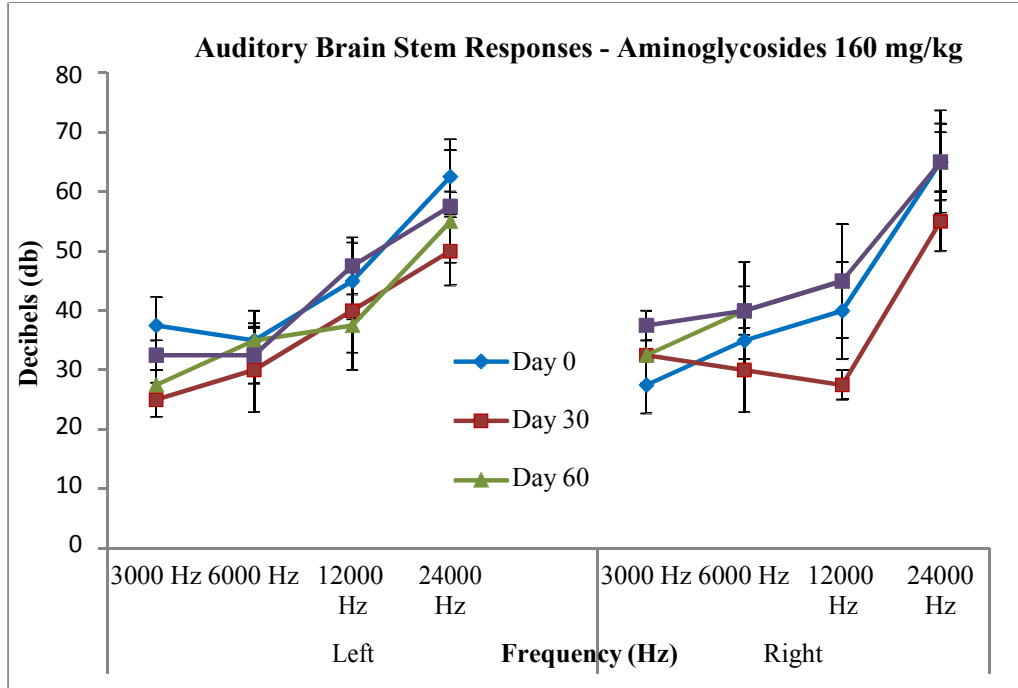
Data on Auditory brain stem responses were analyzed by the particular frequency level being tested (3000 Hz, 6000 Hz, 12000 Hz, 24000 Hz). Generalized estimating equations (GEE) with an identity link and normal distribution were run on the data with saline administered controls, 80 mg/kg and 160 mg/kg of aminoglycoside doses as a between subjects factor and day (0,180) as a repeated measure, as well as the interaction between dose and day. p-value of ≤ 0.01 was considered significant difference. No statistically significant difference was observed at ultra-high doses of aminoglycosides at all the frequencies tested. Because of small number of animals per dose being used in this experiment, further study in a large group of animals per dose is required to confirm these results. Bars represent standard error.

Figure 27.   Histology of cochlea: H & E staining

A. Control animal:

1. Cochlea at 10 x magnification
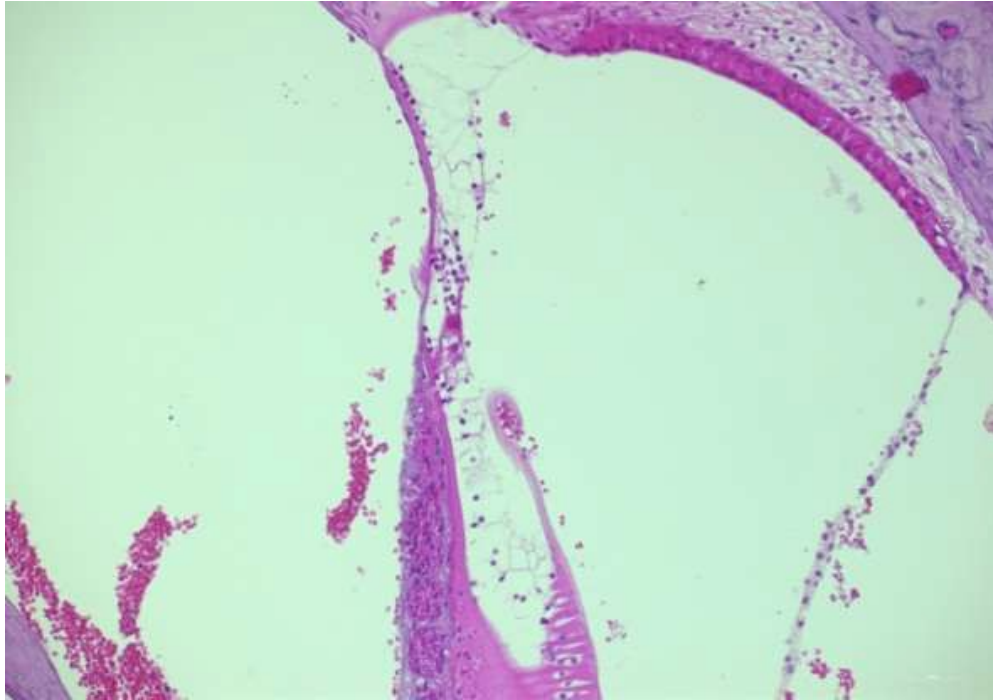
2. Cochlea at 20 x magnification
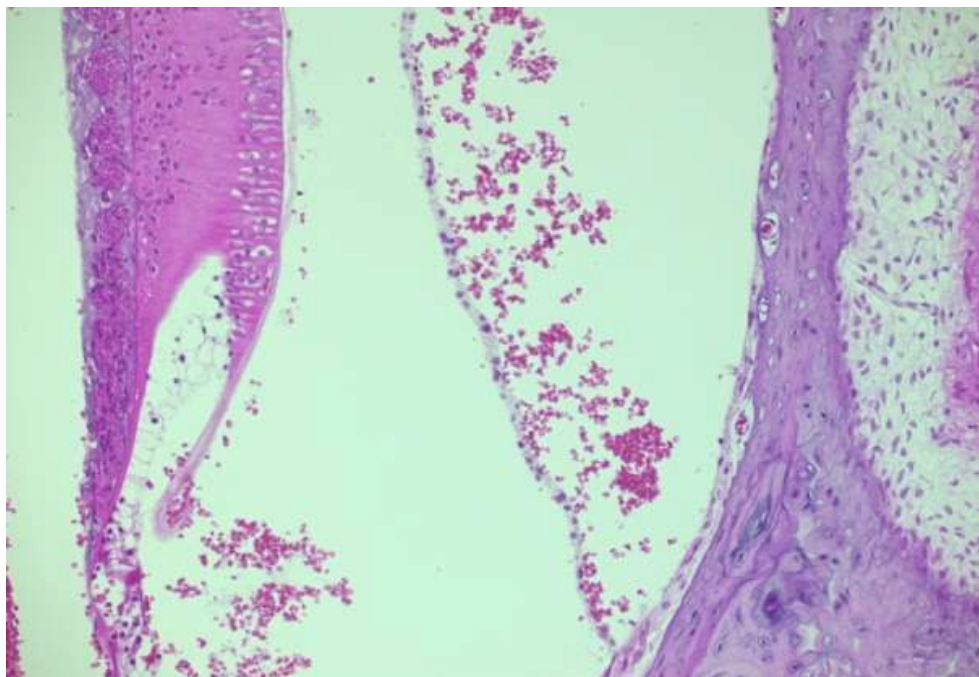
B. Gentamicin 80 mg/kg dosed animal:
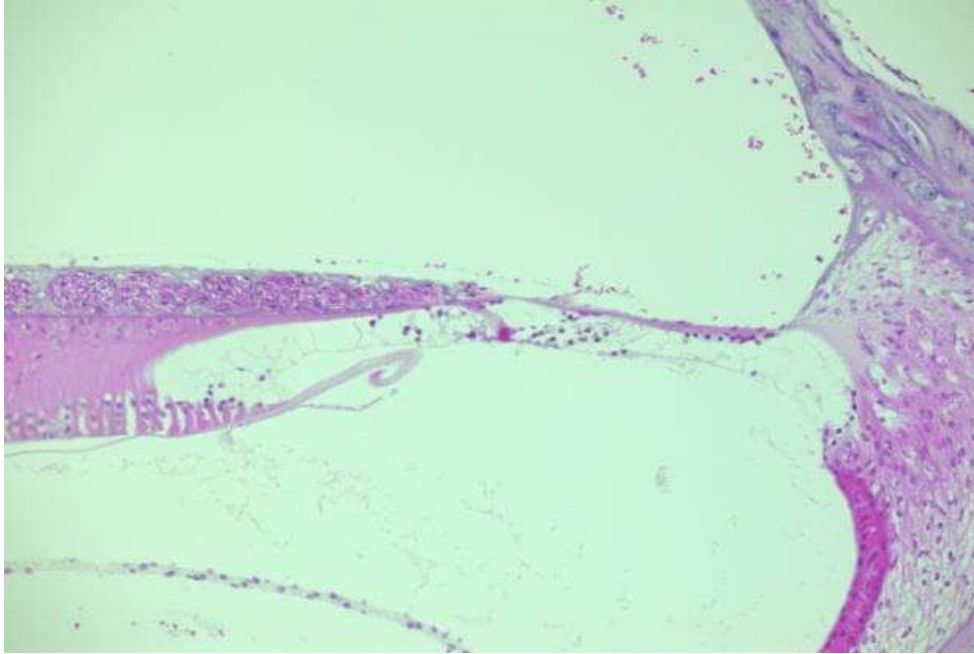
10 X magnification

C. Gentamicin 160 mg/kg dosed animal:
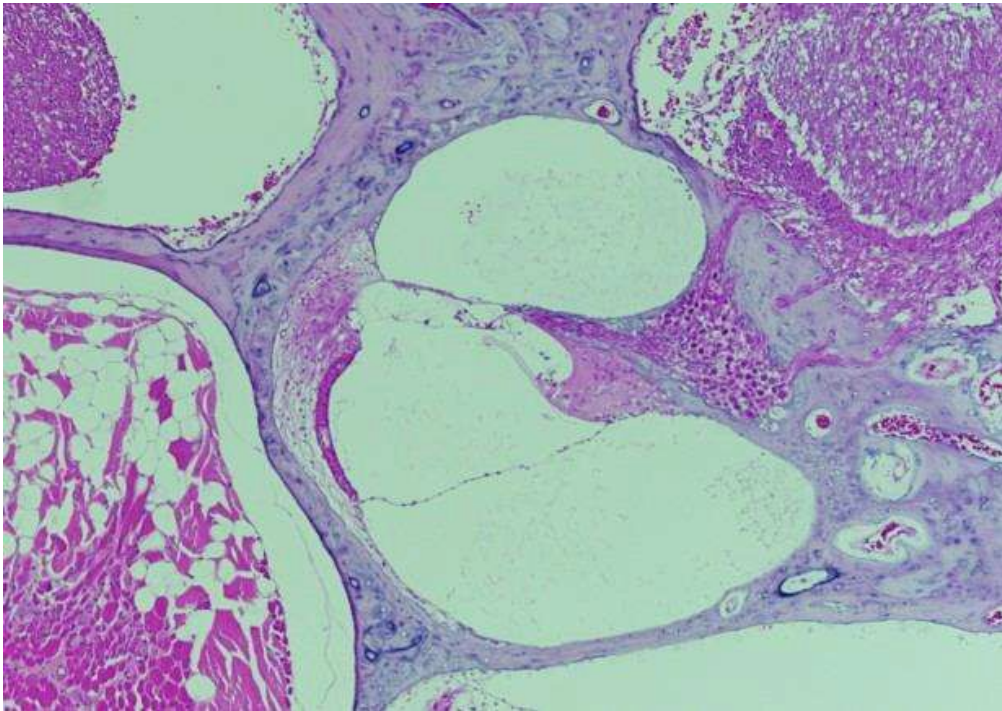
10 X magnification

D. Tobramycin 80 mg/kg dosed animal:

20 X magnification
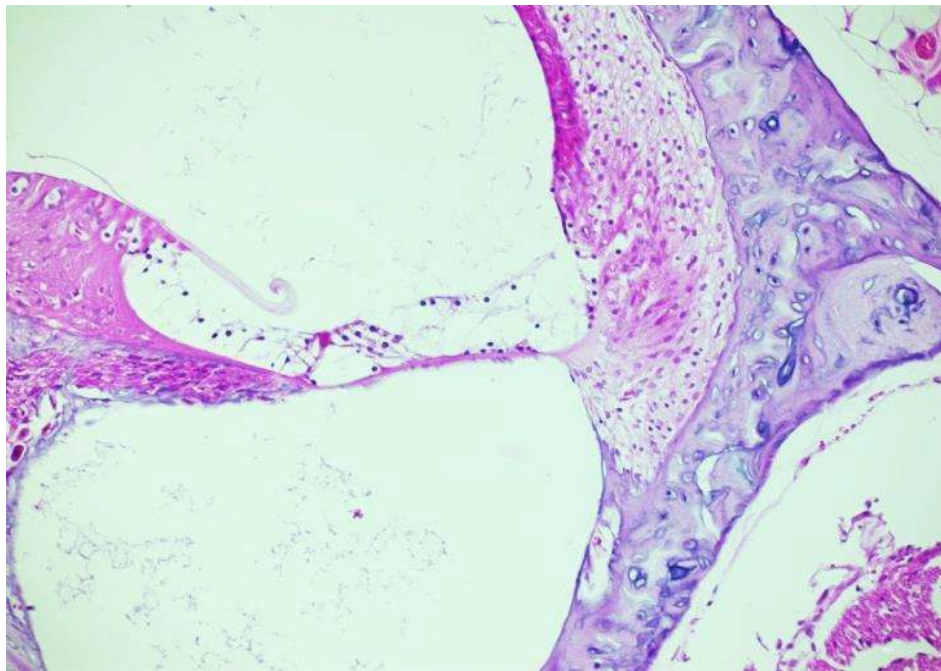
E. Tobramycin 160 mg/kg dosed animal:

10 X magnification
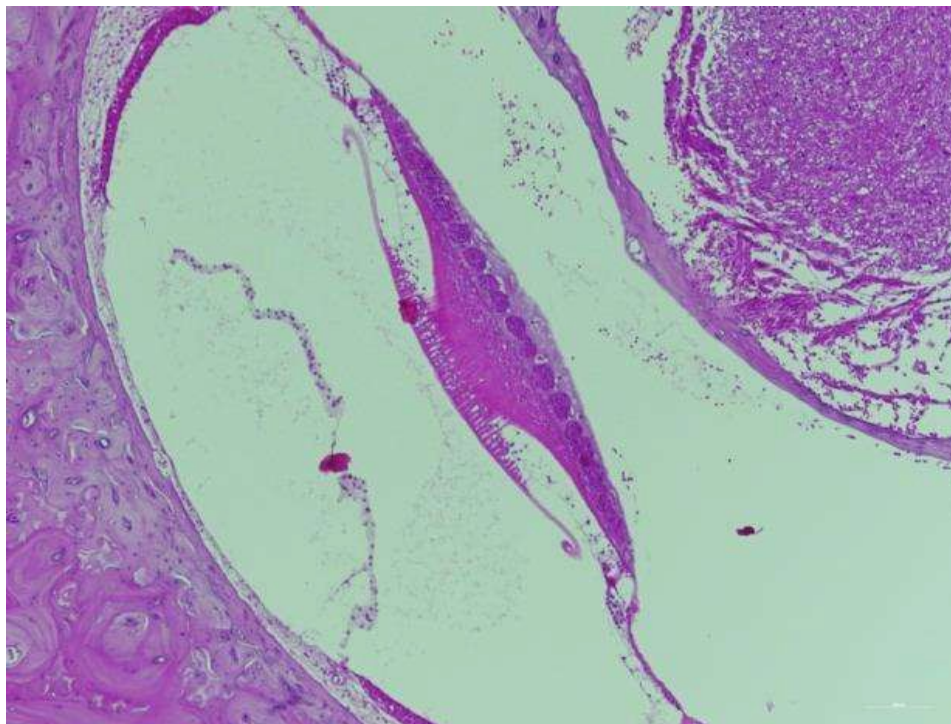
Figure 28. Serum creatinine levels (180 days): N=4 (2 gentamicin + 2 tobramycin/dose)
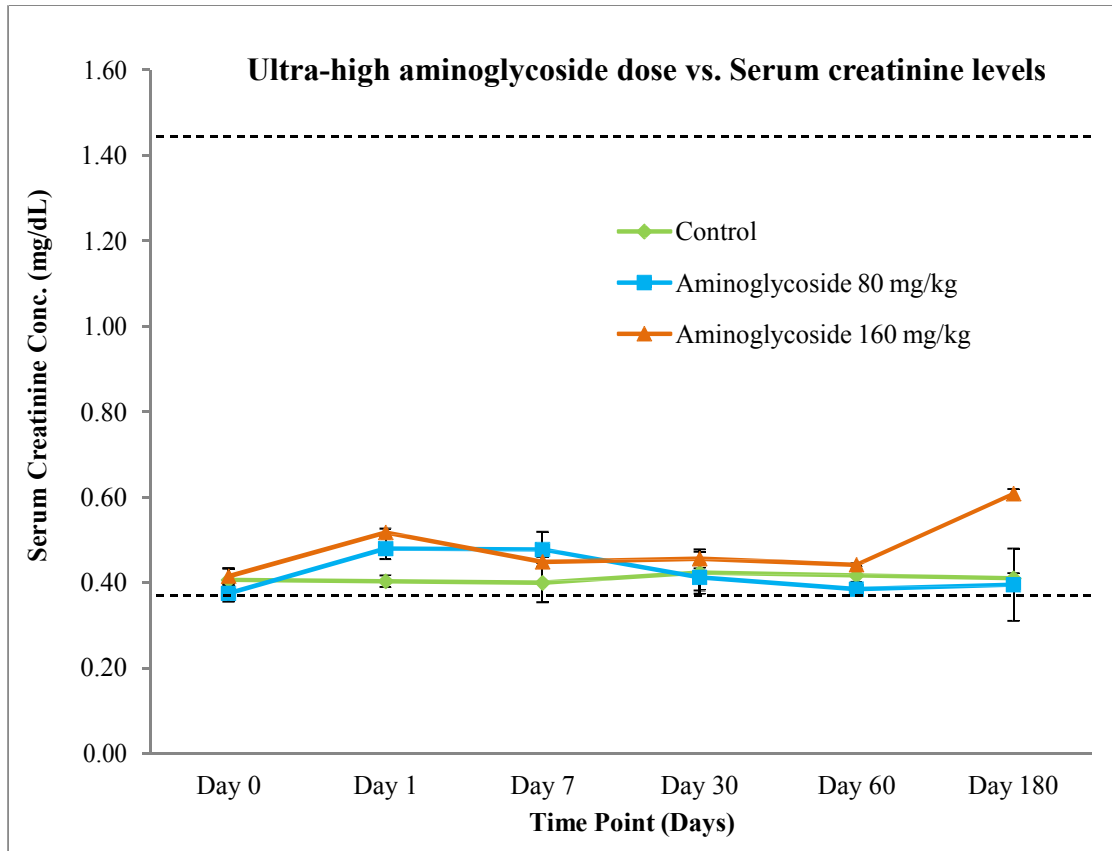
Serum creatinine levels were well in the normal range from day 1 to day 180 in the long-term study. There was no statistically significant difference between the control and treated groups measured by repeated measures ANOVA followed by Dunnett's test.

Dotted lines represent the normal range of serum creatinine.

Bars represent standard error.

Figure 29. Urinary NGAL concentrations after single, ultra-high dose aminoglycoside doses compared to control animals: N = 4 (2 gentamicin + 2 tobramycin per dose)
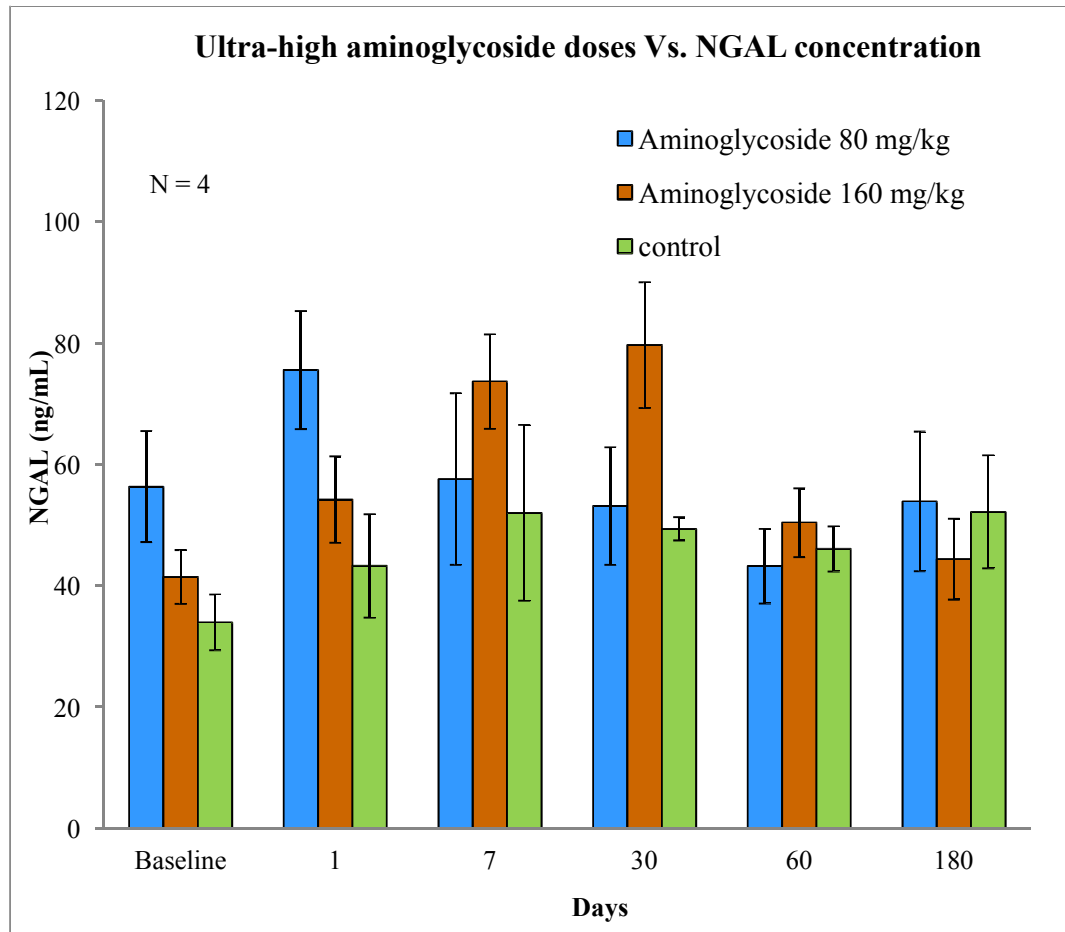
NGAL levels increased from 56.3 ng/mL at baseline to 75.56 ng/mL at 24 h post 80 mg/kg dose of aminoglycosides. With 160 mg/kg dose, at baseline, the urinary NGAL levels were 41.4 ng/mL and after 24 h of therapy the levels were 54.2 ng/mL. In control animals, the NGAL levels at baseline were 33.9 ng/mL and by 24 h time point the levels were 43.3 ng/mL. By day 7 at 80 mg/kg and day 60 at 160 mg/kg dose the NGAL levels became similar to baseline. The NGAL levels did not increase more than one fold throughout the experiment indicating that there was no acute kidney injury. There was no statistically significant difference between the control and treated groups measured by repeated measures ANOVA followed by Dunnett's test. Bars represent standard error.

Figure 30.   TUNEL assay:

a) Positive controls (provided in the kit):

1. Renal cortex: Brown colouration is due to apoptosis of endothelial cells.
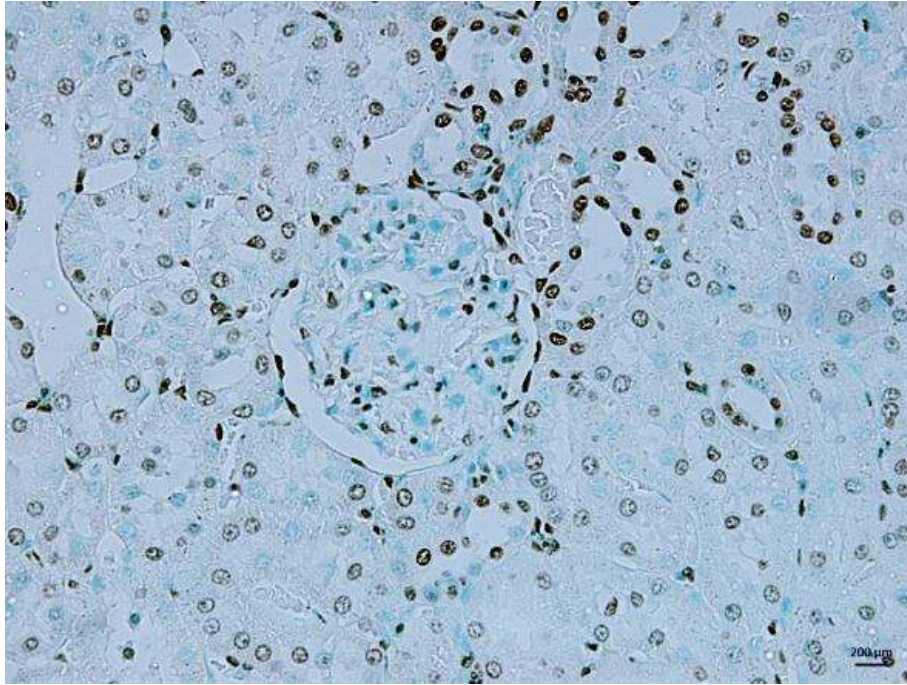
2. Tubular region: Brown colouration is due to apoptosis of endothelial cells.
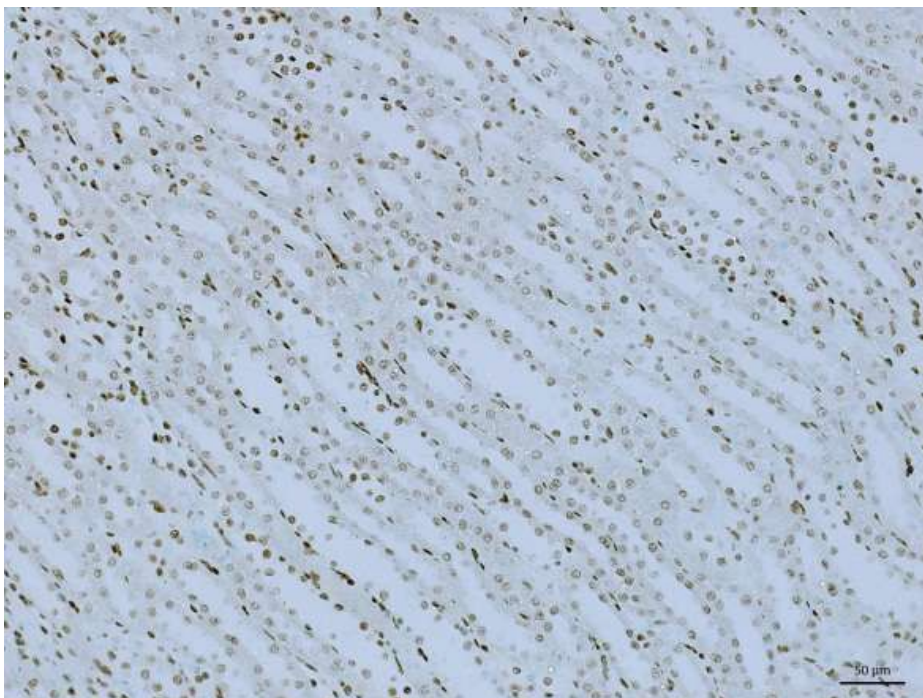
b) Control animals (saline administered):

1. Renal cortex: No apoptosis (green colored)
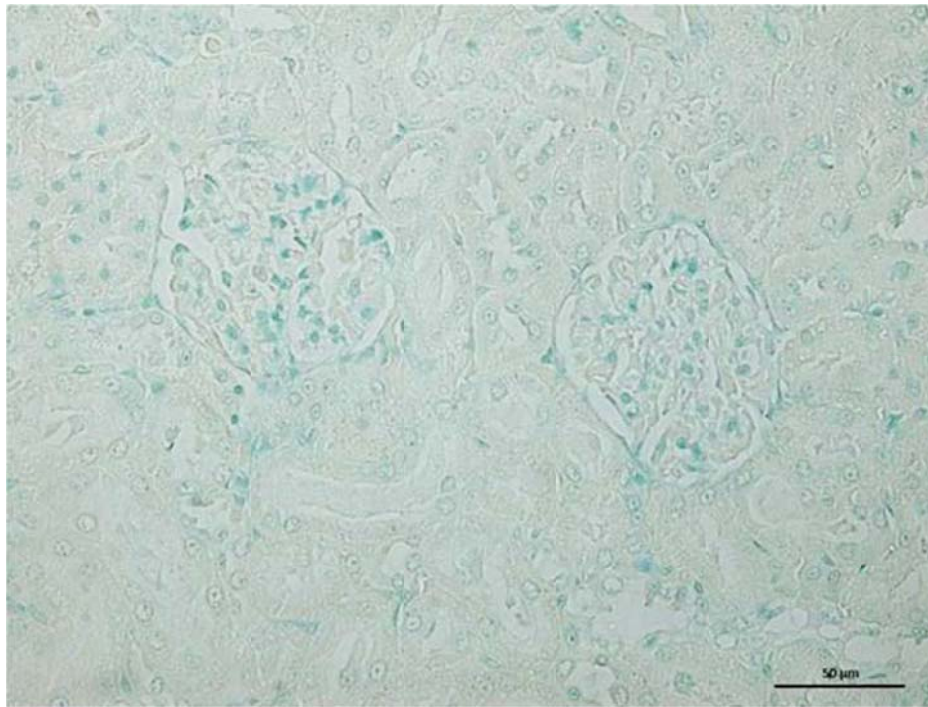
2. Tubular region: No apoptosis
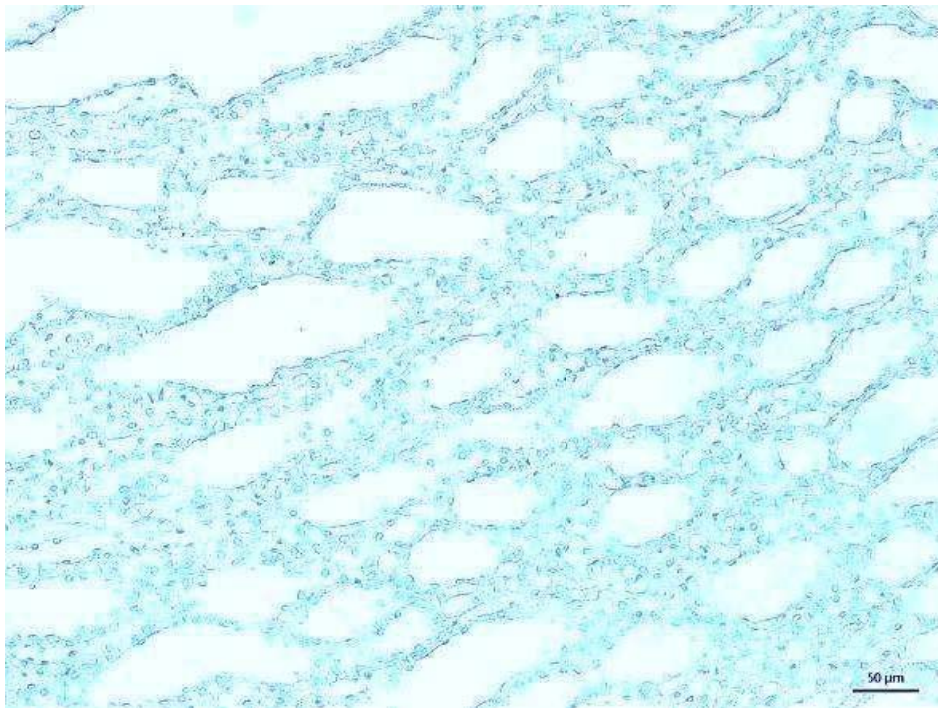
c) Gentamicin 80 mg/kg administered animals:

1. Renal Cortex: No apoptosis
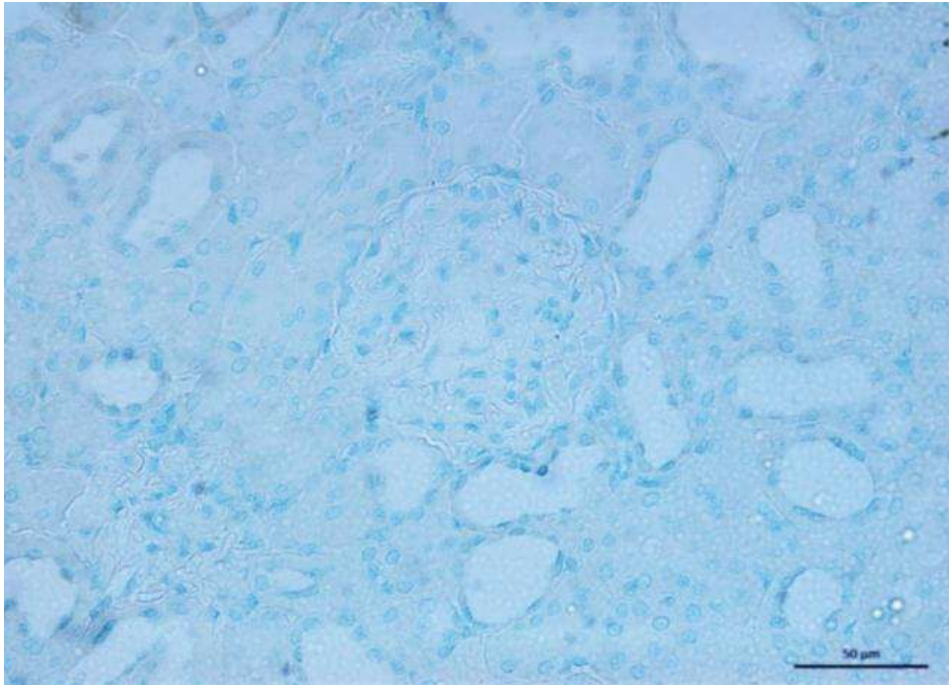
2.Tubular region: No apoptosis
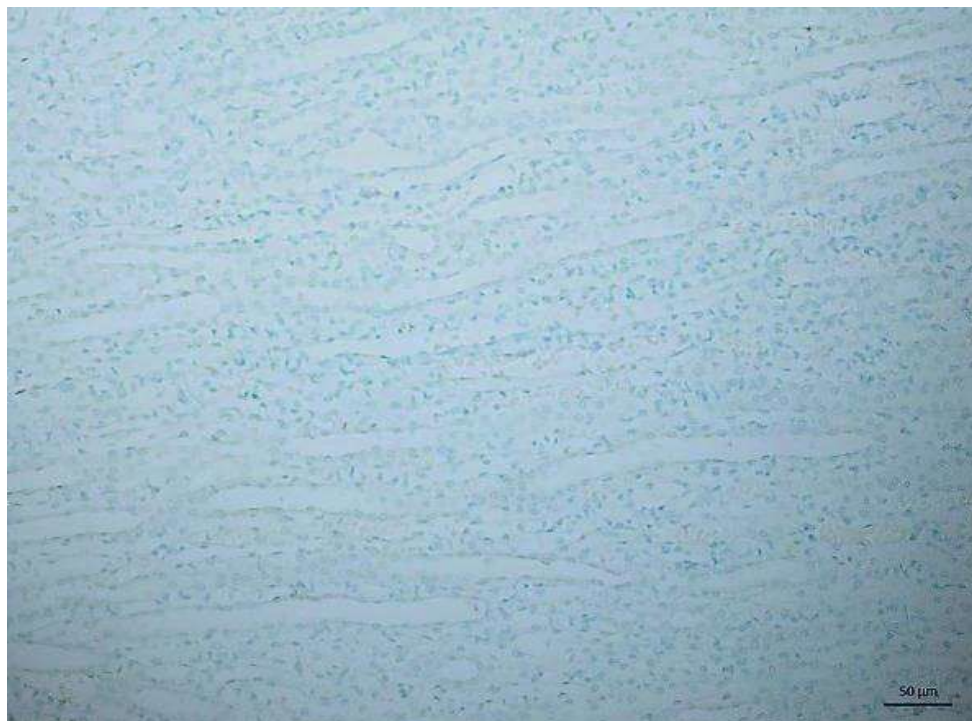
d) Gentamicin 160 mg/kg administered animals:

1. Renal cortex: No apoptosis
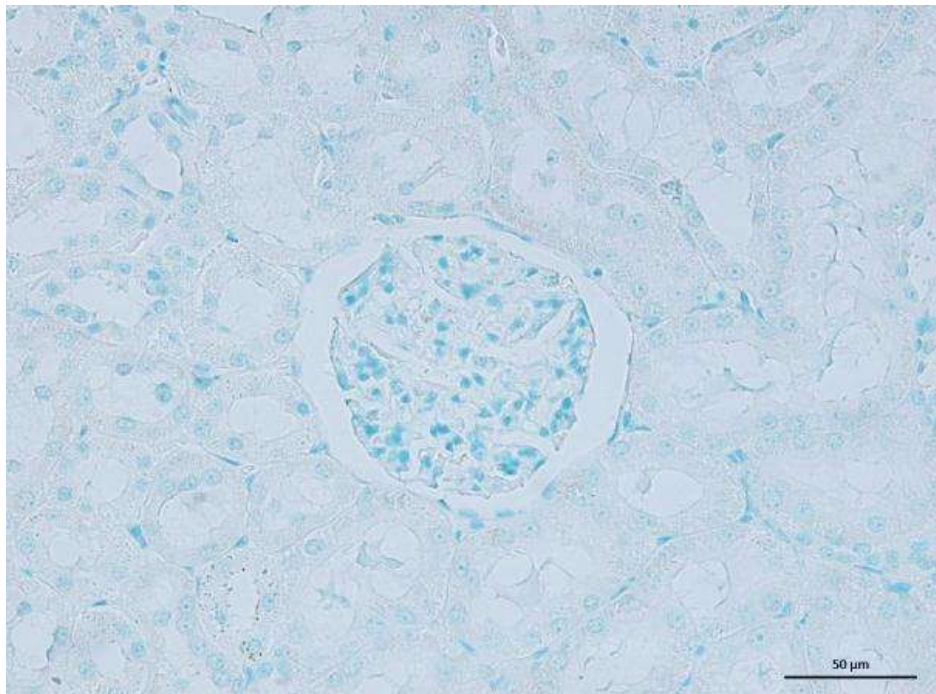
2. Tubular region: No apoptosis
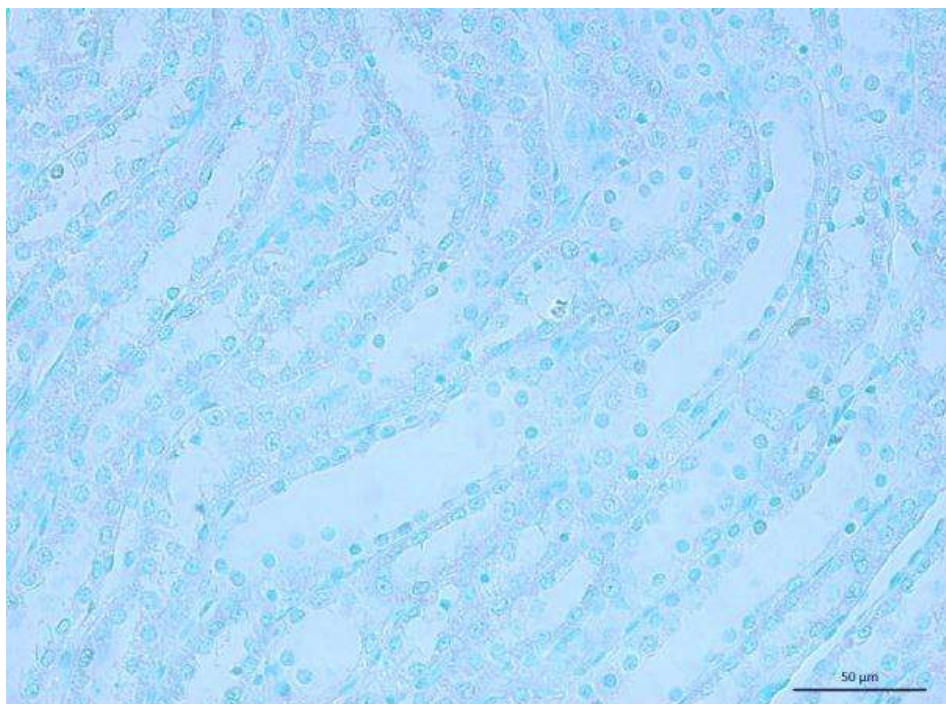
e) **Tobramycin 80 mg/kg administered to animals:**

1. **Renal cortex:** No apoptosis
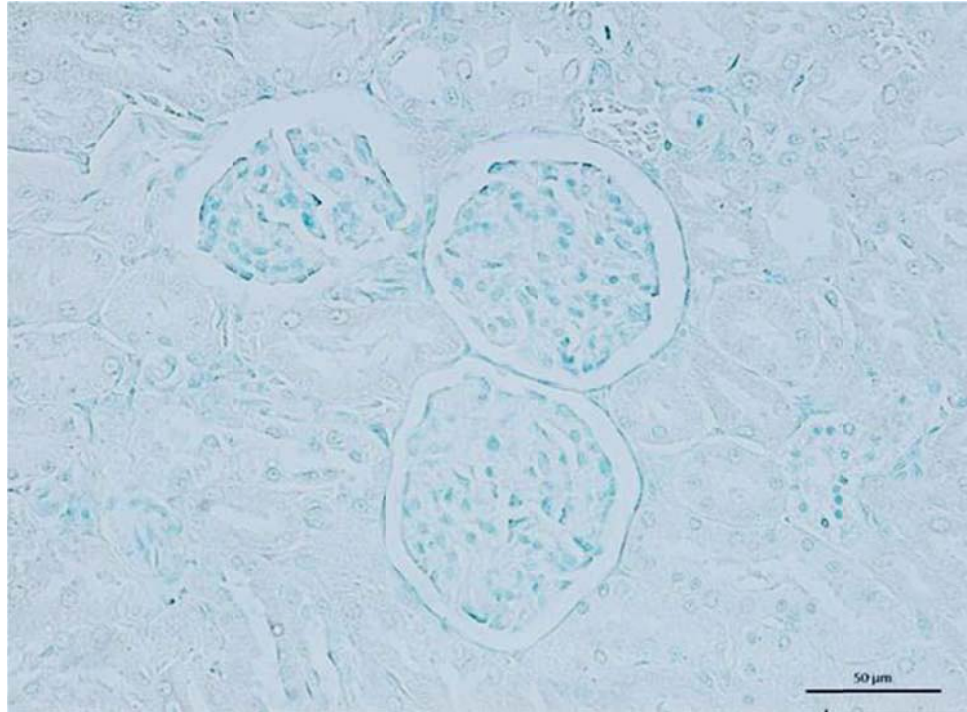
2. **Tubular region:** No apoptosis
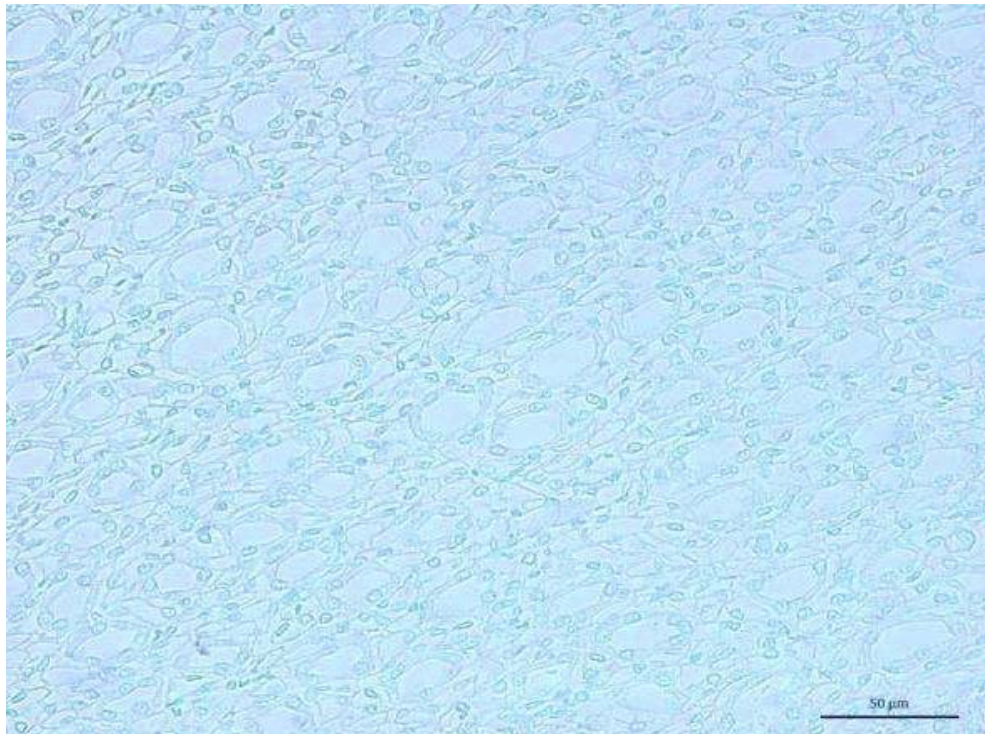
f) **Tobramycin 160 mg/kg administered animals:**

1. **Renal cortex:** No apoptosis (brown color is background of tissue, cells are intact)
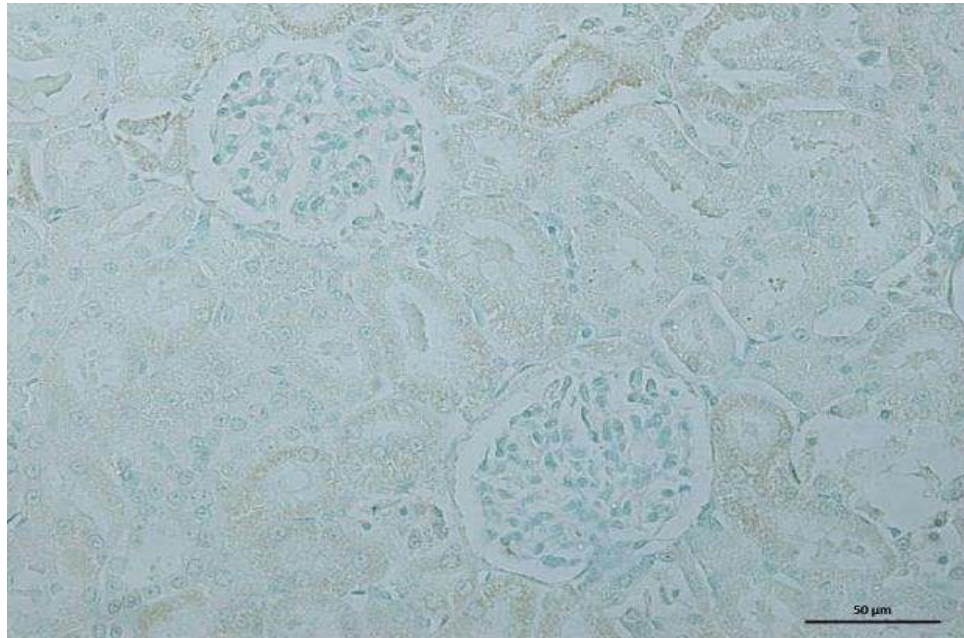
2. **Tubular region:** No apoptosis

Figure 31. Non-invasive study: Pulse oximeter readings

A. Control (saline treated) animals:
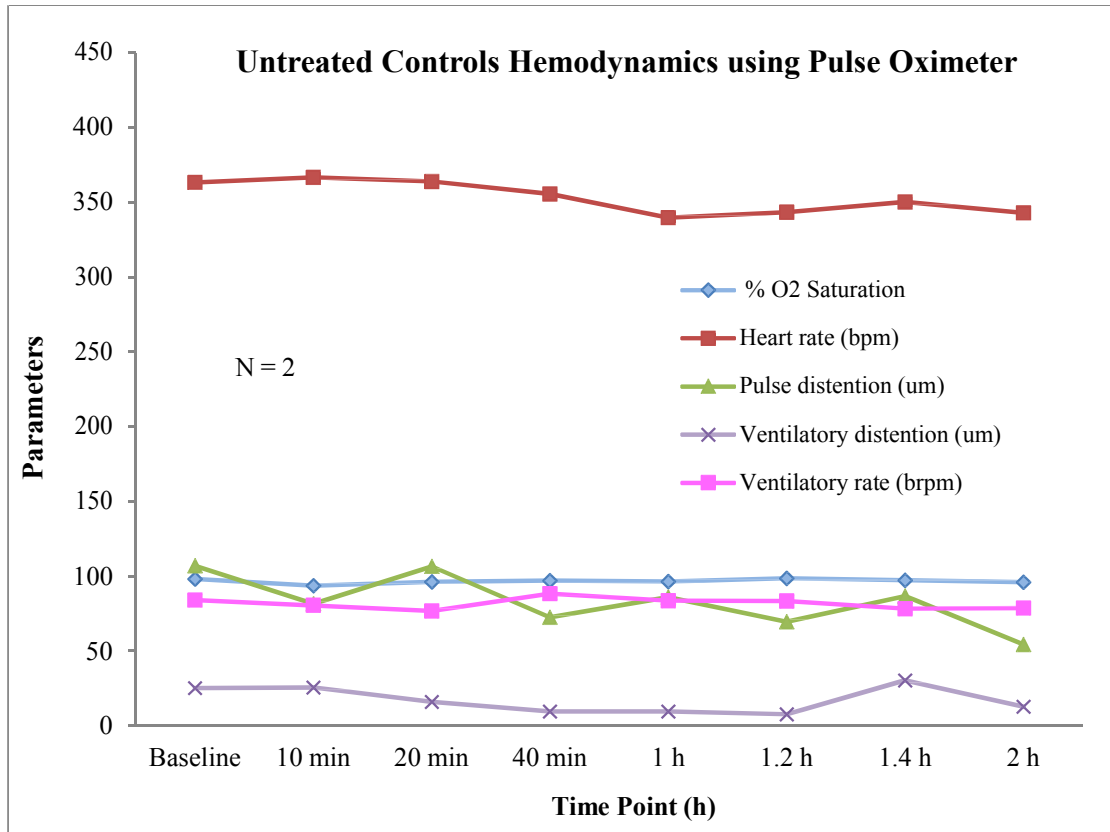
This was a survival experiment; hence hemodynamic parameters were measured for only the first two hours until the animals were ventilator dependent. Once the animals were able to breath on their own the intubation tube was removed and the animals were transferred to empty cages until completely recovered. Later the animals were transferred to their own cages and supplied with food and water *ad libitum* and carefully monitored for 180 days.

B. Aminoglycoside 80 mg/kg dosed animals (2 Animals/gentamicin + 2 Animals/tobramycin):

Aminoglycosides 80 mg/kg - Hemodynamics using Pulse ximeter

N = 4

Legend:
- % O2 Saturation
- Heart rate (bpm)
- Pulse distention (um)
- Ventilatory distention (um)
- Ventilatory rate (brpm)

X-axis: Time Point (h) — Baseline, 10 min, 20 min, 40 min, 1 h, 1.2 h, 1.4 h, 2 h

Y-axis: Parameters (0–450)

This was a survival experiment; hence hemodynamic parameters were measured for only the first two hours until the animals were ventilator dependent. Once the animals were able to breath on their own the intubation tube was removed and the animals were transferred to empty cages until completely recovered. Later the animals were transferred to their own cages and supplied with food and water *ad libitum* and carefully monitored 180 days. Bars represent standard error.

C. Aminoglycoside 160 mg/kg dosed animals (2 Animals/gentamicin + 2

Animals/tobramycin):

Aminoglycosides 160 mg/kg -Hemodynamics using Pulse oximeter

This was a survival experiment; hence hemodynamic parameters were measured for only the first two hours until the animals were ventilator dependent. Once the animals were able to breath on their own the intubation tube was removed and the animals were transferred to empty cages until completely recovered. Later the animals were transferred to their own cages and supplied with food and water *ad libitum* and carefully monitored 180 days. Bars represent standard error.

Figure 32. Histology work: slides from infected controls: (2 capsules)

i.   Kidney: Neutrophil infiltration

1. 40 X magnification of glomerulus - Neutrophil infiltration is pointed out by

    arrows
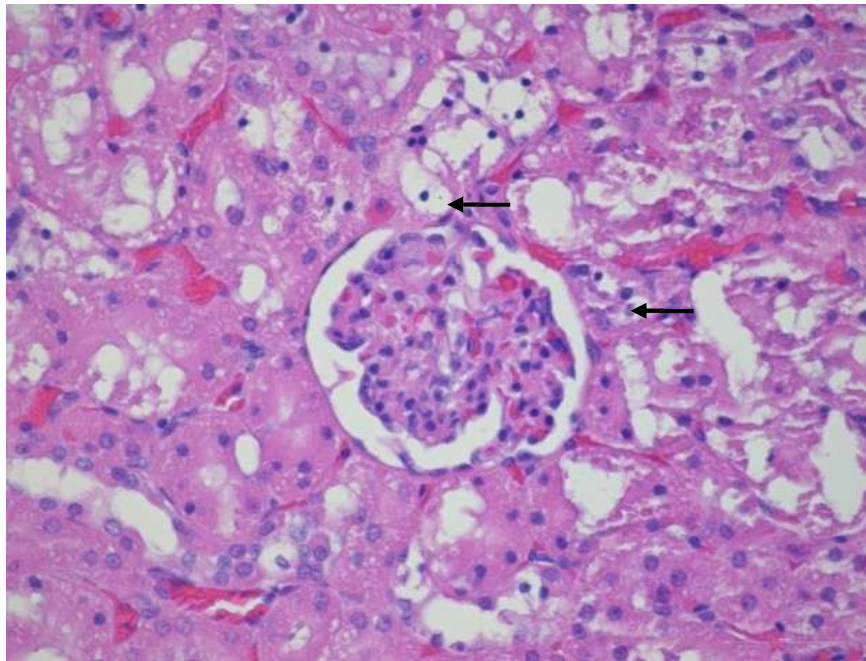
2. 60 X magnification tubular region
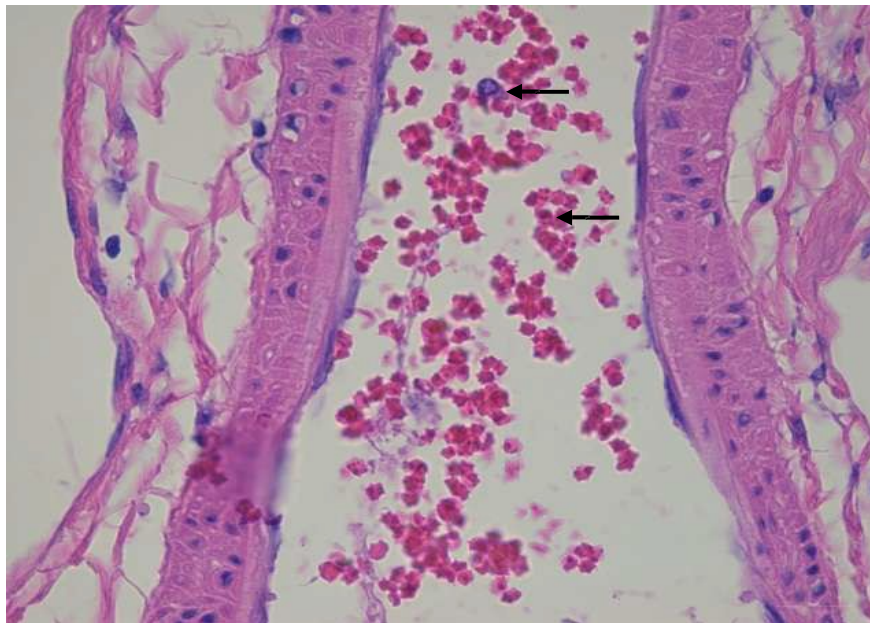
ii. **Spleen:** Neutrophil infiltration and some *E. coli* observed

1. **40 X magnification**
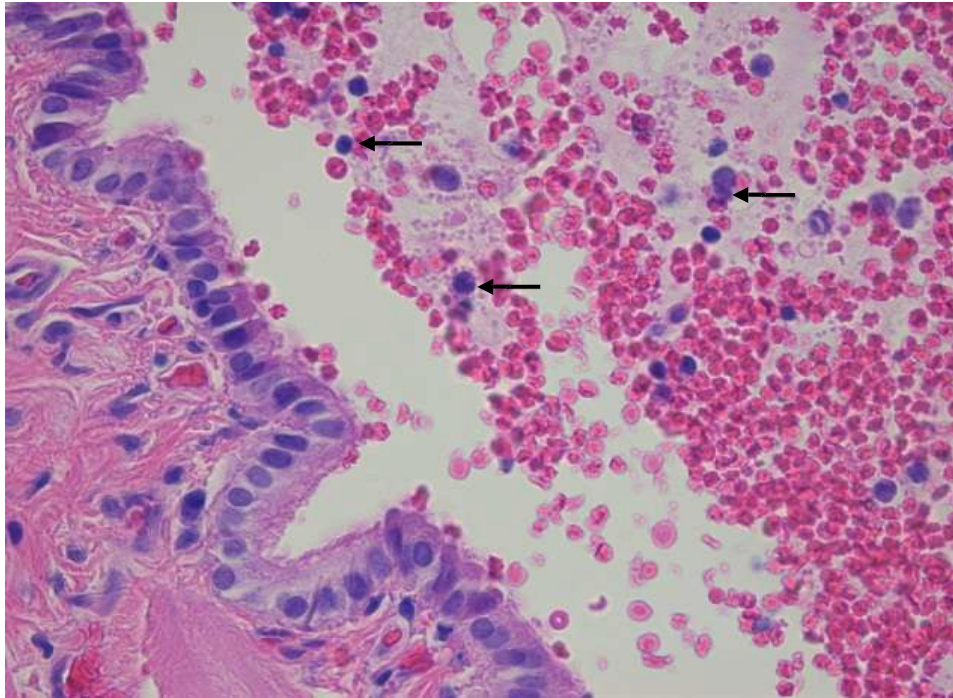
2. **40 X magnification**
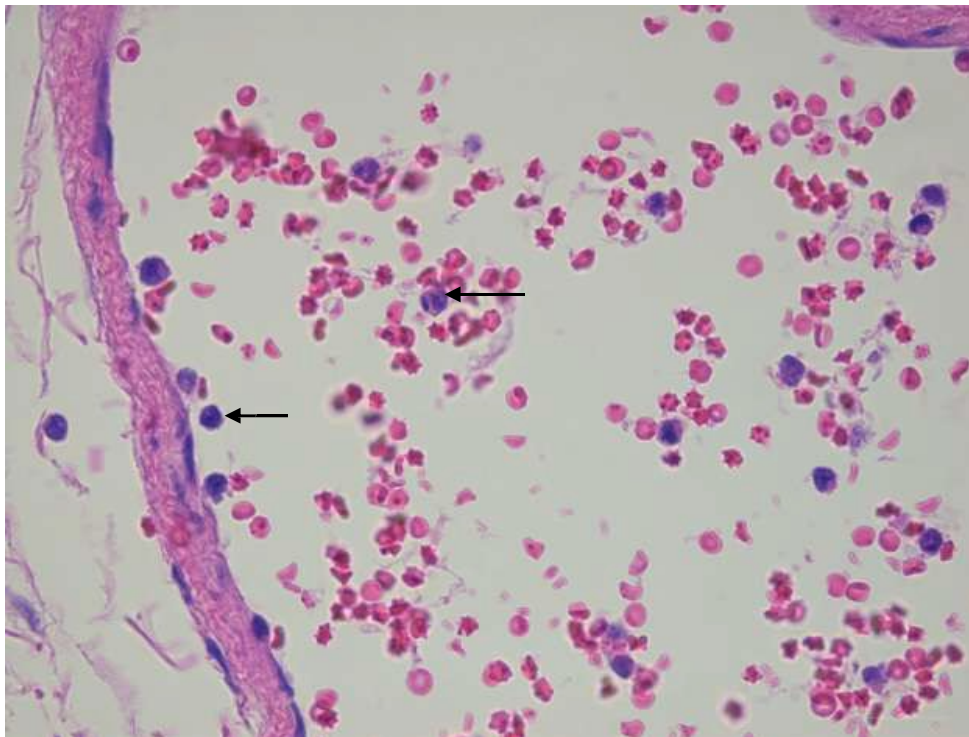
iii. **Liver:** Neutrophil infiltration in high numbers was observed

1. 20 X magnification
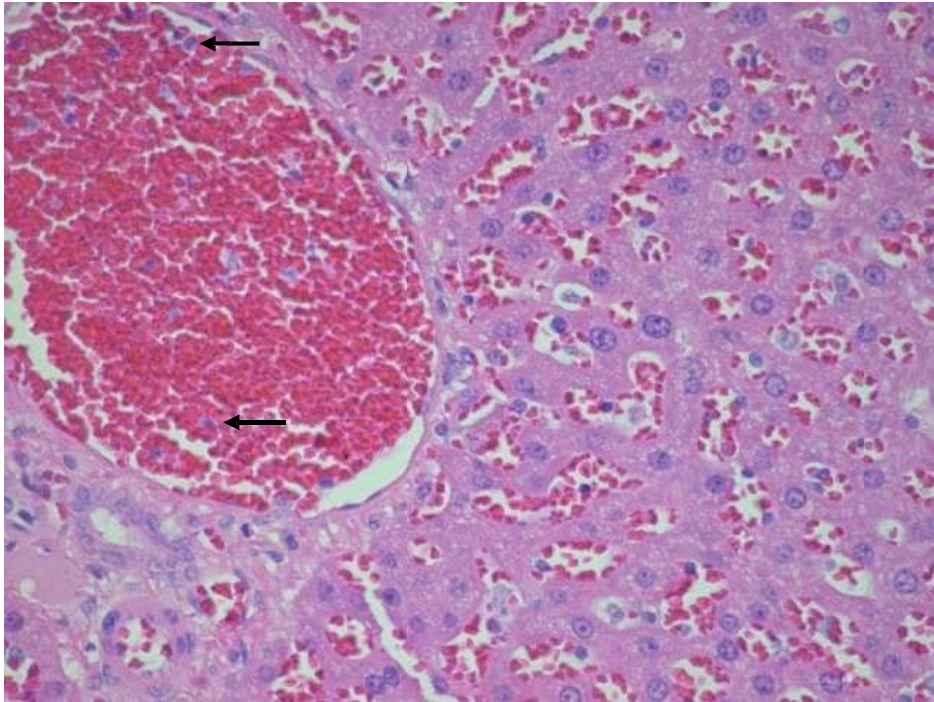
iv. **Lungs:** Neutrophil infiltration and some *E. coli* observed

1. 20 X magnification
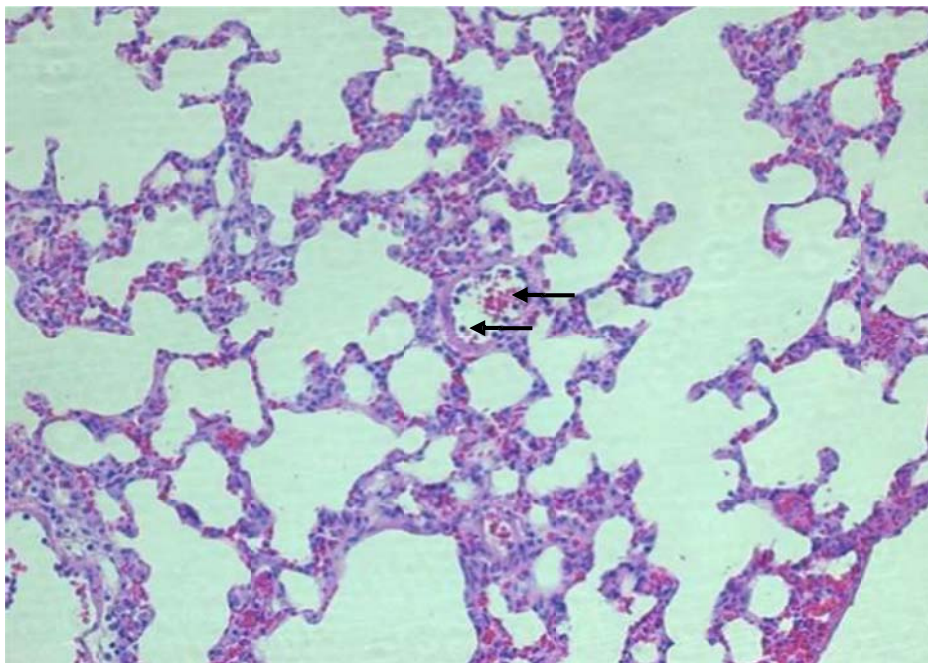
2. 60 X magnification
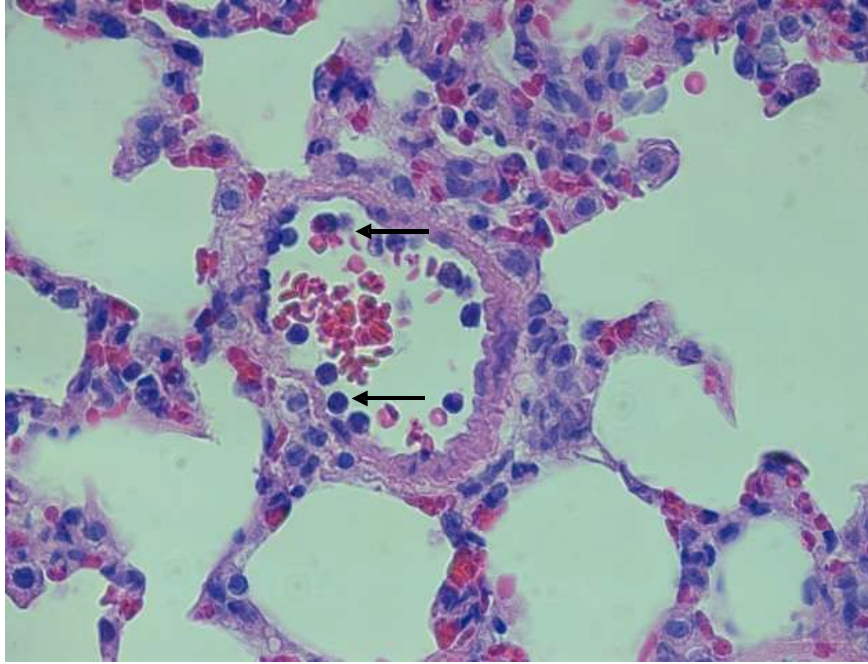
Large number of neutrophil infiltration into all the organs was observed. Rod

shaped bacteria were also confirmed in all the organs.

Figure 33. **Septic shock induced by *E. coli* Bort strain in SD rats: Bacterial load (*E. coli* Bort) in blood 9 h post dosing:**
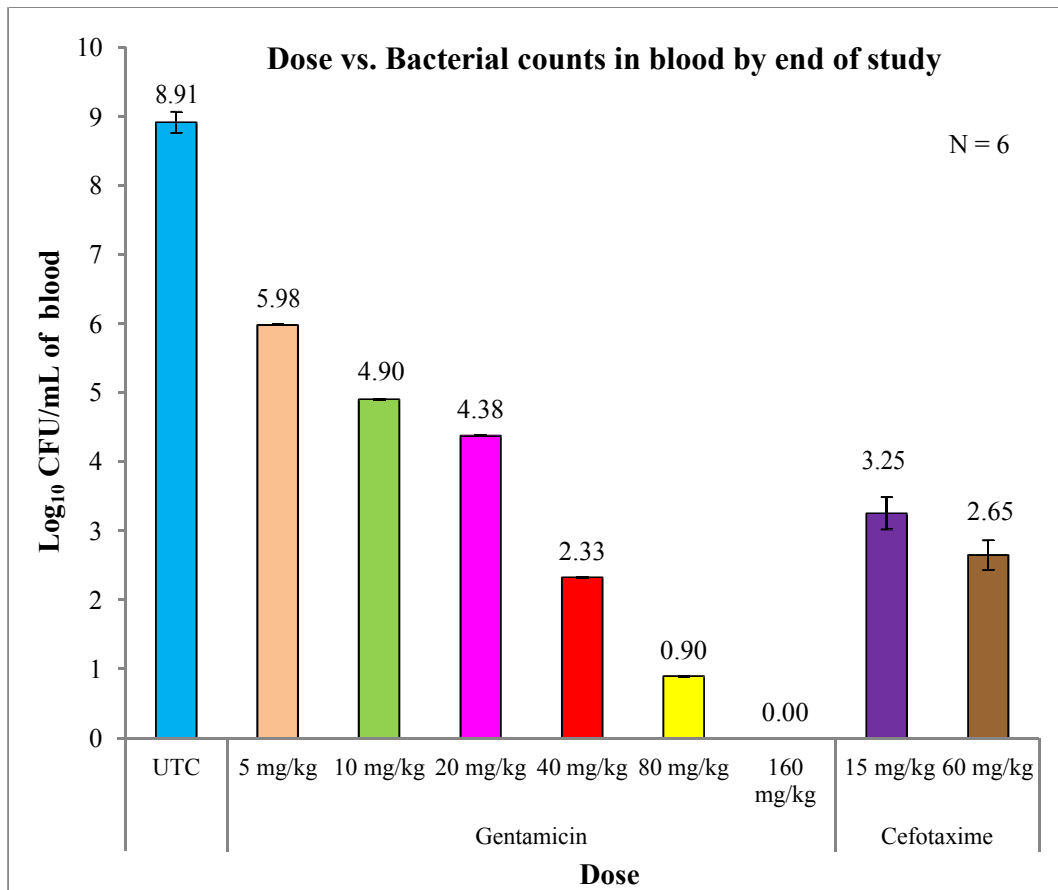
Bacterial load after gentamicin and cefotaxime doses compared to untreated control animals. There was a significant difference between gentamicin treated groups compared to untreated controls (UTC) with a p-value < 0.001 determined by repeated measures ANOVA followed by Dunnettes multiple comparison test. A significant difference in $\log_{10}$ CFU at 9th hour was observed between the treated and untreated animals. Bars represent standard error.

Figure 34. Bacterial load (*E. coli* Bort) in blood at each time point during the experiment:
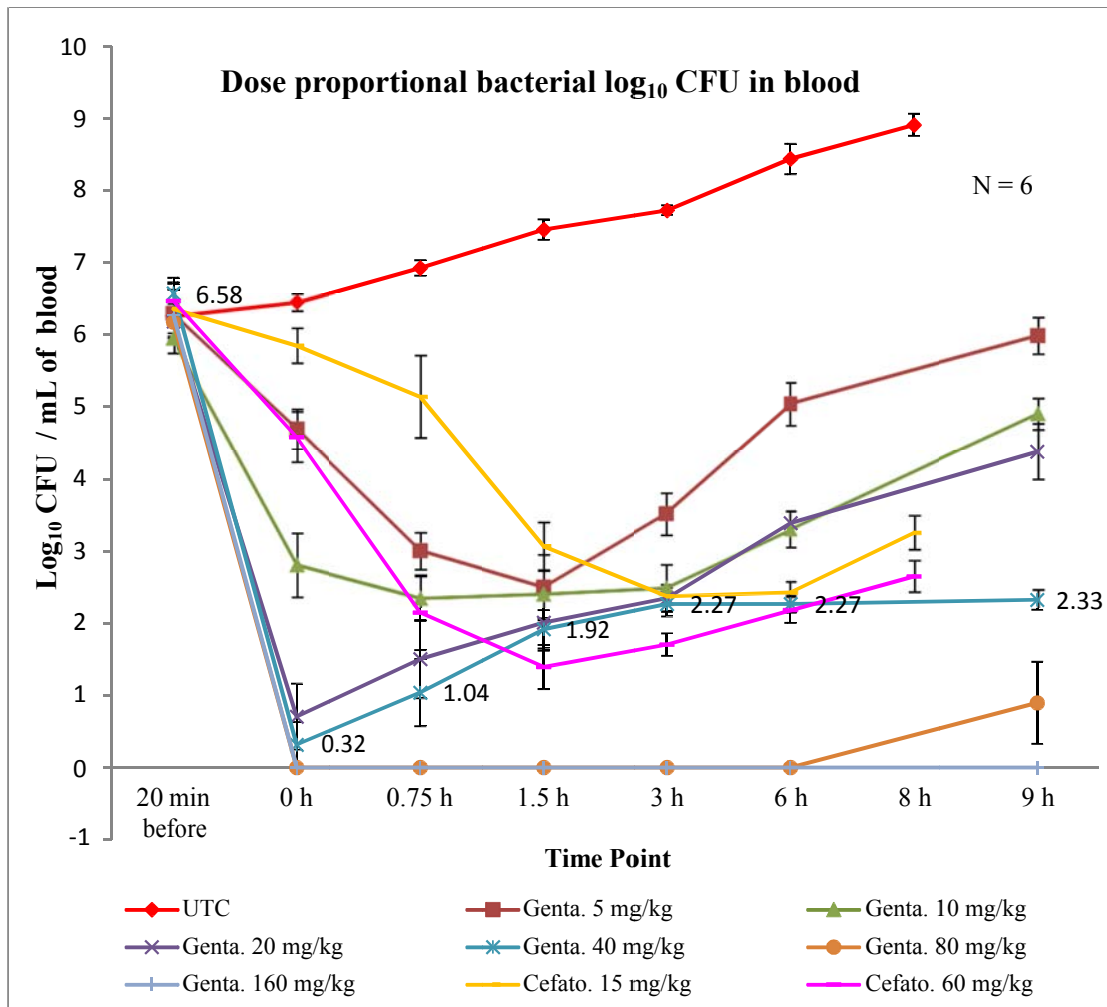
Gentamicin 5 mg/kg: 3 $\log_{10}$ CFU reduction after 45 minutes of dosing.

Gentamicin 10 mg/kg: 3 $\log_{10}$ CFU reduction by 0 h.

Gentamicin 20 and 40 mg/kg: 5.48 and 6.26 $\log_{10}$ CFU reductions by 0 h.

Gentamicin 80 & 160 mg/kg: complete bacterial eradication by 0 h.

There was a significant difference between gentamicin treated groups compared to untreated controls (UTC) (p-value < 0.001)

There was a significant difference between the ultra-high gentamicin dosed groups compared to gentamicin 20 mg/kg dose (p-value < 0.001).

Statistical significance was calculated by repeated measures ANOVA followed by Dunnett's multiple comparison test. Bars represent standard error.

Figure 35. Log₁₀ CFUs from kidneys at the end of the experiment:
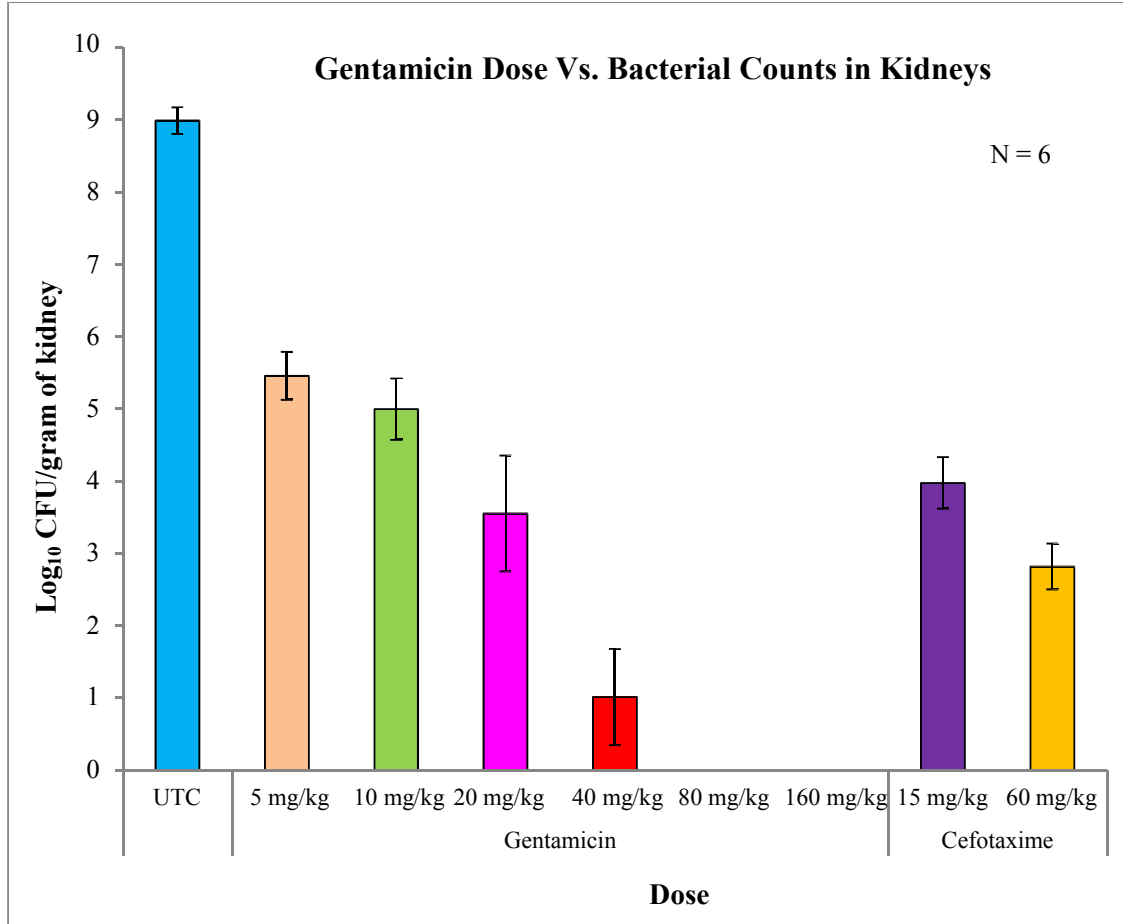
Dose-dependent $\log_{10}$ CFU reduction was observed with gentamicin doses. There was a significant $\log_{10}$ CFU reduction in the gentamicin treated groups compared to untreated control animals. Bacteria were below the detection limits at 80 and 160 mg/kg doses by the end of the experiment. Statistically significant difference was observed between gentamicin treated and untreated controls (UTC) with a p-value < 0.0001 by one-way ANOVA followed by Dunnett's multiple comparison test. There was a statistically significant difference between gentamicin 20 mg/kg compared to ultra-high gentamicin doses with a p-value of < 0.001 by one-way ANOVA followed by Dunnett's multiple comparison test.

Bars represent standard error.

Figure 36. Log₁₀ CFUs from spleen at the end of the experiment:
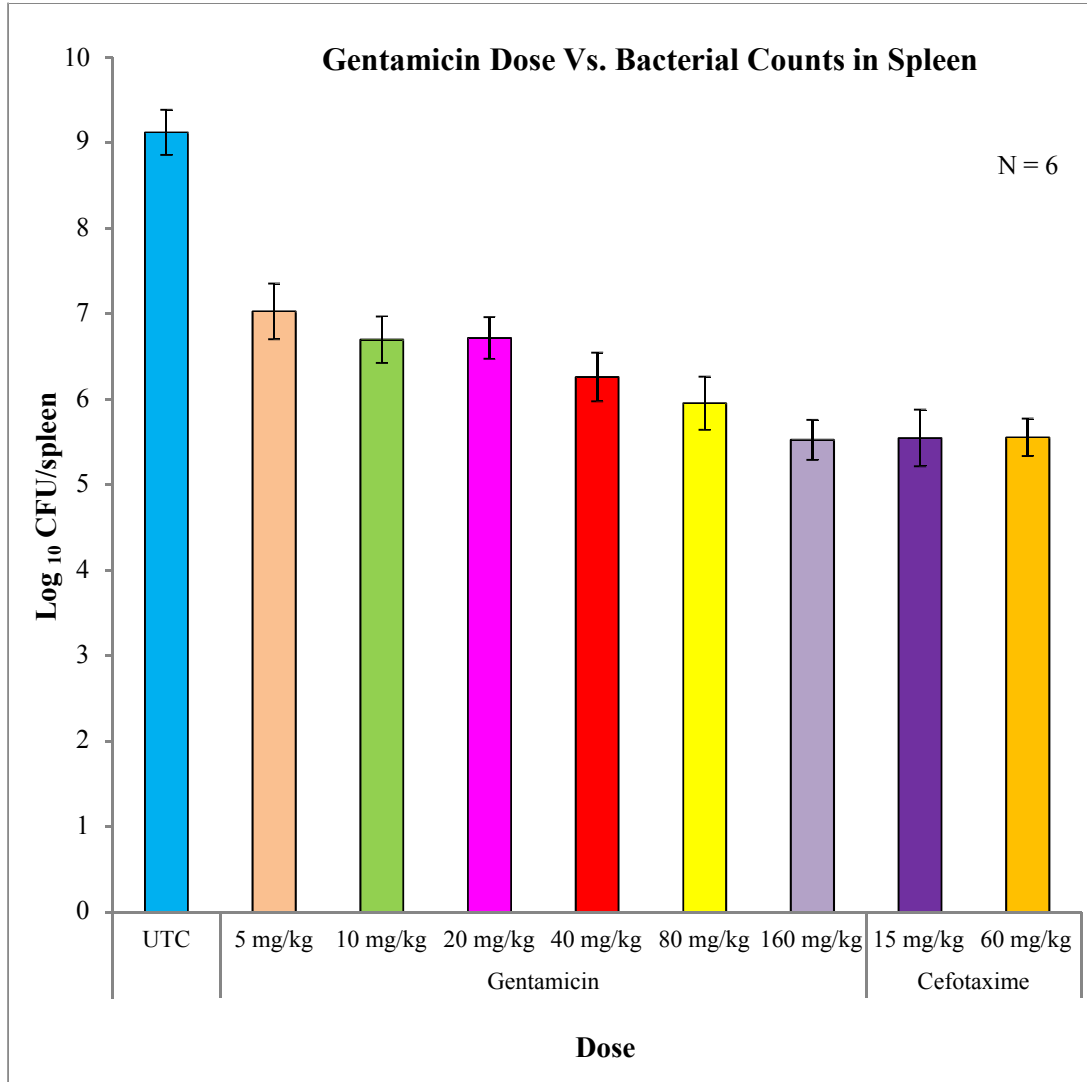
Statistically significant difference was observed between treated and untreated controls (UTC) with a p-value of < 0.0001 by one-way ANOVA followed by Dunnett's multiple comparison test. There was a no statistically significant difference between gentamicin 20 mg/kg compared to 40 and 80 mg/kg doses. Statistically significant difference was observed with a p-value of < 0.01 between gentamicin 20 mg/kg and 160 mg/kg groups.

Bars represent standard error.

Figure 37. Log$_{10}$ CFUs from liver compared to UTC at the end of the experiment:
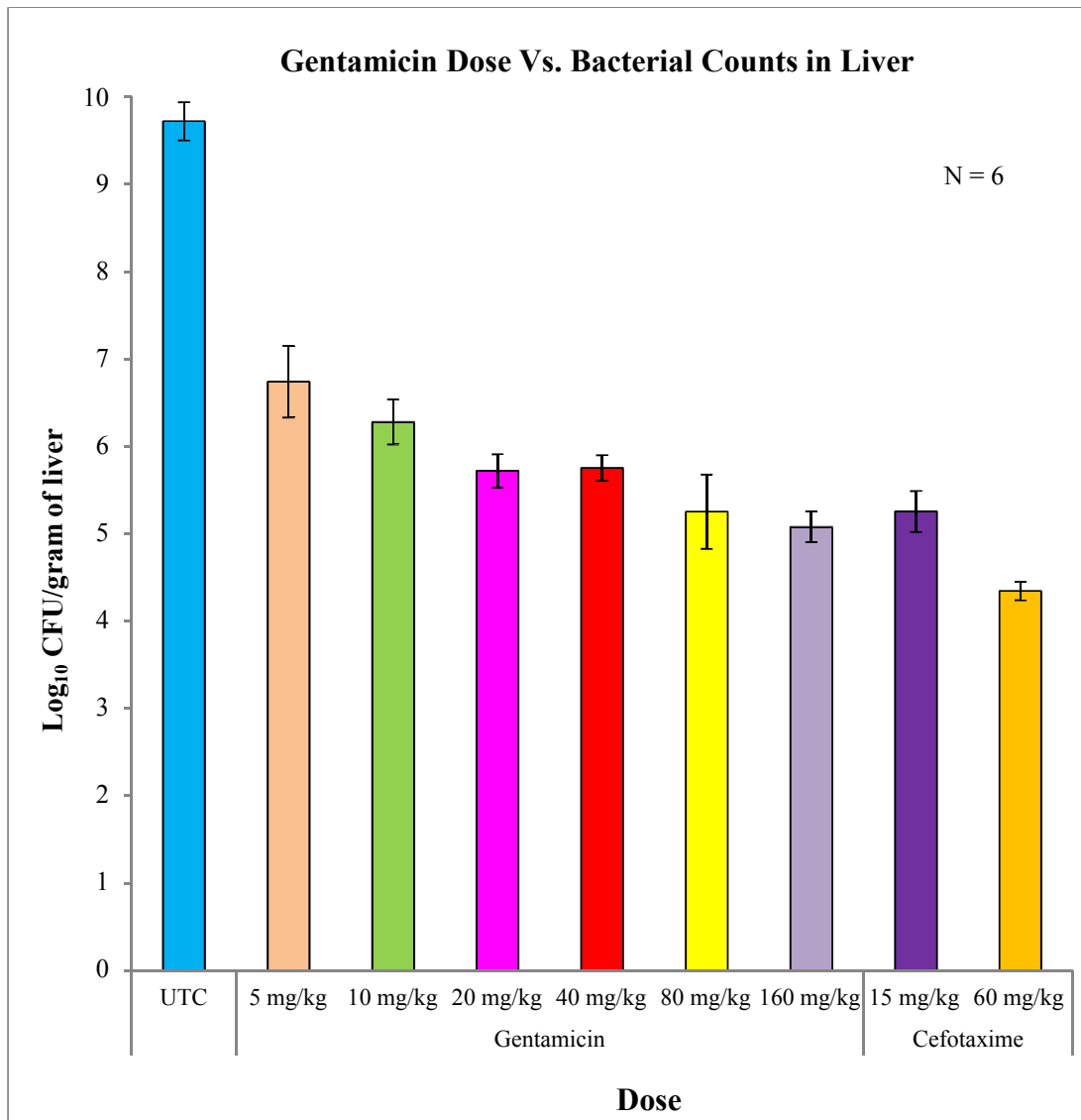
Figure caption: Gentamicin Dose Vs. Bacterial Counts in Liver; y-axis: Log$_{10}$ CFU/gram of liver; x-axis: Dose; N = 6

Statistically significant difference was observed between treated and untreated controls (UTC) with a p-value < 0.01 by one-way ANOVA followed by Dunnett's multiple comparison test. No significant difference was observed between gentamicin 20 mg/kg and ultra-high gentamicin doses.

Bars represent standard error.

Figure 38. Log$_{10}$ CFUs from lungs at the end of the experiment:
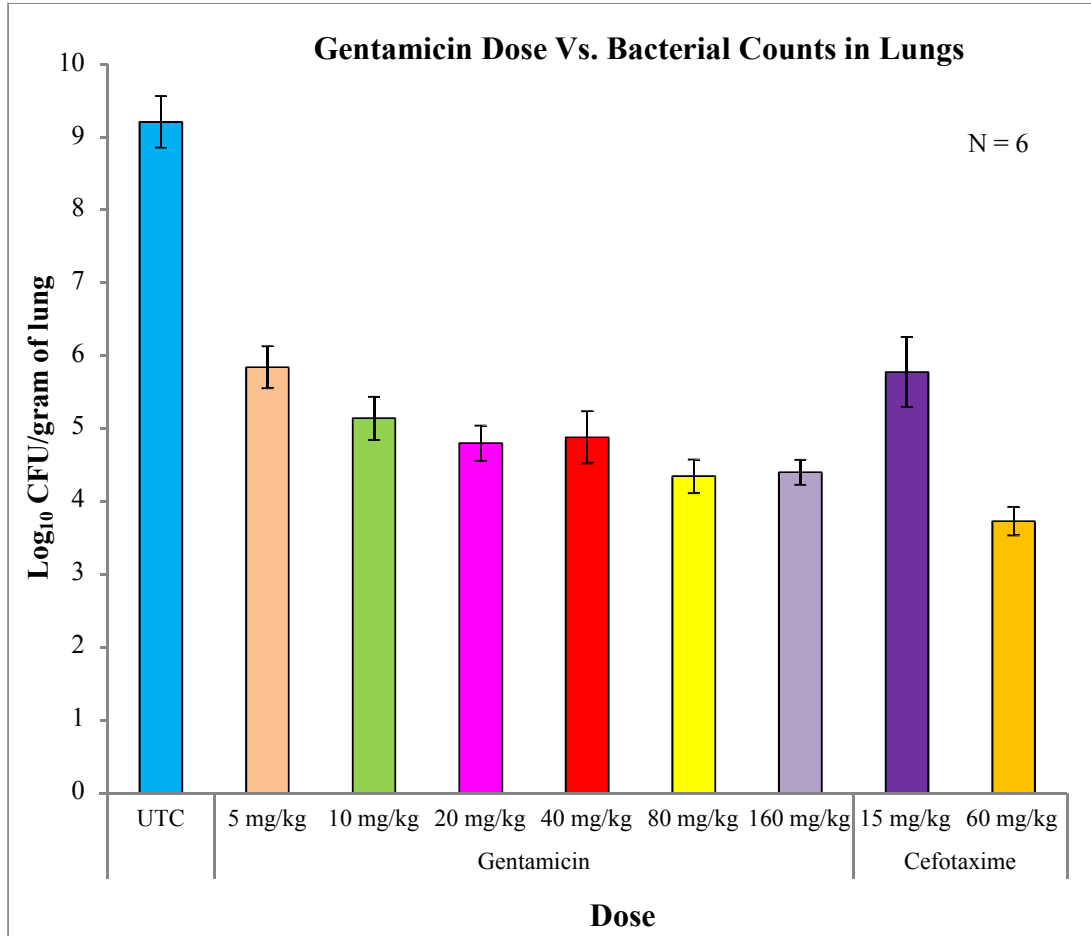
Statistically significant difference was observed between treated and untreated controls (UTC) with a p-value < 0.0001 by one-way ANOVA followed by Dunnett's multiple comparison test. There was no statistically significant difference between gentamicin 20 mg/kg compared to ultra-high gentamicin doses.

Bars represent standard error.

Figure 39.   Log₁₀ CFUs from peritoneal fluid at the end of the experiment:
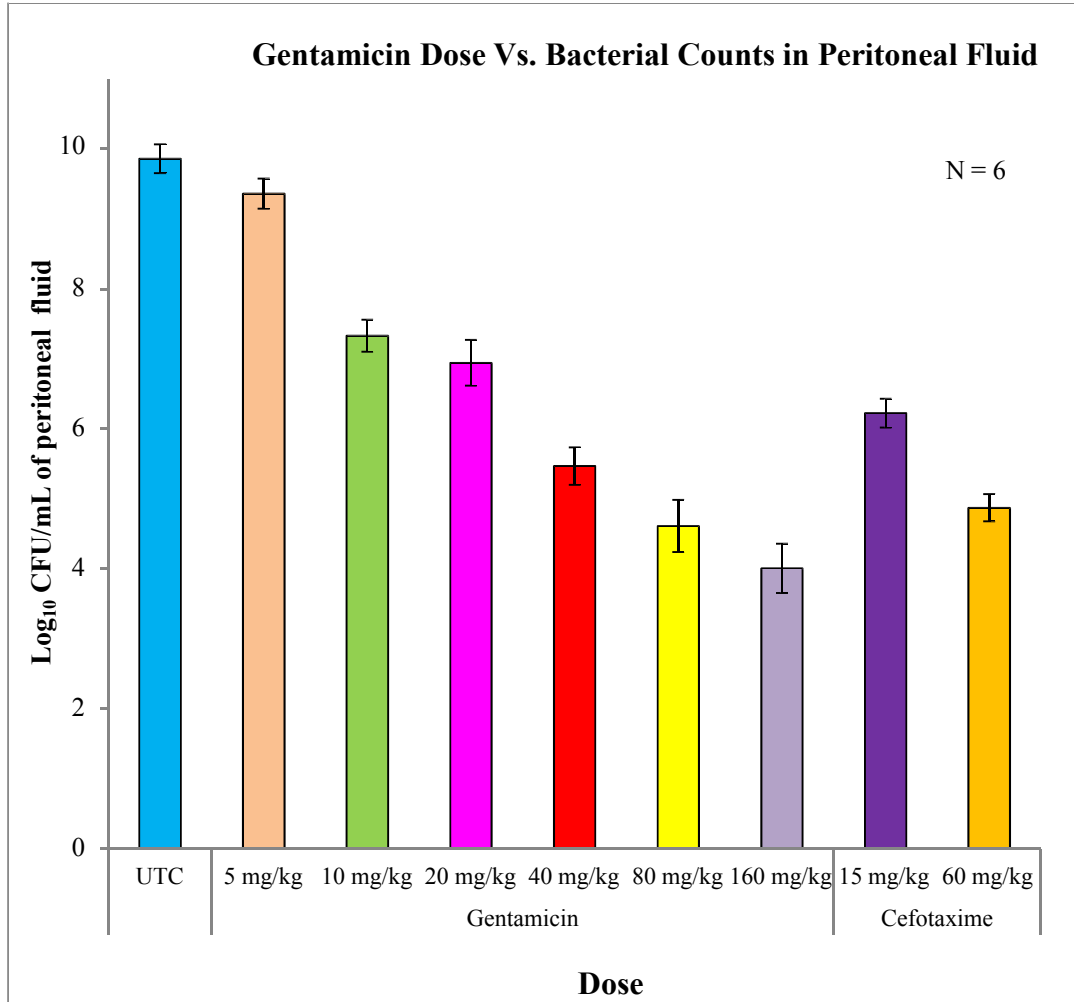
Statistically significant difference between the treated animals compared to an untreated

control group with a p-value of < 0.0001 except for the 5 mg/kg dose.

There was a significant difference between ultra-high gentamicin dosed animals compared

to gentamicin 20 mg/kg dose with a p-value of < 0.0001

Bars represent standard error.

Figure 40. Serum creatinine levels in septic shock induced and treated animals:
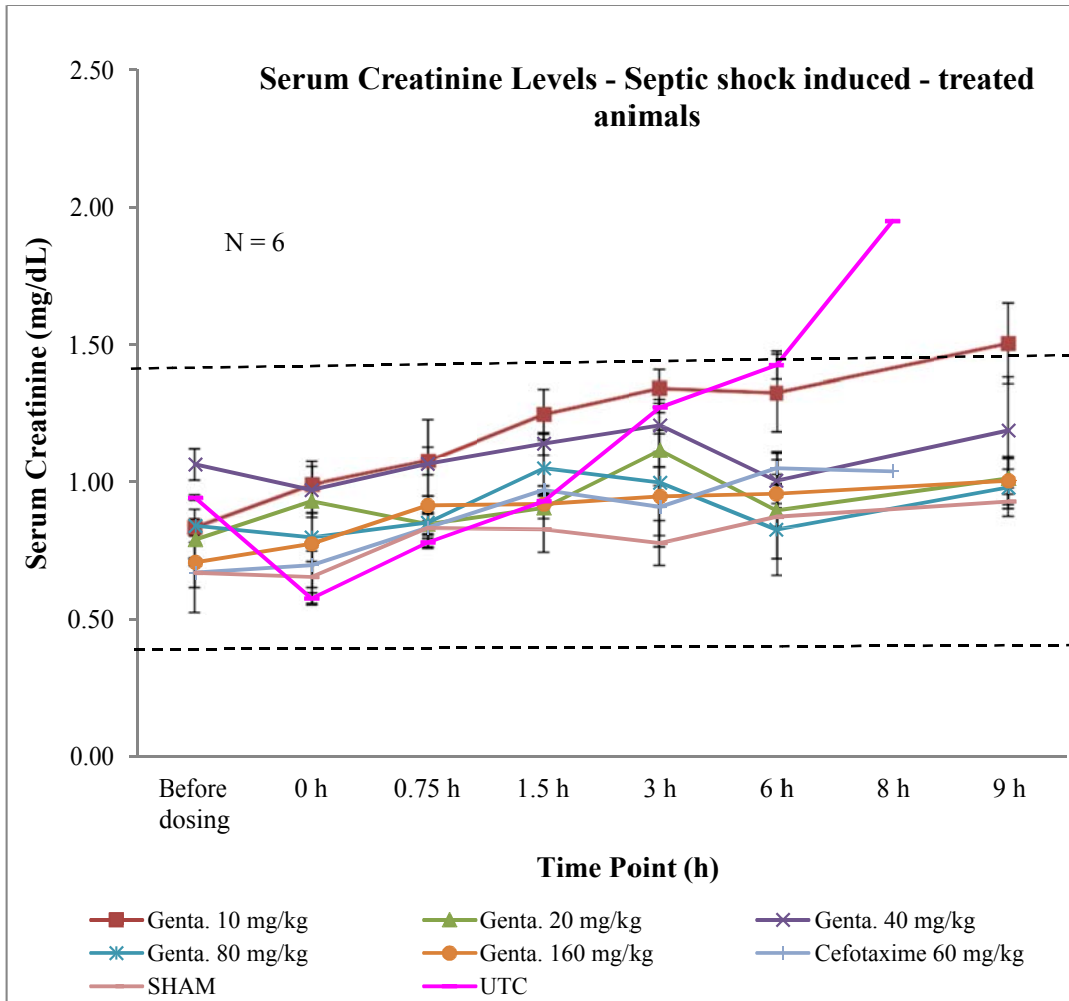
Serum creatinine levels were above the normal limits in UTC and 10 mg/kg dose of gentamicin from 3rd hour onwards. There was a significant difference in the serum creatinine levels between UTC compared to all the treated (except 10 mg/kg dose) and Sham animals with a p-value of < 0.01 determined by repeated measures ANOVA followed by Dunnett's multiple comparison test.

Dotted lines represent the normal range of serum creatinine in animals.

 Bars represent standard error.

Figure 41. Serum lactate levels from treated and untreated animals (N = 2):
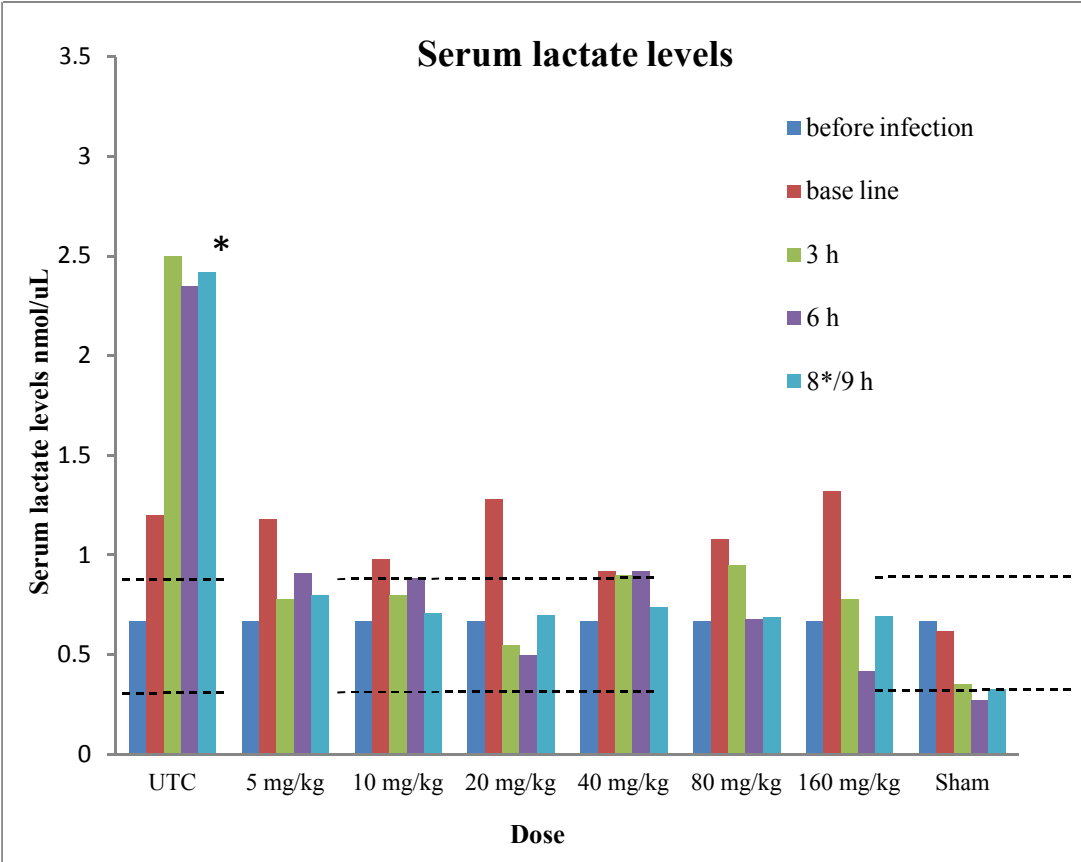
Increase in serum lactate levels was observed in UTC compared to before infection.

Dotted lines represent the normal range of serum lactate in animals.

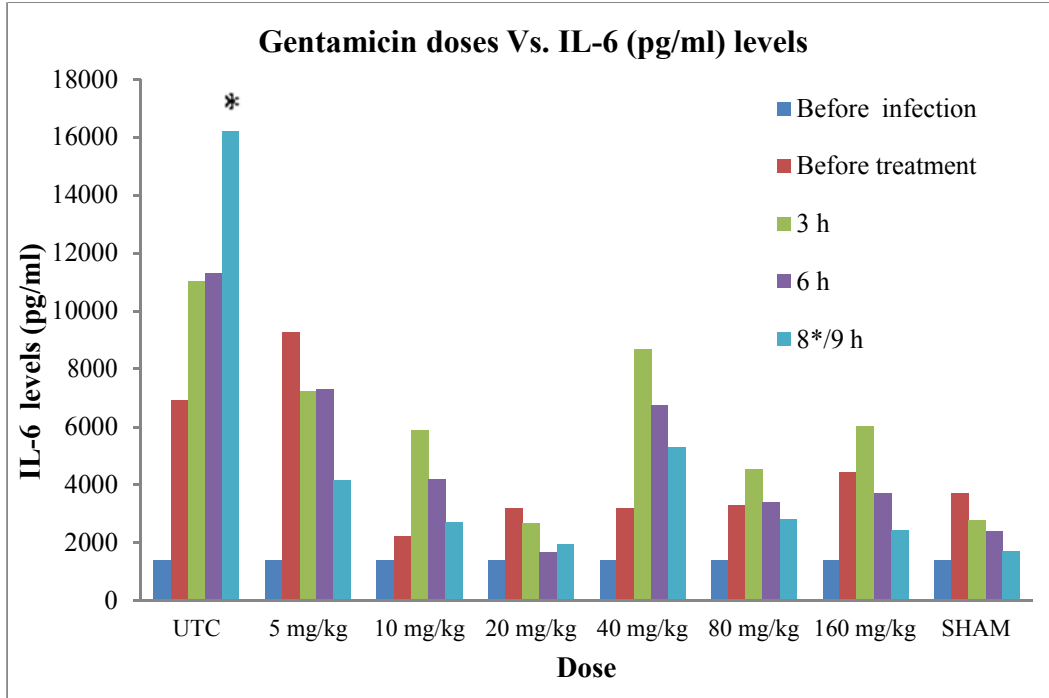**Figure 42. Serum pro-inflammatory cytokine levels in septic shock: with and without antimicrobial therapy:**
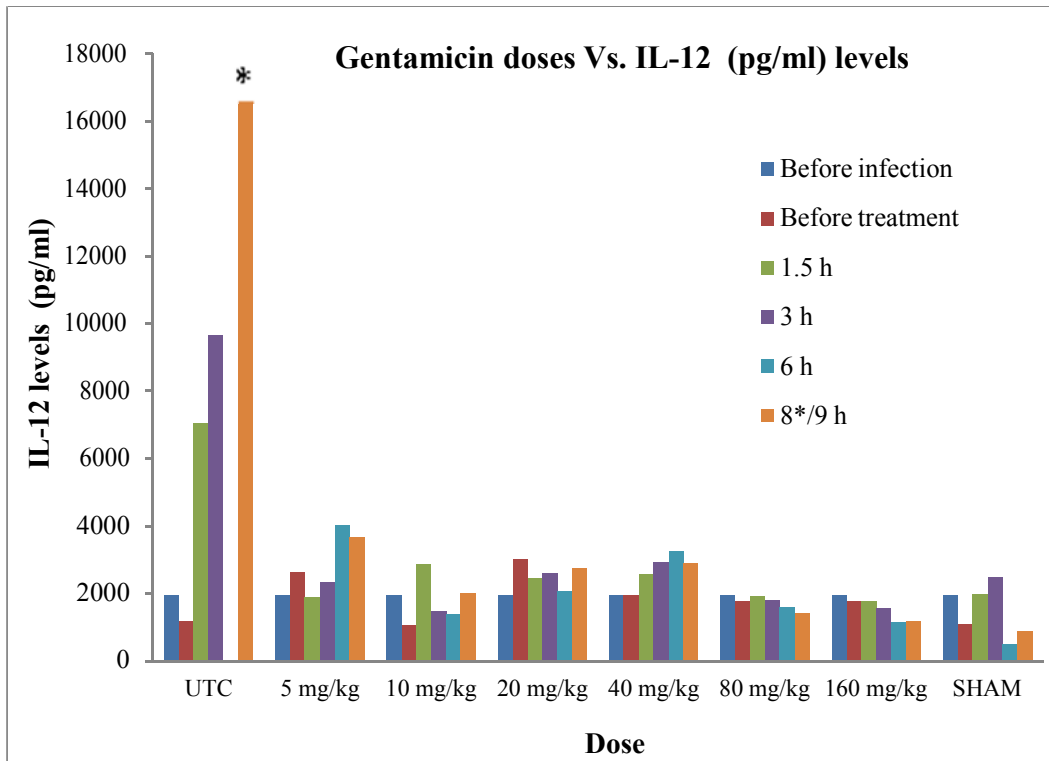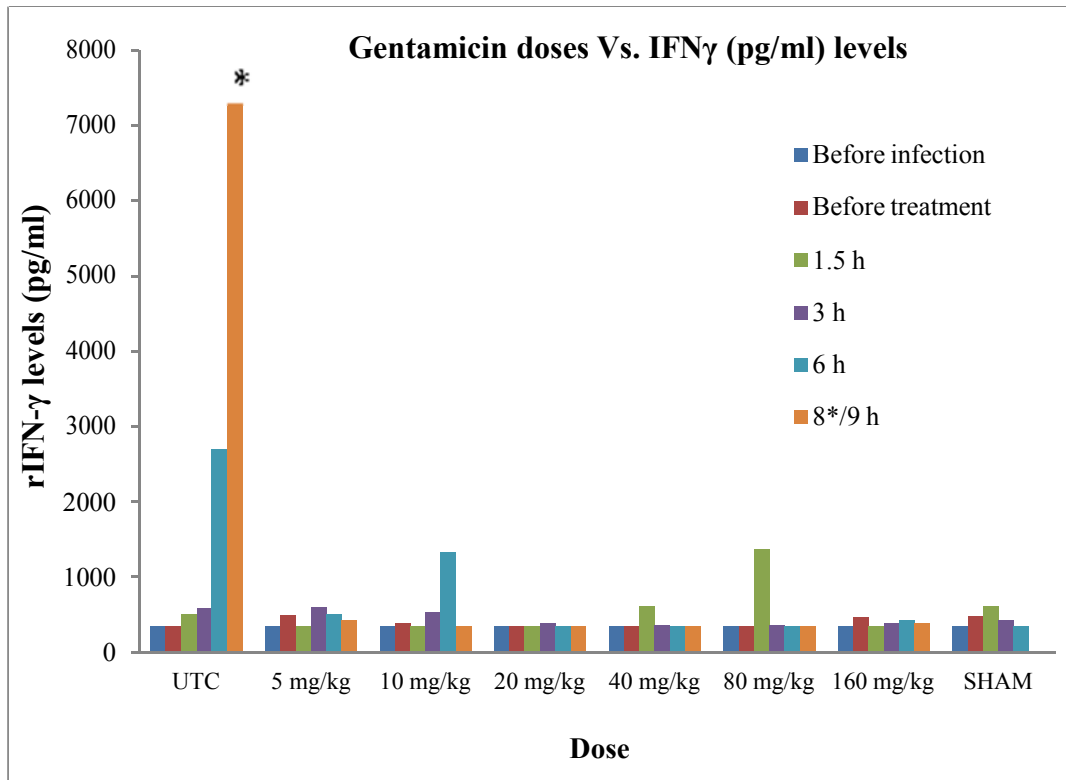
a.   IL-1β levels: (N = 2)
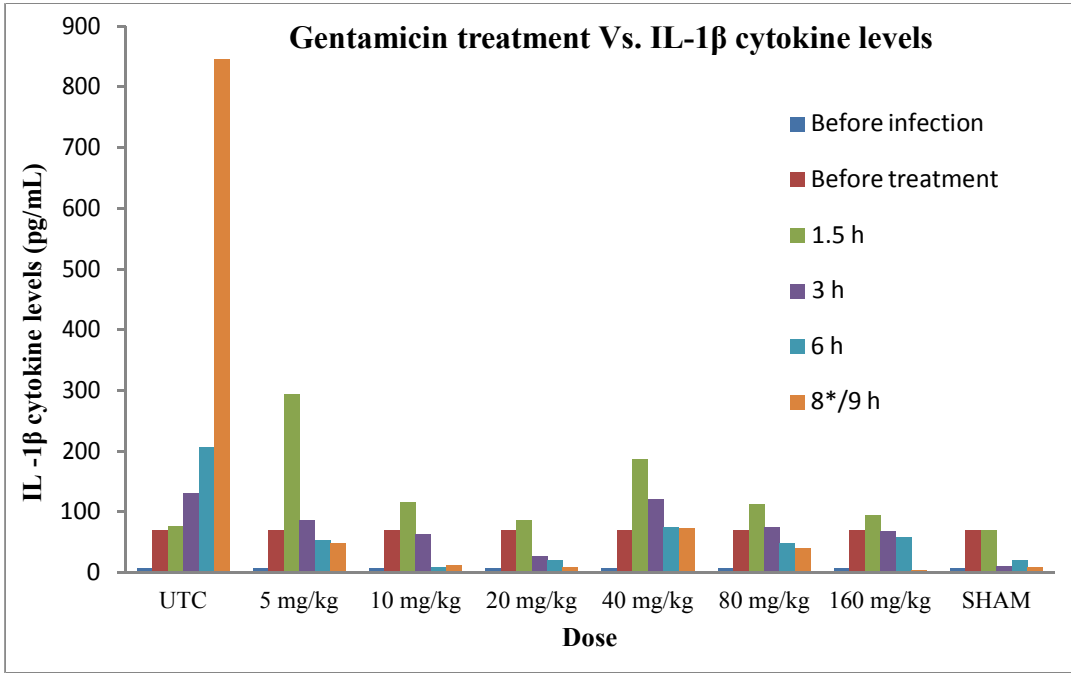
b. TNF-α levels: (N = 2)
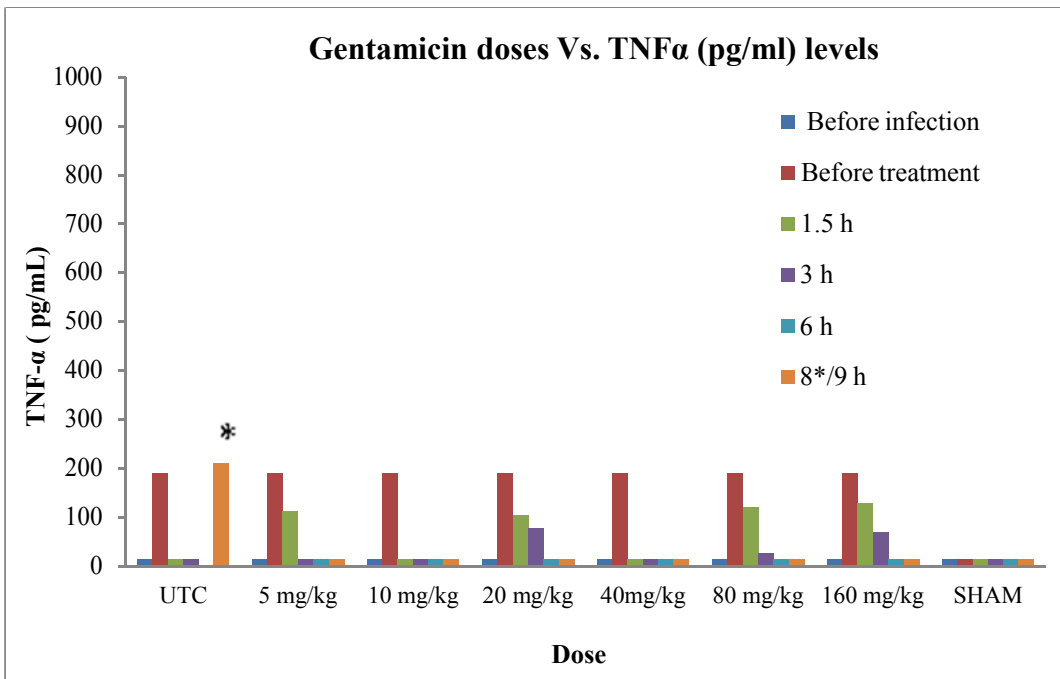
c. IL -6 levels: (N = 2)
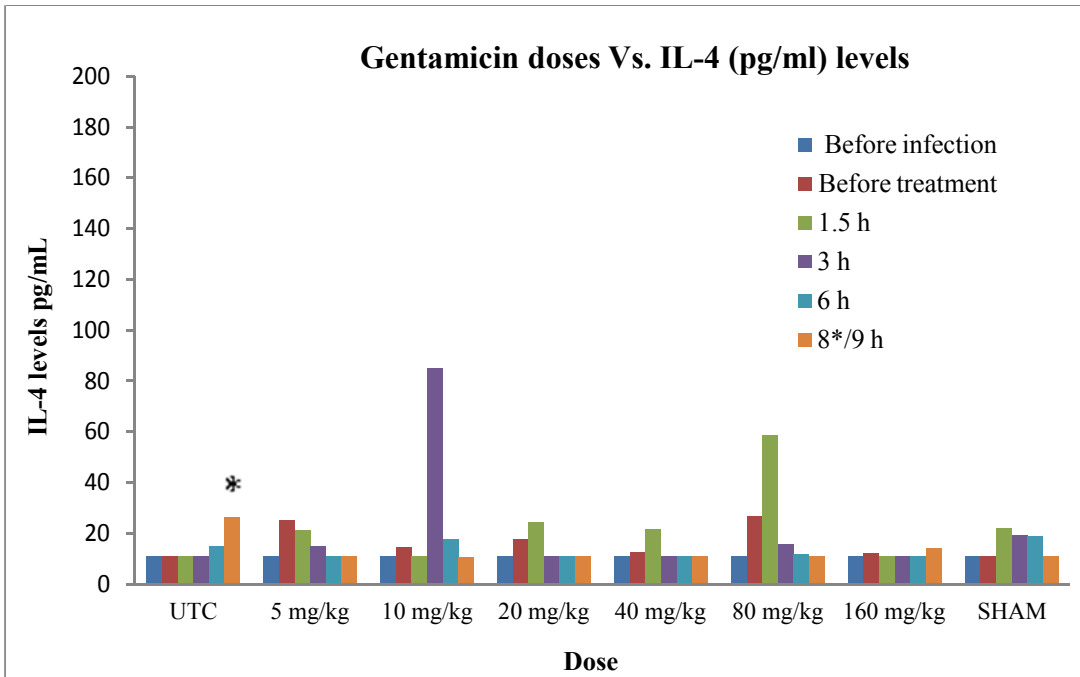

d. IL - 12 levels: (N = 2)
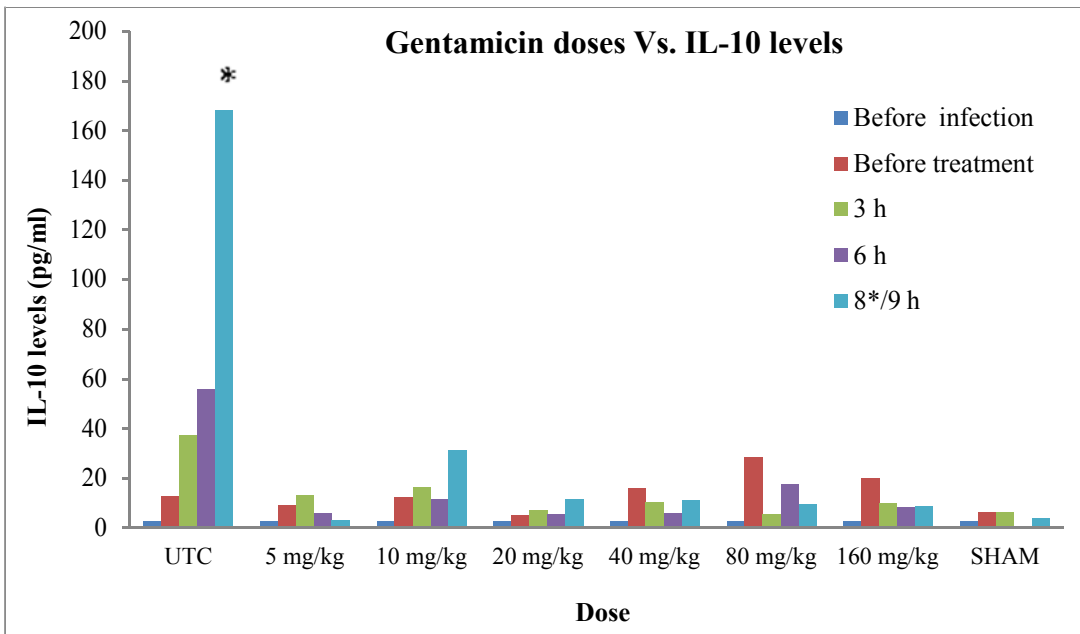
e. IFNγ levels: (N = 2)

a – e: All the pro-inflammatory cytokine levels were found to increase in the UTC. The levels of these cytokines were slightly elevated but came down to lower levels in the gentamicin treated groups.

Figure 43.   Serum anti-inflammatory cytokine levels

a.   IL – 4 levels: (N = 2)

b.   IL – 10 levels: (N = 2)

Increase in anti-inflammatory cytokine levels in UTC compared to treated animals.