2014

THERAPEUTIC EFFICACY OF CERIUM OXIDE NANOPARTICLES AGAINST SEPSIS INDUCED MULTI-ORGAN DYSFUNCTION SYNDROME IN SPRAGUE DAWLEY RATS

Nandini Durga Prasanna Kumar Manne
manne@marshall.edu

THERAPEUTIC EFFICACY OF CERIUM OXIDE NANOPARTICLES AGAINST SEPSIS INDUCED MULTI-ORGAN DYSFUNCTION SYNDROME IN SPRAGUE DAWLEY RATS

A Dissertation submitted to the Graduate College of

Marshall University

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Biomedical Sciences

by

Nandini Durga Prasanna Kumar Manne, BVSc & A.H, M.S.

Approved by

Dr. Eric R. Blough, Ph.D., Committee Chairperson

Dr. Todd L. Green, Ph.D.

Dr. Monica A. Valentovic, Ph.D.

Dr. Elsa I. Mangiarua, Ph.D.

Dr. Robert O. Harris, Ph.D.

Department of Pharmacology, Physiology and Toxicology

Joan C. Edwards School of Medicine

Marshall University, Huntington, WV

August 2014

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor Dr. Eric R. Blough for his invaluable guidance and support in my research project. No words are big enough to express my gratitude for all the patience he took in guiding me until this step. I am really fortunate to have such a wonderful mentor and guide who always gave me freedom to pursue my own ideas and I am deeply indebted to him for spending a part of his life towards my Ph.D. degree. His valuable guidance at the time where I thought it was all over helped me push forward and reach this stage. Dr. Blough made my life at Marshall University very easy without whom it would have been very long difficult years. He helped me not only in my academic career but also guided me towards the path of being a good person.

I would like to thank my committee members Drs. Todd Green, Monica Valentovic, Elsa Mangiarua and Robert Harris for their fine constructive ideas and excellent guidance. I am deeply grateful to them for all the support they have given me. Their valuable suggestions at many critical junctures of my work helped me reach this step. I would also like to thank Drs. Gary Rankin, Richard Egleton, Nalini Santanam and Travis Salisbury for their excellent teaching without which I could not have reached this stage.

Although words are not enough, I would like to express my heartfelt thanks to my parents Prasad Babu and Bhavani for providing me with a wonderful life. Their continuous support and help made me achieve my dreams of obtaining a higher degree in United States. I would also like to express my gratitude to my sister Deepthi and my brother-in-law Sharath Babu for their continuous encouragement and moral support.

I would like to extend my heartfelt thanks to a special person, Ravikumar Arvapalli who helped me during all the stages of my project. His valuable technical assistance helped me learn many things in science. He was always there for me in times of need both in academic and personal career. I would also like to thank Siva Nalabotu without whom I would have not reached this stage. I would also like to thank Kevin Rice for his valuable suggestions in different experiments. It is through constant discussions with him that helped me frame appropriate experiments in my project.

I would also like to thank my lab members Sreenivas Thulluri, Geeta Nandyala, Niraj Nepal, Madhukar Kolli, Sunil Kakarla, Sudarsanam Kundla, Katta Anjiah, Satyanarayana Paturi, Radhakrishna Para, Hari Addagarla, Sravanthi Bodapati, Miaozong Wu, Asano Shinichi, Selvaraj Vellaisamy, Steven Rogers, Venkata Vinay, Bing Ma, Erin Fankenhanel and Melanie Butt for their help all throughout these years. I would not have completed my work without their constant help. I would also like to thanks Drs. Tolou Shokuhfar, Yu Zhao and Kun He for their valuable technical help.

Finally I would like to Marshall University BMS program for giving me the opportunity to earn a doctoral degree and pursue my career in scientific research. I would also like to thank all my friends at Marshall University who made me feel like at home.

DEDICATION

I would like to dedicate my work to my parents Manne Prasad Babu and Manne Bhavani for

their love and endless support in making my dreams come true.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

$\bullet O_2^-$	Superoxide anions
$\bullet OH$	Hydroxyl radicals
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AGE	Advanced glycation end-product
AKI	Acute kidney injury
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine transaminase
APP	Acute phase proteins
AST	Aspartate transaminase
ANOVA	One way analysis of variance on ranks
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
Ca^+	Calcium
CARS	Compensatory anti-inflammatory response syndrome
CeO_2	Cerium oxide
EELS	Electron energy loss spectroscopy
EDS	Energy-dispersive X-ray spectroscopy
EpCAM	Epithelial cell adhesion molecule
ELISA	Enzyme linked immunosorbant assay
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glob	Globulin
H_2O_2	Hydrogen peroxide
HMGB1	High mobility group box protein 1
ICAM-1	Intercellular adhesion molecule-1
ICP-MS	Inductively coupled plasma-mass spectrometry
IL	Interleukin
IRAK-4	IL-1 receptor-associated kinase-4
i.p.	Intra peritoneal
i.v.	Intravenous
JNK	Jun-N-terminal kinase
K^+	Potassium
KIM-1	Kidney injury molecule 1
KRB	Krebs-Ringers Buffer Solution
LIF	Leukemia inhibitory factor
LLOQ	Lower limit of quantitation
LPS	Lipopolysaccharide
LPS-BP	Lipopolysaccharide binding protein
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MDC	Macrophage derived chemokine
MIP	Macrophage inflammatory protein
MODS	Multi-organ dysfunction syndrome
Na^+	Sodium

NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL	Neutrophil gelatinase-associated lipocalin
NSAID	Non-steroidal anti-inflammatory drug
PAMP's	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.5% tween
PKA	Protein kinase A
PMN	Polymorphonuclear
PPR's	Pattern-recognition receptors
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SOCS3	Suppressor of cytokine signaling protein-3
SOD	Superoxide dismutase
SIRS	Systemic inflammatory response syndrome
STAT-3	Signal transducer and activator of transcription-3
STEM	Scanning transmission electron microscope
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.5% tween
TEM	Transmission electron microscope
TIRAP	Toll-Interleukin 1 receptor domain containing adaptor protein
TIMP	Tissue inhibitor of matrix metalloproteinase

TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TP	Total protein
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WBC	White blood cells
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

ABSTRACT

THERAPEUTIC EFFICACY OF CERIUM OXIDE NANOPARTICLES AGAINST SEPSIS INDUCED

MULTI-ORGAN DYSFUNCTION SYNDROME AND DEATH IN SPRAGUE DAWLEY RATS

Sepsis is a generalized term that signifies the presence of a pathogen in the blood stream to which the body responds by eliciting a systemic inflammatory response. Although sepsis is the leading cause of death in non-coronary intensive care units in United States, there are currently no FDA approved therapeutic drugs to treat this disorder. Cerium oxide nanoparticles (CeO_2) have been shown to exhibit anti-oxidant, anti-inflammatory, and anti-bacterial properties both *in vitro* and *in vivo.* Whether CeO_2 nanoparticles can be used for the treatment of sepsis is currently unclear.

To investigate whether CeO_2 nanoparticles can be used to treat moderate sepsis, twelve week old male Sprague Dawley rats were randomly divided into one of four different groups: control, CeO_2 only, sepsis, and sepsis + CeO_2. Moderate sepsis was induced by the intraperitoneal injection of cecal material (400mg/kg). The CeO_2 nanoparticle treated animals received an intravenous injection of CeO_2 nanoparticles (3.5mg/kg) at the time of sepsis induction. Treatment significantly decreased sepsis induced mortality. Treatment associated increases in animal survivability were associated with a significant decrease in serum IL-6, growth regulated alpha protein (KC/GRO), macrophage inflammatory protein-1 beta (MIP-1 β) macrophage derived chemokine (MDC), monocyte chemotactic protein-3 (MCP-3), myoglobin, macrophage inflammatory protein-3 beta (MIP-3β), eotaxin, leptin, macrophage inflammatory protein-2 (MIP-2), interferon gamma induced protein-10 (IP-10), tissue inhibitor of

metalloproteinases-1 (TIMP-1), plasminogen activator inhibitor-1 (PAI-1) and blood urea nitrogen (BUN).

In an effort to further investigate whether there is a similar degree of response with smaller size and dose of CeO_2 nanoparticles on the attenuation of severe sepsis, twelve week old male Sprague Dawley rats were randomly divided into one of four groups: control, CeO_2 only, sepsis and sepsis + CeO_2. Severe sepsis was induced by the intraperitoneal injection of cecal material (600mg/kg). The CeO_2 nanoparticle treated animals received an intravenous injection of CeO_2 nanoparticles (0.5mg/kg) at the time of sepsis induction. Treatment with CeO_2 nanoparticles significantly decreased animal mortality and sepsis-induced hypothermia. Treatment associated increases in animal survivability were associated with evidence of diminished Erk 1/2 phosphorylation, Jak/Stat-3 activation, P-selectin levels and expression of vascular cell adhesion molecule-1 (VCAM-1) in the heart. Changes in cardiac signaling appeared to coincide with decreased serum IL-6, leukemia inhibitory factor, myoglobin and creatine kinase. In the liver, CeO_2 nanoparticle treatment reduced sepsis-induced increases in hepatic superoxide levels, inducible nitric oxide synthase, and protein nitrosylation. Treatment associated increases in liver function were accompanied by diminished levels of serum reactive oxygen species (ROS) and several inflammatory markers.

CeO_2 nanoparticles were also found to attenuate sepsis-induced renal damage by preserving the renal brush border and attenuating the incidence of tubular dilatation. These changes in kidney morphology were accompanied by decreases in levels of serum β-2 microglobulin, kidney injury molecule-1, cystatin-C, osteopontin, BUN, glucose, sodium, and potassium. At the cellular level, CeO_2 nanoparticles attenuated the sepsis-induced activation of

Stat-3 and the cleavage of caspase-3. Taken together the data from this current study indicate that CeO$_2$ nanoparticles can be used for the prevention of sepsis induced multi-organ dysfunction syndrome and death. Additional studies using other sepsis models or interventional time points may be warranted to determine the potential efficacy of using CeO$_2$ nanoparticles to treat sepsis.

<center>**Chapter 1**</center>

Introduction

Sepsis is a pathological condition characterized by systemic inflammatory response syndrome (SIRS) that is triggered by host immune system as a countermeasure to an infectious agent in the blood [1]. Severe sepsis though curable when diagnosed early, can rapidly progress to a medical emergency, multi-organ dysfunction and death if not treated properly. Sepsis is not new. The term sepsis was first used by Hippocrates in the late 4th century BC to describe a process by which flesh rotted and lead to a foul smell [2]. In the late 1800's Louis Pasteur discovered the link between microscopic organisms and infectious disease. Later on, Lister, Lennhartz, Asbough and others demonstrated that sepsis does not necessarily develop due to the presence of an infectious agent, but rather that it is the interaction between the host immune system and the agent that initiates the onset. As we have learned more about this disorder, the previously held notion of sepsis being primarily a disease of bacterial etiology has begun to expand to also incorporate the complex multi-factorial interaction of the host immune system.

Sepsis is the leading cause of death in non-coronary intensive care unit and ranks among the top ten mortality causing diseases in United States [3]. It is estimated that sepsis is responsible for ~60,000 deaths annually [4]. The costs for treating sepsis in 2008 alone were almost $15 billion and are expected to increase by >10% in each passing year [5]. Clearly new and better methods to treat this disorder are needed.

Sepsis is the systemic reaction to an over-activated immune system

<center>**Chapter 1**</center>

Introduction

Sepsis is a pathological condition characterized by systemic inflammatory response syndrome (SIRS) that is triggered by host immune system as a countermeasure to an infectious agent in the blood [1]. Severe sepsis though curable when diagnosed early, can rapidly progress to a medical emergency, multi-organ dysfunction and death if not treated properly. Sepsis is not new. The term sepsis was first used by Hippocrates in the late 4th century BC to describe a process by which flesh rotted and lead to a foul smell [2]. In the late 1800's Louis Pasteur discovered the link between microscopic organisms and infectious disease. Later on, Lister, Lennhartz, Asbough and others demonstrated that sepsis does not necessarily develop due to the presence of an infectious agent, but rather that it is the interaction between the host immune system and the agent that initiates the onset. As we have learned more about this disorder, the previously held notion of sepsis being primarily a disease of bacterial etiology has begun to expand to also incorporate the complex multi-factorial interaction of the host immune system.

Sepsis is the leading cause of death in non-coronary intensive care unit and ranks among the top ten mortality causing diseases in United States [3]. It is estimated that sepsis is responsible for ~60,000 deaths annually [4]. The costs for treating sepsis in 2008 alone were almost $15 billion and are expected to increase by >10% in each passing year [5]. Clearly new and better methods to treat this disorder are needed.

Sepsis is the systemic reaction to an over-activated immune system

Sepsis usually arises as a secondary medical condition to an already existing localized infection of lungs, liver, kidney, or some other internal structure. When the localized infection is left untreated, it can progress to the circulation and become systemic. A systemic infection occurs when the blood becomes contaminated with bacteria. Once this occurs, the immune system is set on high alert and may become over-activated which can result in the development of the systemic inflammatory response syndrome (SIRS). If not properly managed, SIRS can rapidly progress to sepsis, a state of multi-organ failure, sepsis shock and death. The molecular events which govern the transition from infection to SIRS, sepsis, and death are not yet understood although it is thought that the innate immune system may play a role.

Upon invasion of the pathogens into the blood stream, host immune cells such as the circulating macrophages and neutrophils recognize the pathogen associated molecular patterns (PAMP's) that are present on the cell surface which elicit the release of several inflammatory cytokines [6]. If this initial response overwhelms the invader, recovery usually ensues and there are no further problems. However, if the initial response is not sufficient, the immune system becomes further activated which can lead to the development of SIRS and sepsis.

The inflammatory cascade is characterized by the early release of tumor necrosis factor –alpha and IL-1β that are thought to mediate the release of other pro-inflammatory cytokines including IL-6, IL-12, HMGB1 and prostaglandins [7]. In addition to these inflammatory mediators, the potent vasodilator nitric oxide is also released into circulation which can cause severe hypotension [8]. Decreases in pressure can lead to diminished blood flow velocity, which facilitates the interaction and binding of circulating macrophages and lymphocytes to endothelial selectins and extravasation into the extracellular matrix [9]. Increased extravasation

in turn, can lead to tissue edema and impaired tissue oxygenation, tissue hypoxia and organ dysfunction.

The early and accurate diagnosis of sepsis is a goal that has yet to be met. Sepsis diagnosis is difficult due to the presence of several non-specific symptoms such as fever, rashes, anorexia and weakness that make it difficult to differentiate from diseases with similar symptoms until it is too late. The current first line treatment for sepsis is antibiotics but the effectiveness of these agents is often hindered by the presence of inflammatory cytokines. As such there is an immediate need for the development of new and better treatment modalities.

Cerium oxide has a fluorite lattice structure with vacant electrons in its outermost orbital shells [10] which permits the cycling of ceria between its fully oxidized (Ce^{+4}) and reduced (Ce^{+3}) states [11]. Although cerium oxide is currently used in the manufacture of sun screens, polishing agents and as a fuel cell catalyst [12-14], recent studies have suggested that this molecule could also be utilized for biomedical applications. For example, studies have shown that CeO_2 nanoparticles can protect cardiac progenitor cells from ROS induced oxidative stress [15] and normal human breast cells from radiation-induced apoptotic cell death *invitro* *[16]*. Similarly, others have shown that CeO_2 nanoparticles can confer protection against carbon tetrachloride-induced oxidative stress in mice [17]. It has been also been shown that CeO_2 nanoparticles can act as an anti-bacterial agent by impairing the membrane integrity of *E. coli* [18, 19]. Whether CeO_2 nanoparticles can help to prevent sepsis induced multi-organ dysfunction and death has to our knowledge, not been investigated.

Specific aims

Severe sepsis is characterized by a systemic inflammatory response that involves complex interactions between the host immune system and the pathological agent that usually results in a profound level of cellular damage to the host. Current treatment modalities are, unfortunately, not beneficial during the advanced stages of sepsis. Recent studies *in vitro* and *in vivo* have shown that CeO_2 nanoparticles possess anti-bacterial, anti-inflammatory and anti-oxidant properties [20-22]. Whether CeO_2 nanoparticles can be used to prevent sepsis induced multi organ damage is not yet known.

The central focus of this current study is to determine whether CeO_2 nanoparticles can attenuate sepsis induced organ damage and death in the laboratory rat. We hypothesize that CeO_2 nanoparticle treatment will be associated with diminished sepsis-induced organ damage, which will result in improved animal survivability.   This hypothesis will be tested by pursuing the following three specific aims:

Specific Aim I: To determine whether CeO_2 nanoparticles can attenuate the systemic inflammatory response syndrome during septic insult in male Sprague Dawley rats.

Specific Aim II: To investigate if CeO_2 nanoparticles can attenuate severe sepsis-induced inflammatory damage to the heart and liver during a septic insult in male Sprague Dawley rats.

Specific Aim III: To determine whether CeO_2 nanoparticles can attenuate severe sepsis-induced inflammatory damage to the kidney during a septic insult in male Sprague Dawley rats.

Chapter 2

2.1 Review of literature

A review of the pertinent literature concerning the present study will be presented in this chapter. The following areas will be addressed: 1. Etiology and epidemiology of sepsis, 2. Animal models of sepsis, 3. Pathophysiology of sepsis, and 4. Use of CeO_2 nanoparticles for biomedical application.

2.2 Sepsis and systemic inflammatory response syndrome

Sepsis is characterized by a state of uncontrolled immune response to the presence of an infectious agent or their toxic metabolites [23]. Sepsis usually begins with the entry of pathogens or its toxins into the body. Upon entry, the host immune cells attempt to eliminate the infectious agent by direct ingestion and destruction or by the secretion of antibodies that bind to the pathogens making them targets to other host immune cells. During this process, the cells of immune system also release cytokines that act in autocrine or paracrine fashion to stimulate other cells. If the infection is not contained, cytokine release can proceed unabated giving rise to systemic inflammation or a systemic inflammatory response syndrome (SIRS) [24] (Figure 2-1). The diagnostic criteria for SIRS are:

1. An increase in core body temperature above 38.0 °C or decrease in core body temperature below 36.0 °C.

2. An increase in heart rate or tachycardia > 90 beats/minute.

3. An increase in respiratory rate or tachypnea > 20 breaths/minute.

4. An increase in number of white blood cells (WBC) > 12×10^9 cells/L or decrease in WBC < 4×10^9 cells/L.

Although sepsis is characterized by SIRS, one should carefully evaluate the symptoms of disease as other pathological conditions including burns, cancer, pancreatitis can also give rise to the SIRS. As such, the important criterion for the diagnosis of sepsis is the documented presence of an infectious agent or its toxin.

Figure 2-1. Progression of sepsis

Source: https://my.vanderbilt.edu/sepsismonitor/progress-reports/
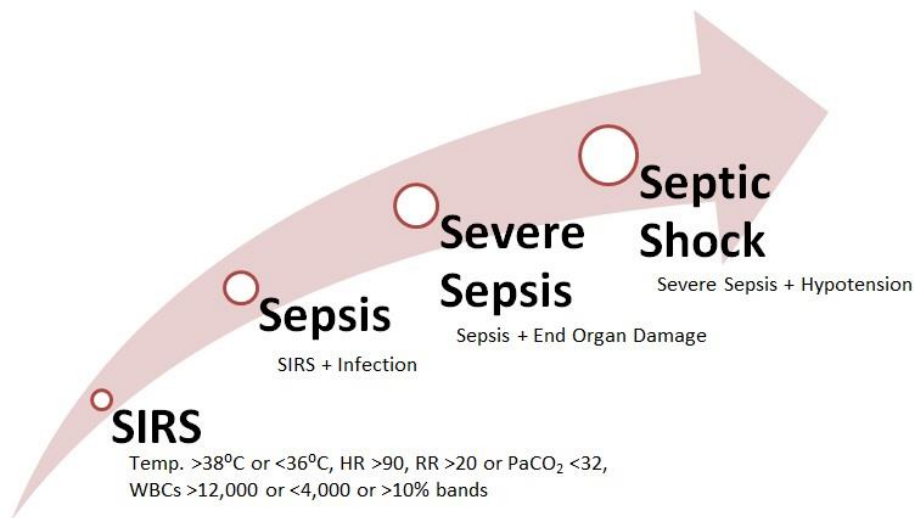
2.3 Etiology and epidemiology of sepsis

Sepsis is a complex disease of multifactorial etiology that can result in death within a few hours to days depending on the immune status of the individual. Sepsis syndrome involves characteristics of several diseases including hemorrhagic/hypovolemic shock, and ischemia.

6

Although the occurrence of sepsis has been known for over 3000 years, the etiology and pathogenesis of sepsis is still not well understood [25].

Severe sepsis is one of the leading causes of death in United States and is the number one cause of death in non-intensive care coronary units [26]. According to a recent report from Center for Disease Control and Prevention, the incidence of sepsis has more than doubled over the last decade likely because of increase in aging population with chronic illness or greater increase in invasive surgical procedures, chemotherapy and immunosuppressive drugs. The costs for treating sepsis rose from almost $15 billion in 2008 to over $20 billion in 2011 making sepsis the most expensive disease to be billed to Medicare in 2011 [27]. Perhaps most telling, sepsis is thought to account for 60-80% of all deaths in developing countries, which is almost 6 million deaths annually [28]. New and better methods to treat this disorder are clearly needed.

2.4 Animal models of sepsis

Understanding the mechanism(s) of sepsis-induced MODS is of paramount importance to developing new therapeutic modalities. Unfortunately, the study of patients already in sepsis is oftentimes complicated by a lack of proper case history, the presence of existing comorbidity, and differences in septic stage. Moreover, people with sepsis are usually admitted to the hospital during the terminal stages of sepsis which can make it extremely difficult to tease out the mechanisms mediating sepsis progression. Given these and other ethical reasons, animals have been widely used to study this disorder. Although there are several animal models of sepsis currently in use, it should be noted that no one model adequately replicates the human condition. Animal models of sepsis include:

1. Lipopolysaccharide injection

2. Injection of live bacteria

3. Cecal ligation and puncture (CLP)

4. Colon ascendens stent peritonitis (CASP)

5. Polymicrobial sepsis model

The injection of lipopolysaccharide (LPS) into animals to study the progression of sepsis and its underlying mechanisms was first documented in the early 1970's with the isolation of endotoxin [29]. LPS is part of the outer membrane of gram-negative bacteria and is a primary cause of host toxicity. The LPS molecule (Figure 2-2) consists of three distinct subunits- a) outermost O-antigen b) a middle core region made of polysaccharide and c) an inner lipid A portion that is highly conserved and a key cause of bacterial toxicity [30]. While the use of LPS to understand the pathophysiology of sepsis has been vital to understanding SIRS, the LPS model as a whole does not fully model the sepsis seen in humans. First, LPS does not represent the whole bacteria and in addition, it does not explain why gram-positive bacteria, fungi, or viruses can also cause sepsis. Secondly, there is a vast difference in the degree of response to LPS between humans and animals. For example, studies have shown that the dosage of LPS in mice has to be, on average, two hundred and fifty fold higher to produce the same degree of cytokine response seen in humans [31]. Reasons for the discrepancy in the sensitivity to LPS to provide the same degree of immune response in humans and rodents might probably be attributed due to changes in balance of leukocyte subsets, NK inhibitory receptor families or the toll receptors [32]. Thirdly, the clinical course of sepsis progression in humans is much slower when compared with animal models [33].

Figure 2-2. Basic Structure of LPS

Source: Doi:10.1038/nrmicro1068

The injection of live bacteria into animals has paved the way for better understanding the mechanisms behind host-pathogen interactions. Experiments performed using different strains of bacteria, bacterial loads, loading types (bolus or slow and sustained release using infusion pumps), and routes of administration (intraperitoneal, intravenous or subcutaneous) where either one or several of these parameters were varied in different combinations have greatly added to our knowledge on progression of sepsis. Nonetheless, like the LPS model, this preparation has been widely criticized as it is thought that the injection of a single bacterial strain poorly mimics the polymicrobial infections seen in humans and secondly, because the

injected bacteria oftentimes fail to colonize the host as is seen with a natural infection. To overcome this barrier, studies employing polymicrobial agents have come into existence.

One of the first developed, and perhaps most common model of polymicrobial sepsis is the cecal ligation and puncture (CLP) procedure. The CLP model is currently regarded as the gold standard among all sepsis models [34]. This model involves ligation at ileocolic junction followed by a single or multiple punctures to the cecum to control the severity of sepsis (Figure 2-3) [35]. This model of sepsis is highly favored among many investigators due to its ability to manifest the hemodynamic changes seen in clinical conditions [36]. In addition, this model is also thought to mimic the clinical presentation seen with abdominal trauma injuries. Nonetheless, one major downfall of this model is a lack of repeatability within and between different laboratories.

Figure 2-3. Model of cecal ligation and puncture
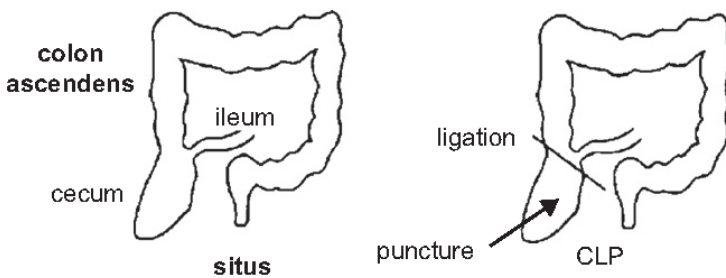
Source: DOI: 10.1016/j.ddmod.2011.10.002

A newer variation of the CLP model which is the colon ascendens stent peritonitis (CASP) (Figure 2-4) model of sepsis was developed by Zantl and collagues [37]. In this procedure, a stent is placed into the ascending colon which allows the passage of cecal

contents into the peritoneal cavity. One significant advantage of the CASP model over the CLP

procedure is that this model is more rapid in eliciting an early and Toll like receptor -4 (TLR4)

driven immune response than one that is driven by TNF-α. Whether this newly developed

model will someday replace the older, and much more variable, CLP procedure as the model of

choice for sepsis research is still unknown.

Figure 2-4. Model of colon ascendens stent peritonitis
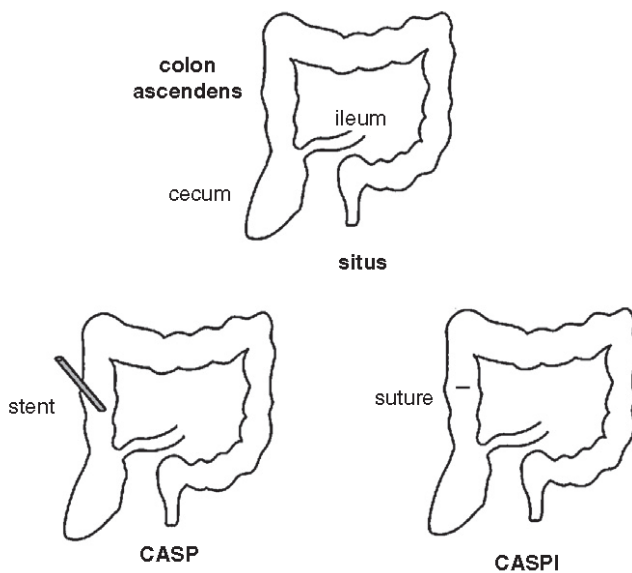
Source: DOI: 10.1016/j.ddmod.2011.10.002

The third model of polymicrobial sepsis, and perhaps the newest of all, is the cecal

inoculum procedure in which the cecal contents from a healthy donor animal are injected into

three or more other animals to induce the sepsis response [38]. A major advantage over the LPS

model is that this procedure uses a mixture of both gram positive and negative whole bacteria

instead of just the LPS molecule. In contrast with CLP and CASP, the degree of variability is

minimized among animals as the amount of cecal material is fixed. Nonetheless, like all models, disadvantages also exist with the cecal inoculum procedure. In particular, the bolus injection of cecal contents creates a rapid immune response which differs from the presentation typically seen in clinical cases.

2.5 Pathophysiology of sepsis

The pathophysiology of sepsis is a very complex process that begins with the entry of the infectious agent into the body and its subsequent interaction with the host immune system which results in release of inflammatory mediators. Overt stimulation of immune system results in the excessive production of inflammatory mediators which include the cytokines, chemokines, prostaglandins and other vasoactive substances. This initial response, if excessive, can result in the systemic inflammatory response.

Activation of the immune system begins with the binding of LPS to the lipopolysaccharide binding protein (LPS-BP) [39] which then binds to CD14, a glycosylphosphatidylinositol-anchored protein [40] that helps in recognition of the LPS by TLR4-MD2 complex [41]. Upon recognition of LPS, the TLR4 undergoes oligomerization to propagate intracellular signaling via its adaptor proteins [42]. Downstream signaling is broadly divided into either MyD88 dependent (TIRAP pathway) or MyD88 independent (TRAM pathway) pathways (Figure 2-5). Upon stimulation of TLR4 by LPS, the Myd88 becomes activated and recruits IL-1 receptor-associated kinase-4 (IRAK-4) [43]. IRAK-4 then activates its downstream protein TRAF6 which forms a complex with ubiquitin conjugating enzymes and the subsequent activation of TAK1. It is thought that TAK1 is responsible for the phosphorylation and activation of

downstream IKK and MAPK proteins [44]. The activation of IKK leads to phosphorylation of IKB alpha that removes the inhibitory effect on nuclear transcription factor NF-kB [45] which allows it to translocate to the nucleus and cause the transcription of inflammatory cytokines [46].

The MyD88 independent pathway [47] involves the activation of TRAM via the TLR4-LPS complex and the recruitment of downstream TRAF3 to IRF3. This recruitment is necessary for late phase activation of NF-kB and MAPK proteins [48]. In addition, activation of IRF3 is also necessary for the production of interferons [49] which are important defense mechanism against both virus and bacteria [50].

Figure 2-5. Mechanism of LPS induced production of inflammatory mediators

Source: http://dx.doi.org/10.1016/j.cyto.2008.01.006
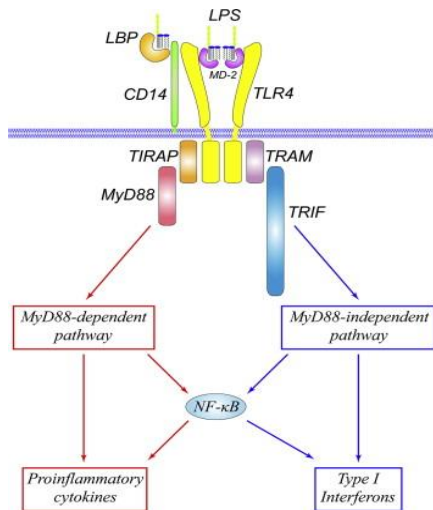
It is thought that inflammatory mediators released through the activation of macrophages, neutrophils and other immune cells act in a paracrine and autocrine fashion on

different cell types [51]. Cytokines can increase the generation of intracellular levels of ROS through activation of NADPH oxidase or cause the synthesis of nitric oxide through induction of iNOS gene expression [52-54]. Reactive oxygen species (ROS) can lead to impaired cell function by activating or inhibiting proteins that are involved in cell signaling along with formation of advanced glycation end products [55-57]. ROS can also cause peroxidation of lipids that affect the integrity of cellular membranes [58] while nitric oxide reacts with superoxide and causes formation of peroxynitrite [59] and protein nitrosylation [60]. In addition, nitric oxide can also cause vessel vasodilation, hypotension, and marked circulatory impairment [61]. Although the effects of ROS and inflammatory mediators are systemic, the degree of insult oftentimes varies by organ.

2.6 Multi-organ dysfunction syndrome

The liver produces C-reactive protein, ceruloplasmin, ferritin, haptoglobin and coagulation and complement factors as part of the acute inflammatory phase [62]. These proteins are involved in xenobiotic opsonization, the entrapment of bacteria in blood clots and the inhibition of iron uptake leading to inhibition of bacterial replication [63]. Also involved in this acute response are the liver Kupffer cells which are responsible for a large portion of the cytokines produced during sepsis and the phagocytosis of bacteria [64]. This cytokine release, if excessive, can lead to impairments in glucose metabolism [65], decreased cytochrome p450 activity, and hepatic dysfunction [66]. Histologically, hepatic failure is characterized by increased infiltration of polymorphonuclear (PMN) cells, centrilobular necrosis, sinusoidal dilation, and hepatocyte congestion [67]. Coincident with these changes in tissue morphology,

biochemical changes include increased levels of glutathione S transferase (GST), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) [68].

While the liver is heavily involved in marshalling the acute response, the brunt of the insult is most likely felt most by the kidney as it is highly susceptible to the hypoxia oftentimes seen after changes in circulatory volume or severe vasodilation [69]. Within the kidneys, the proximal tubules are more susceptible to sepsis induced hypoxic damage than the distal tubules which can rely on glycolysis to provide energy [70]. Progressive renal failure is associated with increased blood levels of blood urea nitrogen (BUN) and creatinine that can lead to systemic organ failure [71]. Sepsis induced renal damage is histologically characterized by the loss of tubular brush border, increased tubular dilatation and vacuolization. In some instances, renal tubules also show the presence of sloughed endothelial cells. These changes result in intratubular obstruction that can progress to complete renal failure [72].

Along with the renal system, the lungs also exhibit signs of inflammatory damage through increased accumulation of neutrophils and monocytes that results in acute respiratory distress syndrome (ARDS) [73]. ARDS is physiologically characterized by pulmonary edema, arterial hypoxia and impaired excretion of carbon dioxide [74]. Accumulated PMN secrete inflammatory cytokines and vasodilators that result in decreased velocity of blood flow and impairment of the vascular endothelial barrier through the disruption of adherens junction proteins such as VE-cadherin [75].

Figure 2-6. Mechanism of sepsis induced cardiac failure
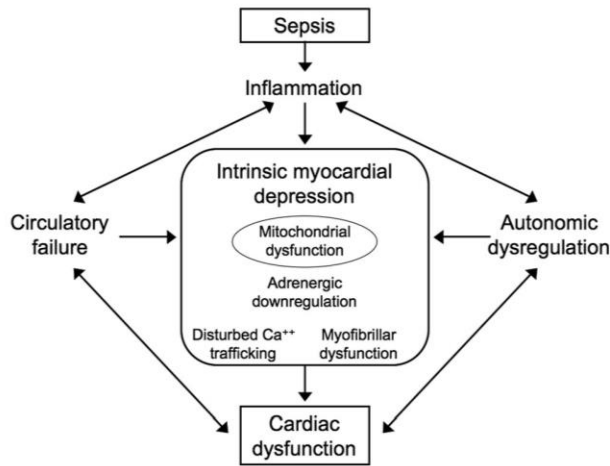
Finally, during the advanced stage of sepsis there are marked cardiovascular complications including myocardial depression, impaired left ventricular diastolic function, and reduced ejection fraction (Figure 2-6) [76-78]. The underlying causes of sepsis-induced cardiac failure are multifactorial in nature but are usually attributed to the following: Increased cytokines which cause myofibrillar destruction, high nitric oxide levels which result in systemic hypotension, peroxynitrite formation and myofibrillar protein damage, infiltration of monocytes, impaired oxygenation due to failure of the coronary circulation, and increased prostaglandins which adversely affect coronary endothelial function. Histologically, sepsis induced cardiac failure is typically characterized by contraction band necrosis and the presence of increased sarcolemmal rupture [79].

Figure 2-7. Schematic representation of sepsis induced multi-organ dysfunction
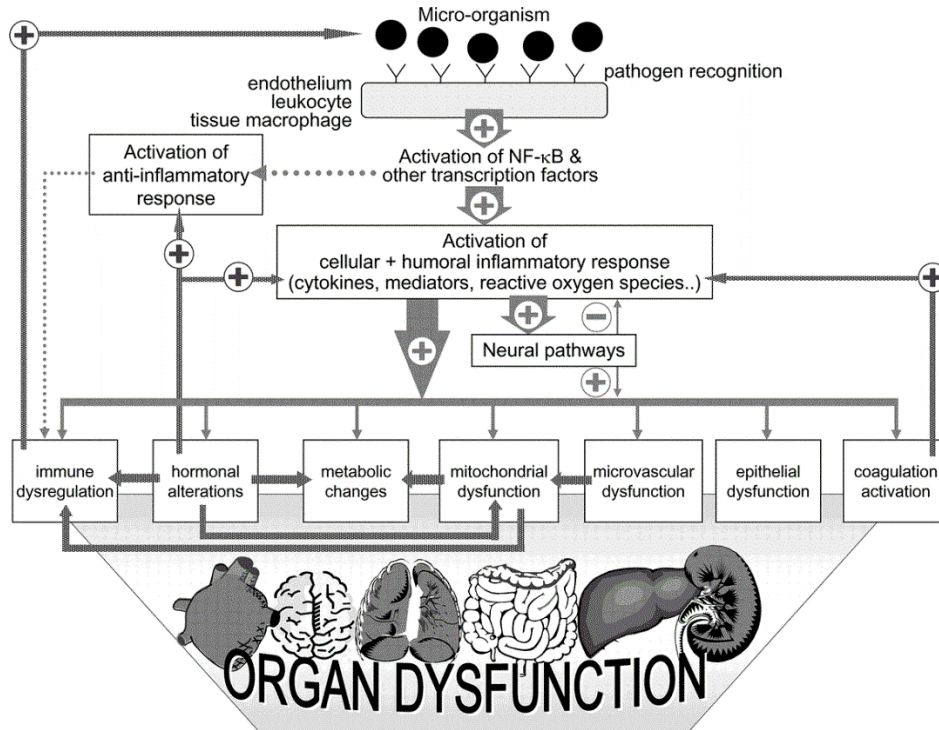
2.6 Current therapies for sepsis

The clinical progression of sepsis in humans is usually characterized by an early hyperdynamic phase that later transitions to a hypodynamic phase. The hyperdynamic phase is characterized by increased body temperature, respiratory rate and hyperglycemia. Conversely, the hypodynamic phase is associated with decreased body temperature, hypoglycemia and marked hypotension. The 2013 Guidelines from the International Surviving Sepsis Campaign suggest the following treatment strategy [80]:

1. Recognize sepsis

2. Administer broad spectrum antibiotics and obtain blood and serum for microbial culture and estimation of lactate levels.

3. Maintain blood pressure by administration of isotonic crystalloid fluids and achieve a urine output of 0.5ml/kg/hr.

4. Administer vasopressor such as norepinephrine if the patient has not responded to early fluid administration and if there is still a marked hypotension.

5. Finally measure patient's blood oxygen saturation and try to identify the causes behind its alteration and restore them to base values.

As a supportive treatment, a mild sedation without neuromuscular block can be indicated. Similarly, the administration of insulin is advised if subsequent glucose readings measure above 180mg/dl and hemodialysis for renal failure patients are suggested [80]. While all the therapeutic agents are geared towards minimizing the alterations in normal homeostasis, none of them actually addresses the underlying causes for sepsis induced mortality.

2.7 Nanotechnology

Nanotechnology can be described as the manipulation of matter at atomic or molecular scale to enhance its physical and chemical properties [81]. The word nanotechnology was first coined by Dr. Norio Taniguchi to describe processes related to manufacture of semiconductors [82]. The physical, chemical, and electro-magnetic properties of different substances can often vary considerably at the nanoscale [83]. Researchers and industrialists are now exploiting these properties for the design and manufacture of new therapeutic agents [84]. It is currently estimated that more than 800 products that utilize nanoparticles are being manufactured by

different companies [85]. Nanomedicine is an emerging branch of nanotechnology that is concerned with the development of therapeutic drugs for diseases such as cancer, diabetes and infectious diseases [86]. The most important contribution of nanomedicine is the development of therapeutic drugs that have enhanced permeation and retention (EPR) effects which allow them to successfully treat tumors that are inaccessible to commercial therapeutic agents [87]. In addition, recent animal studies have shown that the drugs designed through nanotechnology can be used to treat atherosclerosis, prevent age-associated macular degeneration and promote wound healing (Table 2-1) [88-90].

Table 2-1. List of FDA approved nanomedicines

PRODUCT	COMPOSITION	INDICATION	APPROVED
Table 1. Select FDA-Approved Agents Utilizing Nanomedicine			
Lipid-Based Nanoparticles			
Abelcet	Lipid complex formulation of amphotericin B	Invasive fungal infections	1995
AmBisome	Liposomal preparation of amphotericin B	Fungal and protozoal infections	1997
DaunoXome	Liposomal preparation of daunorubicin	HIV-related Kaposi's sarcoma	1996
DepoCyt	Liposomal formulation of cytarabine	Lymphomatous meningitis	1999
DepoDur	Liposomal formulation of morphine sulfate	Relief of postsurgical pain	2004
Doxil/Caelyx	PEGylated liposomal formulation of doxorubicin	Various cancers	1995
Inflexal V	Liposomal influenza vaccine	Influenza	1997
Visudyne	Liposomal formulation of verteporfin	Wet age-related macular degeneration	2000
Polymer-Based Nanoparticles			
Adagen	PEGylated adenosine deaminase enzyme	Severe combined immunodeficiency disease	1990
Cimzia	PEGlyated Fab' fragment of a humanized anti-TNF-alpha antibody	Crohn's disease, rheumatoid arthritis	2008
Copaxone	Polymer composed of L-glutamic acid, L-alanine, L-lysine, and L-tyrosine	Multiple sclerosis	1996
Eligard	Leuprolide acetate and PLGH polymer formulation	Advanced prostate cancer	2002
Macugen	PEG-anti-VEGF aptamer	Neovascular age-related macular degeneration	2004
Mircera	Chemically synthesized ESA, methoxy PEG-epoetin beta	Symptomatic anemia associated with chronic kidney disease	2007
Neulasta	Conjugate of PEG and filgrastim	Chemotherapy-induced neutropenia	2002
Oncaspar	PEGylated formulation of L-asparaginase	Acute lymphoblastic leukemia	1994
Pegasys	PEGylated interferon alfa-2a	Hepatitis C	2002
PegIntron	PEGylated interferon alfa-2b	Hepatitis C	2001
Renagel	Polyamine (polymer loaded with amine groups)	Chronic kidney disease	2000
Somavert	PEGylated human growth hormone receptor antagonist	Acromegaly	2003
Protein-Based Nanoparticles			
Abraxane	Albumin-bound paclitaxel (*nab*-paclitaxel)	Breast cancer	2005

Source: http://www.uspharmacist.com/content/s/197/c/33020/

Currently, several types and formulations of nanoparticles are being investigated to effectively treat several different pathological conditions. Nanoparticles made of gold, silver platinum and cerium oxide have been used to treat breast cancer, viral infections, and diabetes [91-93]. Multifunctional nanoparticles with increased stability, biocompatibility, and high intracellular penetration that can deliver therapeutic drugs are being used in preclinical trials to

treat cancer [94], for gene therapy [95], and for new types of multimodal imaging [96] (Figure 2-8). Generally, nanoparticles such as iron oxide nanoparticles coated with specific antibody are used for targeted therapeutic approach as well as for diagnostic purposes.

Figure 2-8. Schematic image of a multifunctional nanoparticle
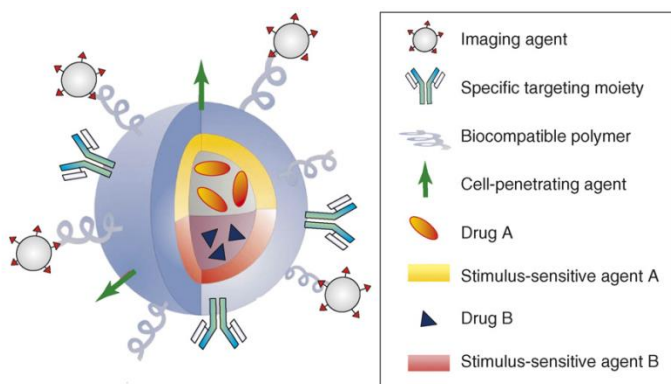
Source: Doi:10.1016/j.tibtech.2008.04.005

2.8 Cerium oxide nanoparticles and its prospective applications in medicine

Cerium oxide (CeO_2) is a rare earth metal oxide with a fluorite lattice structure that can cycle between oxidized (Ce^{+4}) and reduced (Ce^{+3}) states due to vacancies in the outermost orbital shells (Figure 2-9) [17]. It is thought that the ratio of Ce^{+3}/ Ce^{+4} determines the ability of the CeO_2 nanoparticles to act in the oxidation or reduction of other substances. One of the main qualities that distinguishes CeO_2 nanoparticles from other elements in the lanthanoid series is the presence of partially filled subshells with electrons in orbitals 4f and 5d, which makes it a potential tool for biomedical applications [97]. At the nanoscale, CeO_2 nanoparticles have increased chemical reactivity due to increase in surface to volume ratio. CeO_2 nanoparticles are used in the manufacture of cosmetics, polishing agents, diesel fuel additives,

and fuel cells [98-100]. Investigation into the use of CeO_2 nanoparticles for biomedical applications is just beginning.

Recent data has suggested that CeO_2 nanoparticles can inhibit the progression of ovarian cancer by inhibition of angiogenesis without cytotoxic effects on different organs. [101]. Arya and coworkers have shown that CeO_2 nanoparticle treatment can attenuate cellular ROS levels and protect primary cortical cells from apoptosis by acting to stabilize mitochondrial membrane potential [102]. Estevez and colleagues have shown that CeO_2 nanoparticles can protect the mouse hippocampus from ischemic damage through reduction in levels of superoxide and nitric oxide [103]. Similarly, other studies have shown that CeO_2 nanoparticles protect rodents from hypoxia induced oxidative stress [104] and that they can also protect endothelial cells, cardiac progenitor cells and pancreatic islets against H_2O_2 induced oxidative damage [15, 93, 100].

Figure 2-9. Structure of Cerium oxide (CeO_2)
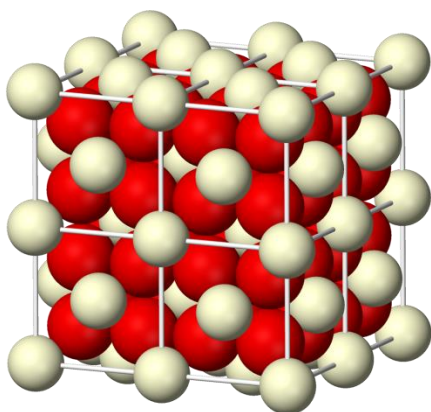
Source: http://en.wikipedia.org/wiki/Cerium(IV)_oxide

Interestingly, researchers have also exploited the use of CeO_2 nanoparticles as a potent anti-infectious agent. Thill and coworkers were among the first to formulate the idea of using CeO_2 nanoparticles as an anti- bacterial agent against *E.coli.* In this work, they demonstrated that the nanoparticles are positively charged at neutral pH which allows the particles to bind to the negatively charged bacterial wall and induce bacterial cytotoxicity [18]. Independent studies by other researchers have demonstrated similar findings for *B.subtilis* [105]. In addition to its use as an anti-bacterial agent, other work has shown that CeO_2 nanoparticles protect L929 cells from the vesicular stomatitis virus by inhibiting its replication [106]. Whether CeO_2 nanoparticles can function as an anti-infectious agent *in vivo* is still unclear and awaits investigation.

2.9 Summary

Severe sepsis is a life threatening disease with high mortality rate. The symptoms of sepsis are oftentimes confused with signs of general fever or flu making diagnosis difficult and delaying the initiation of medical intervention. Current sepsis treatments have failed to decrease the mortality rate as they are directed towards eliminating the bacteria but not the inflammatory mediators generated through host-bacterial interaction. *In vitro* and *in vivo* studies have shown that CeO_2 nanoparticles exhibit potent anti-inflammatory, anti-oxidant and anti-infectious properties [22, 107, 108]. Whether CeO_2 nanoparticles can be used to treat sepsis and its associated complications *in vivo* is not known.

Chapter 3

The current chapter is focused on the hypothesis of this study and is set as three different

papers each corresponding to a specific aim as stated in chapter-1.

Paper-1

The following paper corresponds to specific aim-1 and deals with the hypothesis whether CeO_2

nanoparticles can attenuate the systemic inflammatory response syndrome during septic insult

in male Sprague Dawley rats.

Therapeutic applications of cerium oxide nanoparticles in treatment of polymicrobial sepsis induced systemic inflammatory response syndrome and associated mortality

Running title- Treatment of sepsis induced systemic inflammatory response syndrome with CeO$_2$ nanoparticles.

Key words- Sepsis, cerium oxide nanoparticles, systemic inflammatory response syndrome, interleukin-6

Abstract

Sepsis is a disease of medical emergency that is characterized by severe oxidative stress and system inflammation which leads to multi-organ failure and subsequent death. Cerium oxide nanoparticles (CeO_2) have been shown to exhibit anti-oxidant and anti-inflammatory properties. This study tested whether administration of CeO_2 nanoparticles could be beneficial in the treatment of sepsis. Male Sprague-Dawley rats received a septic insult by intraperitoneal injection of cecal inoculation (400 mg/kg). A single dose of CeO_2 nanoparticles (3.5mg/kg) or vehicle was administrated to rats immediately after cecal inoculation. Treatment of sepsis animals with CeO_2 nanoparticle significantly increased the survival rate along with decrease in major inflammatory cytokine, interleukin-6. Treatment associated increases in animal survival were associated with decreased serum KC/GROα, MIP1β, MDC, MCP3β, MIP3, Eotaxin, TIMP1, myoglobin and blood urea nitrogen. Taken together, the findings of this preliminary study indicate CeO_2 nanoparticles may be useful as a therapeutic agent for treatment of sepsis.

Background

Sepsis is associated with multiple-organ failure and is the leading cause of death in critically ill patients [109]. Despite decades of clinical research, the incidence of sepsis and the number of deaths resulting from sepsis continue to rise [110]. Current sepsis treatments are largely supportive in nature and consist of antibiotics, NSAIDS, intravenous fluids, and mechanical ventilator support where warranted [111]. Sepsis is characterized by a systemic inflammatory state due to the overproduction of reactive-oxygen species (ROS) that appears to be mediated, at least in part, by the activation of monocytes and macrophages. Indeed, it is thought that macrophage derived nitric oxide (NO), tumor necrosis factor (TNF-α), interleukin (IL)-1β and IL-6 play a major role in both the innate and acquired immune responses to bacterial infection [112, 113].

Given the potential damaging effects of increased ROS on cellular structure and function some have hypothesized that anti-oxidant drugs may be useful for the treatment of sepsis [114, 115]. To date, most *in vivo* interventional studies examining the effects of anti-oxidant therapies have focused on the use of traditional pharmacological treatments [116, 117]. Although promising for certain applications, pharmacological intervention can be hindered due to poor bio-distribution and the requirement of multiple daily dosing since each antioxidant molecule is typically capable of scavenging only one free radical [118]. In an effort to overcome these limitations, some researchers have begun investigations into the use of nanomaterials for biomedical applications. Among the different nanomaterials under development, the use of cerium oxide (CeO_2) nanoparticles may be of particular interest. Cerium oxide is a rare earth element of the lanthanide series which is widely used as an agent for ultraviolet absorption,

27

oxygen sensing and automatic catalytic converters [119]. Cerium oxide appears to exhibit the ability to transition between the Ce^{3+} and Ce^{4+} oxidative states which results in an auto regenerative redox cycling [120]. Although biomedical applications still await analysis of biocompatibility, recent data has indicated that these particles possess potent anti-bacterial activity, anti-oxidant activity [18, 105], and cytoprotective properties [121-124].

Whether CeO_2 nanoparticles can be used for the treatment of sepsis has, to our knowledge, not been investigated. Therefore the purpose of this study was to investigate the effect of CeO_2 nanoparticle treatment on sepsis induced systemic inflammatory response syndrome and associated mortality in a laboratory model of severe sepsis. Our findings suggest that treatment with CeO_2 nanoparticles may provide therapeutic benefit for the management of polymicrobial sepsis.

Methods

Characterization of CeO_2 nanoparticles

NanoActive CeO_2 (99.9% purity as determined by ICP-MS; Lot #06-0118) was purchased from NanoScale corporation (Manhattan, KS, USA). The stock suspensions were prepared in deionized water by sonication using a Vibra Cell Sonicator (Sonics & Materials, Inc) at 600 W for 2 min at room temperature.

The hydrodynamic size and size distribution of CeO_2 nanoparticles were evaluated in deionized water using a Particle Size Analyzer (HORIBA, Model-LB-550) equipped with a He-Ne

laser (633nm) using back scattered light. Experiments were performed utilizing triplicate runs performed on three different days with freshly prepared samples.

Particles were imaged in their native state using a MultiMode-8, Atomic Force Microscope (Bruker, Ewing, NJ) in the tapping mode. Briefly, a few drops of CeO_2 nanoparticles were placed on freshly pealed mica substrate and allowed to dry in a petri dish floating in sonicator water bath (VWR, 50D, Radnor, PA) for 30 min at 240 W. Images were recorded in topography mode. Width and height measurements of adsorbed nanoparticles were made using the microscope's section analysis software.

For scanning electron microscopy, the particles were filtered with a 0.2 micron nucleopore filter. The filters were then trimmed and one quarter was placed on an aluminum stub with double-stick carbon tape. The sample was then sputter coated with gold/palladium and viewed on a Hitachi S4800 field emission scanning electron microscope. For transmission electron microscopy, the particles were diluted in double distilled filtered water and a drop was placed on a formvar-coated copper grid to dry. The sample was viewed on a JEOL 1220 transmission electron microscope.

Induction of polymicrobial sepsis

Ninety two male Sprague-Dawley rats weighing 390-440 g were obtained from Hill Top laboratories. Rats were housed two per cage and maintained in accordance with the guidelines provided by the Marshall University Institutional Animal Care and Use Committee (IACUC), and The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in a 12:12- h dark-light cycle at a temperature of 22 ± 2°C. Animals were provided food and water

ad libitum, and acclimatized for at least 14 days prior to experimentation. The polymicrobial sepsis procedure was performed as outlined previously [38]. Briefly, animals were randomly assigned to one of four groups: sham control, CeO_2 nanoparticle only, sepsis, and sepsis + CeO_2 nanoparticle groups. After being anesthetized with ketamine HCL: xylazine (45 mg/kg / 5 mg/kg i.p.) , the mid ventral surface is cleanly shaved and disinfected with 70% ethyl alcohol and a small 0.5 cm vertical midline abdominal incision was made. Animals in the sepsis and sepsis + CeO_2 group received an i.p. injection of cecal inoculum (400mg cecal material/kg @ 5ml/kg in 5% dextrose water). The cecal inoculum was prepared by mixing cecal contents obtained from donor rats. Fresh inoculum was prepared each day. The animals in control and sepsis group received sterile 5% dextrose water @ 5ml/kg i.p. All incisions were closed with interrupted silk sutures (3-0), and the abdomen was gently massaged to distribute the injectate. After the surgical procedure, animals in sham control and sepsis groups received 100 µl of sterile distilled water while animals in CeO_2 and sepsis+CeO_2 groups received 3.5 mg/kg of CeO_2 nanoparticles in 100µl of sterile distilled water via tail vein injection. All rats were given free access to food and water after recovery from anesthesia. Animal survival was assessed for 14 days.

In other experiments, animals were randomly assigned to one of the four groups as detailed previously. At 6 or 24 h after study initiation, animals were humanely sacrificed under anesthesia and blood was collected by cardiac puncture. Serum was separated and used for measuring various serum protein markers.

Enzyme linked immunosorbant assay and multiplex immunoassay

ELISA was performed to determine the levels of serum IL-6. The cytokine interleukin-6 (IL-6) was analyzed in different groups using a reagent kit from BD Bioscience (Franklin Lakes, NJ, USA) as outlined by the manufacturer. Multiplexed serum protein markers were analyzed group wise by sending pooled samples to Rules-Based Medicine (Austin, TX, USA) using Rodent MAP® version 3.0 antigen analysis with a Luminex 100 instrument, as described previously [125].

Estimation of serum biochemical markers

Serum biochemical parameters were analyzed using an Abaxis VetScan® analyzer (Abaxis, Union City, CA, USA). Briefly, 100ul of serum was loaded into each vetscan cartridge and analyzed for changes in levels of serum albumin, alkaline phosphatase (ALP), alanine transaminase (ALT), amylase, BUN, calcium, phosphorus, glucose, sodium, potassium, globulin and total protein across different groups.

Statistical analysis

Results are presented as mean ± standard error of mean. For significant differences in survival curves, log-rank test (Mantel-Cox) was performed using Prism 5.0 software (GraphPad Software, La Jolla, CA). One-way analysis of variance (ANOVA) was used for comparison among different groups followed by Student Newman Keuls *post hoc* test to determine statistical significance using Sigmaplot 12 statistical software (Systat software Inc, San Jose, CA). A p-value of < 0.05 was considered to be statistically significant.

Results

CeO$_2$ nanoparticle characterization

The mean hydrodynamic diameter of the CeO$_2$ nanoparticles as determined by dynamic light scattering (DLS) was 140 ± 52.9 nm (Figure 3-1, Panel A). AFM analysis indicated an agglomerate size of 37.75 ± 3.8 nm width and 1.38 ± 0.28 nm height respectively (Figure 3-1, Panel B). SEM and TEM analysis of CeO$_2$ nanoparticles determined the size of individual CeO$_2$ nanoparticles to be approximately between 20-40nm (Figure 3-1, Panels C, D).

CeO$_2$ nanoparticles increased animal survivability in septic animals

Compared to control animals, the animals inoculated with the cecal material exhibited several signs of shock including diarrhea, piloerection, and little or no spontaneous movement. However, sepsis animals that are treated with CeO$_2$ nanoparticles showed significant improvement as seen by their alertness along with greater frequency in consumption of food and water when compared to sepsis group alone. Furthermore treatment of the septic animals with CeO$_2$ nanoparticles increased animal survivability from ~25% to ~85% (Figure3-2).

CeO$_2$ nanoparticles treatment decreased sepsis-induced systemic inflammation

Compared to controls, sepsis animals had increased serum IL-6 levels at 6 and 24 h ($P<0.05$). CeO$_2$ nanoparticle treatment decreased serum IL-6 levels at 6h by 66% when compared with sepsis group ($P<0.05$) (Figure 3-3, Panel A). Although not significant, CeO$_2$ nanoparticles also attenuated the sepsis induced increase in serum IL-6 at 24h. Similarly when

compared to controls, sepsis animals showed higher levels of growth regulated alpha protein (KC/GRO), macrophage inflammatory protein-1 beta (MIP-1 β) macrophage derived chemokine (MDC), monocyte chemotactic protein-3 (MCP-3), myoglobin, macrophage inflammatory protein-3 beta (MIP-3β), eotaxin, leptin, macrophage inflammatory protein-2 (MIP-2), interferon gamma induced protein-10 (IP-10), tissue inhibitor of metalloproteinases-1 (TIMP-1) and plasminogen activator inhibitor-1 (PAI-1) at 6 and 24 h (Figure 4A, 4B). However, treatment with CeO_2 nanoparticle diminished the levels of KC/GRO, MIP-1β, MDC, MCP-3, myoglobin MIP-3β, eotaxin, leptin MIP-2, IP-10, and TIMP-1 by 1 – 3 fold (Figure 3-4, Panels A-D).

The levels of several other analytes including macrophage colony stimulating factor-1 (M-CSF-1), monocyte chemotactic protein-1 (MCP-1), stem cell factor (SCF), vascular endothelial growth factor A (VEGF-A), IL-1α, IL-7, Il-11 and lymphotactin exhibited a trend towards being increased with sepsis and decreased by at least one fold with CeO_2 nanoparticle treatment (Figure 3-4, Panels E, F).

CeO_2 nanoparticles attenuated sepsis induced alterations in serum biochemical parameters

Sepsis is characterized by alterations in serum biochemical parameters such as BUN, sodium, potassium and phosphorus. Treatment with CeO_2 nanoparticles attenuated sepsis induced increase in blood urea nitrogen and changes in sodium levels (Table 3-1) ($p < 0.05$). Although not significant, CeO_2 nanoparticle also attenuated sepsis induced changes in serum alkaline phosphatase, amylase, phosphorus and potassium (Table 3-1).

DISCUSSION

Despite decades of intensive investigation and significant advances in medical technology, management of patient care and antimicrobial therapy, the overall mortality rate in severe sepsis still remains unacceptably high [126]. The primary finding of this study was the observation that a single injection of CeO_2 nanoparticles, in the absence of antibiotic treatment, fluid resuscitation, or other pharmacological intervention, was associated with a ~80% increase in animal survivability in the cecal inoculum model of severe sepsis (Figure 3-2).

Previous studies have shown that the immune response to microbial insult is characterized by release of large amounts of pro-inflammatory cytokines which lead to multiple organ failure [127]. It is thought that elevations in serum IL-6 levels are highly correlated with the survival of the septic patient [128, 129]. Similar to other reports using this model, our *in vivo* studies showed that sepsis was associated with increased serum IL-6 levels [130] and importantly, that our nanoparticle based treatment significantly attenuated these increases (Figure 3-3). In addition to IL-6, it is also known that MODS is caused by other cytokines and chemokines such as eotaxins, MCP-1, MCP-3, etc. [131]. Consistent with previous data demonstrating that CeO_2 nanoparticles can function as an antioxidant [132], the CeO_2 nanoparticle treatment also appeared to attenuate the expression of the serum cytokine and chemokine markers KC/GRO, MIP-1β, MDC, MCP-3β, myoglobin, MIP-3β, eotaxin, IP-10, and leptin (Figure 3-4). Given that many of these cytokines, are derived from the liver Kupffer cells these data suggest that our nanoparticle intervention is also capable of affecting macrophage function.

Sepsis is known to cause acute kidney injury (AKI) in about half of the infected individuals and has a very poor prognosis in comparison with the patients without AKI [133]. Previous studies have shown that sepsis induced AKI is characterized by increased serum levels of BUN and creatinine [134]. These changes are also accompanied by decreased sodium and increased potassium levels [135]. Consistent with previous work, we found that blood urea nitrogen (BUN) levels began to increase from 6 to 24 h, which is suggestive of kidney dysfunction. However, treatment with cerium oxide nanoparticles attenuated the increase in BUN at 6 h significantly (Table 3-1) ($p < 0.05$). Although not statistically significant, cerium oxide nanoparticles appeared to attenuate sepsis induced increases in BUN, sodium potassium and phosphorus at 24 h time point. Similarly cerium oxide nanoparticles appear to increase serum amylase and glucose levels that were attenuated by sepsis at 24 h (Table 3-1).

Conclusion

Sepsis is a state of severe whole body inflammation that rapidly progress to MODS unless treated in time. Current therapeutic regime is largely ineffective in treating patients with sepsis mainly due to the increasing antibiotic resistance along with the lack of a specific anti-inflammatory drug that could attenuate SIRS. The main finding of the study is that a single dose of cerium oxide nanoparticles attenuated sepsis induced mortality and SIRS along with serum biochemical parameters in the absence of any supportive treatment. Future studies aimed at the molecular mechanism of action of cerium oxide nanoparticles at the cellular level against septic insult are warranted.

Acknowledgments

Figures

Figure 3-1. Characterization of CeO$_2$ nanoparticles.

CeO$_2$ nanoparticles are characterized by A) DLS B) AFM C) SEM D) TEM
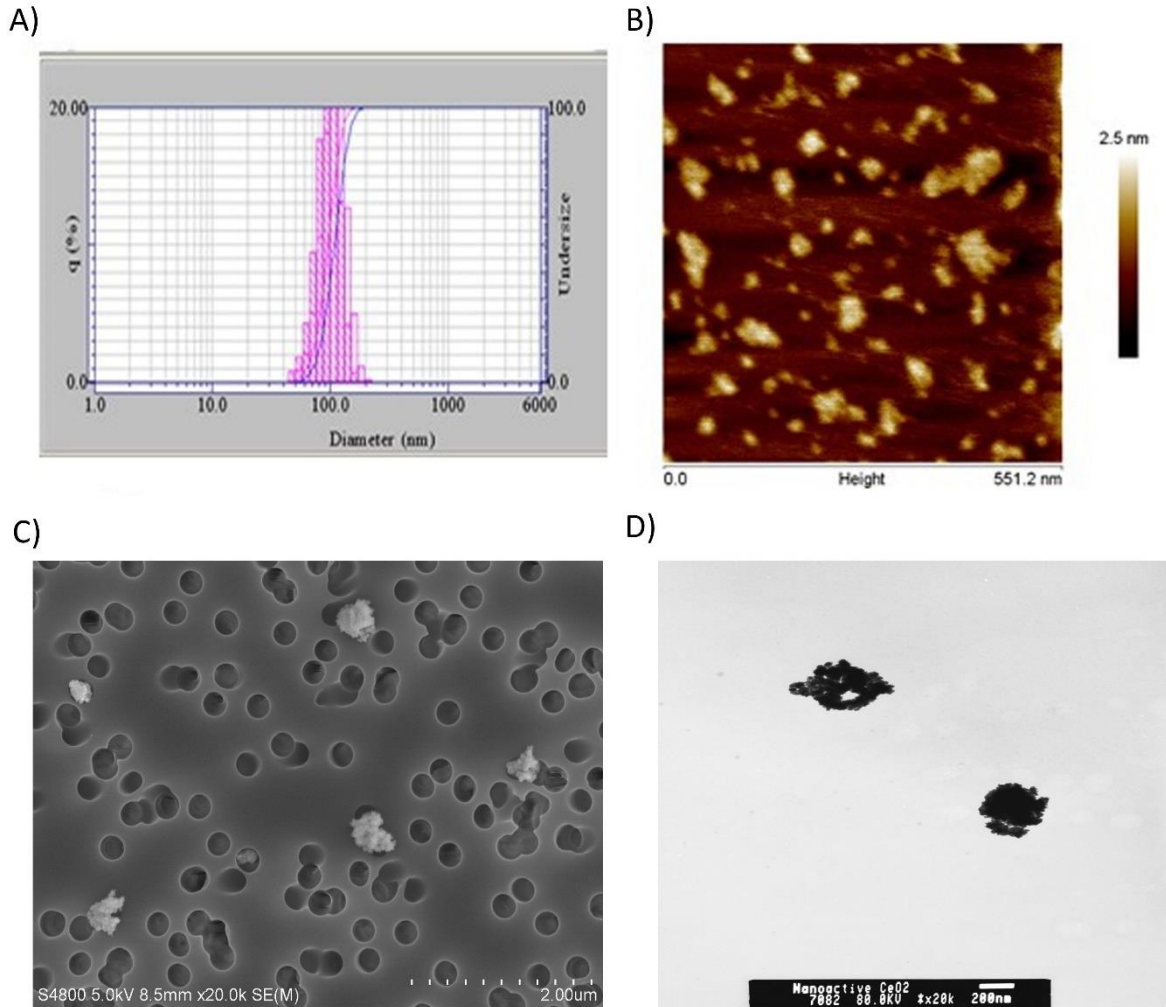
Figure 3-2. CeO$_2$ nanoparticles attenuated sepsis induced mortality.

(n=8/group).
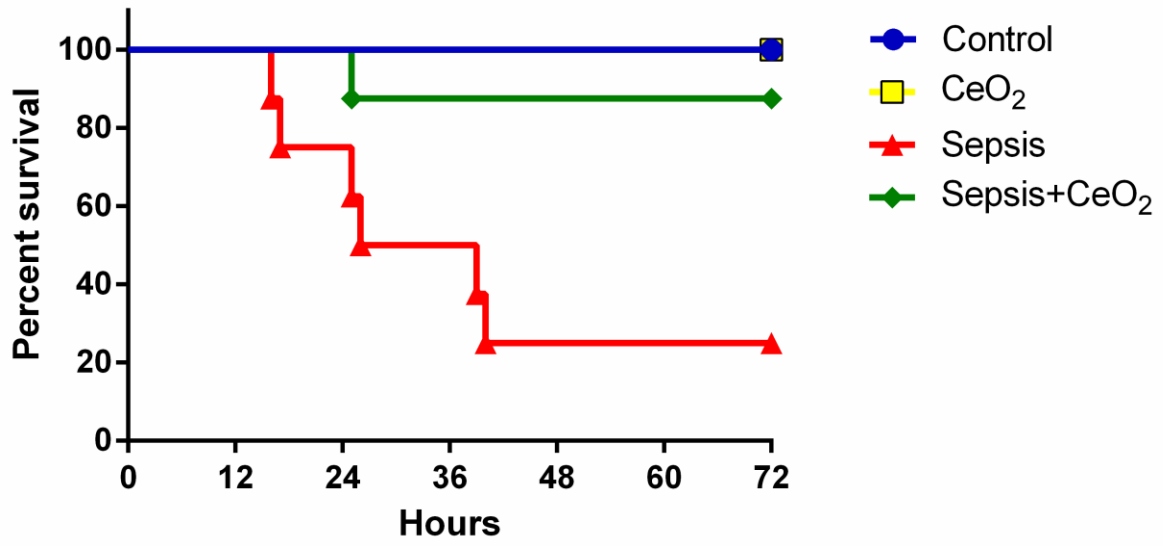
Figure 3-3. CeO$_2$ nanoparticles attenuated sepsis induced increase in serum IL-6.

"*"Significantly different from control group, "$" significantly difference from CeO$_2$ group and "#" significantly difference from sepsis group (p<0.05). (n=6/group)
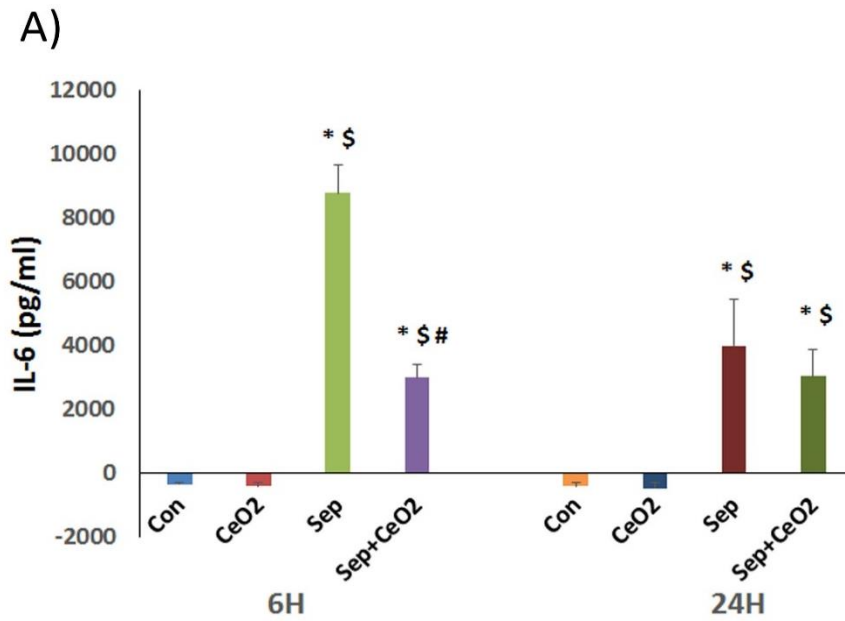
Figure 3-4. CeO₂ nanoparticles prevented sepsis induced SIRS by attenuating serum inflammatory proteins.

(n=6/group).
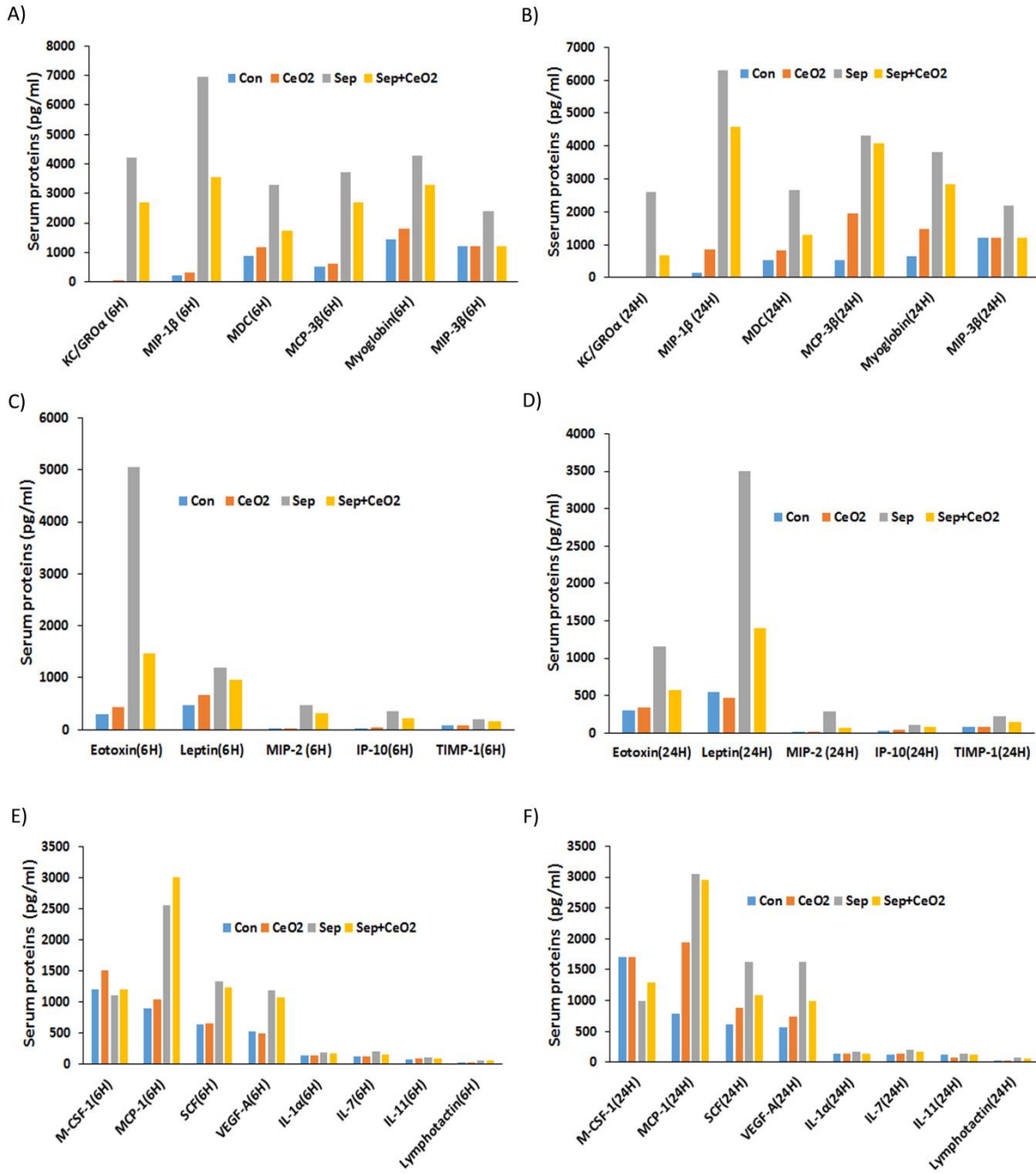
Table 3-1. CeO$_2$ nanoparticles attenuated sepsis induced changes in serum biochemical parameters.

"*"Significantly different from control group, "$" significantly difference from CeO$_2$ group and "#" significantly difference from sepsis group (p<0.05). (n=6/group).

Analyte	6 h Control	6 h Ceo2	6h Sepsis	6h Sepsis+Ceo2	24 h Control	24 h Ceo2	24h Sepsis	24h Sepsis+Ceo2
ALB	4.08±0.06	4.16±0.11	3.78±0.12$^{\$}$	3.86±0.04	3.46±0.05	3.33±0.11	2.80±0.08$^{*\$}$	2.78±0.08$^{*\$}$
ALP	195.00±19.96	196.25±20.78	160.88±15.22	176.13±25.47	265.88±22.02	238.13±36.40	91.29±4.83$^{*\$}$	113.44±6.05$^{*\$}$
ALT	77.63±6.83	66.13±4.85	54.38±2.52*	55.13±3.96*	86.00±15.34	76.00±6.12	69.14±6.14	82.88±11.45
Amylase	920.25±30.64	874.50±17.90	1003.50±57.40	972.25±32.65	863.63±17.89	889.25±66.22	561.71±58.26$^{*\$}$	790.56±108.41
BUN	25.00±1.83	29.25±2.86	39.13±1.38$^{*\$}$	32.13±1.13$^{*\#}$	17.88±0.90	18.88±2.89	71.43±13.40$^{*\$}$	41.22±5.60$^{*\$}$
Ca+	10.75±0.26	10.60±0.22	10.91±0.22	10.478±0.18	10.50±0.14	10.50±0.19	10.19±0.08	10.11±0.21
Phos	11.41±0.79	10.70±0.58	13.86±0.73$^{\$}$	11.78±0.89	9.89±0.26	9.35±0.21	10.77±0.87	9.41±0.40
Glu	376.88±12.44	362.75±16.26	312.88±66.71	283.75±40.21	341.63±11.36	267.63±22.30	101.86±11.88$^{*\$}$	116.67±6.08$^{*\$}$
Na+	142.00±0.82	144.25±10.13	140.88±1.39	138.50±0.66$^{\$}$	143.75±0.73	143.75±1.01	145.00±1.23	140.378±1.15$^{\#}$
K+	7.16±0.25	7.46±0.23	6.98±0.23	6.79±0.16	7.59±0.28	7.63±0.21	8.29±0.25	7.64±0.23
Glob	1.35±0.05	1.44±0.07	1.50±0.06	1.43±0.05	1.80±0.06	2.00±0.09	2.39±0.07$^{*\$}$	2.34±0.07$^{*\$}$
TP	5.41±0.04	5.60±0.09	5.26±0.08$^{\$}$	5.30±0.06$	5.28±0.05	5.31±0.08	5.19±0.13	5.13±0.09

Paper-2

The following paper corresponds to specific aim-2 and deals with the hypothesis whether CeO_2 nanoparticles can attenuate severe sepsis-induced inflammatory damage to the heart and liver during a septic insult in male Sprague Dawley rats.

Therapeutic applications of anti-oxidant and anti-inflammatory properties of cerium oxide nanoparticles in treatment of polymicrobial sepsis induced multi-organ failure

Running title- Treatment of sepsis induced multi-organ failure with CeO$_2$ nanoparticles.

Key words- Sepsis, cerium oxide nanoparticles, cardiac failure, oxidative stress, inflammation

Abstract

Sepsis is a life threatening disease that is associated with high mortality. Existing treatments have failed to improve survivability in septic patients. The purpose of the present study is to evaluate whether cerium oxide nanoparticles (CeO_2) can prevent sepsis-induced mortality by preventing cardiac and hepatic dysfunction in male Sprague Dawley rats. Administration of a single dose (0.5mg/kg) of CeO_2 nanoparticles intravenously to septic rats significantly improved survival rates and functioned to restore core body temperature towards baseline. Treatment-induced increases in animal survivability were associated with reduced hepatic oxidative stress, diminished serum cytokines, and decreased serum chemokine levels. Changes in serum inflammatory markers with treatment were accompanied by decreased vascular leak along with reduced serum creatine kinase activity and myoglobin levels. In the heart, treatment diminished ERK 1/2 MAPK- Stat-3 signaling and endothelial activation. Taken together these data suggest that CeO_2 nanoparticles can be used as a novel therapeutic agent for treatment of polymicrobial sepsis.

44

Introduction

Severe sepsis is a medical emergency that is caused by an excessive systemic inflammatory response to bacteria, fungi, or other xenobiotic. Although easily managed if caught early, diagnosis is often difficult due to non-specific symptoms which can result in delayed treatment and the development of multiple organ failure, septic shock and death. Standard treatment protocols advocate the administration of antibiotics in conjunction with supportive therapy which is largely ineffective once sepsis has developed.

The cardiovascular failure that characterizes the latter stages of sepsis has been largely attributed to the development of systemic hypotension due to severe vasodilatation and the microvascular leak caused by the derangement of the endothelial barrier. As this stage progresses, a state of depressed myocardial contractility occurs that oftentimes leads to irreversible myocardial damage [136].

It is thought that much of the tissue damage seen in sepsis is caused by elevations in tissue reactive oxygen species (ROS) and reactive nitrogen species (RNS) subsequent to the increased release of cytokines that are a defining characteristic of the systemic inflammatory response syndrome (SIRS). Recent data has suggested that the liver Kupffer cells are the primary producer of the cytokines observed during SIRS [137]. Whether strategies designed to quickly attenuate cytokine release by the Kupffer cells can diminish serum cytokine levels and improve animal survivability following a lethal septic insult has, to our knowledge, not been investigated.

Cerium is a lanthanide metal that can undergo redox cycling from Ce^{4+} (oxidized) to Ce^{3+} (reduced) states and is usually found in a complex with oxygen as CeO_2. In nanoparticle form,

CeO_2 has been shown to function as a superoxide dismutase (SOD) mimetic [138], as a preventive measure to combat hypoxia-induced oxidative stress [104], and to protect different cell types against hydrogen peroxide-induced apoptosis [93, 100]. The use of CeO_2 nanoparticles to prevent sepsis-induced cardiovascular dysfunction has, to our knowledge, not been investigated.

Materials and Methods

Characterization of CeO$_2$ nanoparticles

Cerium oxide nanoparticles were purchased from US Research Nanomaterials, Inc (Houston, TX). Dynamic light scattering (DLS) was performed to estimate the mean size of CeO_2 nanoparticles in suspension using LB-550 DLS particle size analyzer (Horiba Scientific, Edison, NJ). Naked particle size of the CeO_2 nanoparticles was characterized by transmission electron microscopy using JEOL JEM-2010 transmission electron microscope (TEM). X-ray diffraction (XRD) was performed by Scintag XDS 2000 powder diffractometer. Scanning transmission electron microscopy (STEM) images were acquired by the Aberration Corrected Analytical Electron Microscope (TEM/STEM JEOL JEM-ARM200CF, Japan) operated at 200 keV. Electron energy loss spectroscopy (EELS) data for CeO_2 nanoparticles were collected by Gatan Enfina.

Induction of polymicrobial sepsis and therapeutic intervention

One hundred twenty eight male Sprague Dawley rats aged 10 weeks were purchased from Hill-Top laboratories and housed in two-per cage at 22 ± 2°C with a 12:12 light-dark cycle

for 2 weeks prior to experimentation. Animals were fed with standard rodent chow and had access to food and water *ad libitum*. All surgical procedures were performed in accordance with the guidelines provided by the Marshall University Institutional Animal Care and Use Committee (IACUC), and The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Briefly, animals were anesthetized under isoflurane and a small mid ventral incision of 0.5 cm was made. Sham controls and CeO_2 only groups were injected with 5ml/kg of 5% sterile dextrose solution intraperitoneally and the incision was closed with 3-0 silk sutures. For the sepsis and sepsis+CeO_2 groups, animals received a cecal inoculum of 600mg/kg BW in 5ml/kg BW of 5% sterile dextrose solution intraperitoneally. Cecal material was obtained from healthy rats and the material from each donor was used to induce sepsis in 4-5 rats. Sham control and sepsis groups received 200 µl of sterile distilled water intravenously via tail vein while the CeO_2 and sepsis+CeO_2 groups received CeO_2 nanoparticles (0.5mg/kg) in 200 µl of sterile distilled water intravenously. Rectal temperature was recorded at 0, 3, 6, 12, 18, 24, and 48h after receiving the cecal inoculum. Animals were observed for mortality for a period of 14 days.

Sample collection and estimation of organ ceria content

Whole blood was collected by cardiac puncture and centrifuged at 5,000 x g for 10 min to collect serum at 3h and 18h time points. Animals were sacrificed under isoflurane anesthesia and the heart and liver were excised and washed in Krebs–Ringer bicarbonate buffer (KRB) to remove any blood, blotted to remove excess moisture, snap frozen in liquid nitrogen, and stored at -80°C for further analysis.

Frozen hearts and livers were sent to Elemental Analysis Inc. (Lexington, KY) for estimation of ceria content by induction coupled plasma-mass spectrometry (ICP-MS) as described elsewhere [125].

Estimation of immune cell number and serum markers of inflammation

The number of white blood cells (WBC), neutrophils, monocytes and lymphocytes were estimated in whole blood and peritoneal fluid collected at 3h and 18h time points using an Abaxis VetScan HM2 hematology analyzer (Abaxis, Union city, CA). Peritoneal fluid was obtained only from sepsis and sepsis and sepsis + CeO_2 groups. Total serum ROS/RNS was measured using OxiSelect™ *in vitro* ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA) as outlined by the manufacturer. Creatine kinase activity was measured in serum using a creatine kinase fluorometric assay kit (Cayman Chemical Company, Ann Arbor, MI) as directed by the kit instructions.

Serum and peritoneal fluid samples from each of the different groups (n=6/group) collected at 3h and 18h time points were pooled and sent to Myriad RBM (Austin, TX) for the analysis of serum growth factors, cytokines and markers of inflammation using rodent MAP® V. 3.0. Pooled samples were run in triplicate for statistical analysis.

Liver histology and superoxide levels

Liver tissue was sectioned (4μm) using Leica CM1950 cryostat onto poly-L-lysine coated slides. Visualization of hematoxylin and eosin staining was performed to evaluate liver morphology using Evos XL microscope (Life technologies, Grand Island, NY).

Levels of hepatic superoxide were estimated using dihydroethidium stain (Life technologies, Grand Island, NY). Briefly, liver sections were washed with PBS for 5 min and stained with 5μM solution of dihydroethidium for 1h at room temperature in the dark. Tissue sections were imaged using Evos FL microscope (Life technologies, Grand Island, NY) after PBS washing (3 x 5 min) and the levels of superoxide were estimated by image analysis using image J analysis software.

SDS-PAGE and immunoblotting

Approximately 100 mg of frozen tissue was taken and pulverized in liquid nitrogen and added to 900ul of T-PER (Pierce, Rockford, IL, USA) containing 1% protease and phosphatase inhibitors (P8340 and P5726, Sigma- Aldrich, St. Louis, MO, USA). Samples were homogenized and centrifuged at 13,000 rpm for 10 min at 4°C to collect the supernatant. Amount of protein in the samples was estimated through 660 nm assay (Pierce, Rockford, IL, USA) and normalized with T-PER and 4x Laemlli buffer to a final equal concentration across all samples. Equal amount of protein was loaded in 10% PAGEr Gold Precast gel (Lonza, Rockland, ME) and transferred to nitrocellulose membranes using standard protocol as detailed elsewhere [139]. Membranes were block with 5% milk in TBST for 1h at room temperature, washed thrice with TBST and probed for detection of ERK 1/2 MAPK , p-ERK 1/2 MAPK (Thr202/Tyr204), Stat-3, p-Stat-3 (Tyr705), GAPDH (Cell Signaling Technology, Danvers, MA), P-selectin, VCAM-1, nitrotyrosine (Abcam, Cambridge, MA) and iNOS (Santa Cruz, Dallas, TX). Membranes were incubated with primary antibody overnight at 4°C, washed with TBST (3 x 5 min), and incubated with secondary anti-rabbit (Cell Signaling Technology, Danvers, MA) or anti-mouse antibody (Santa

Cruz, Dallas, TX) for 1 h at room temperature. Immunoreactive signal was visualized using Supersignal West Pico Chemiluminiscent substrate (Pierce, Rockford, IL, USA) and quantified using Fluorchem 9900 software (Protein Simple, Santa Clara, CA). Protein expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Results are presented as mean ± SEM. The log-rank test (Mantel-Cox) was performed using Prism 5.0 software (GraphPad Software, La Jolla, CA) to determine differences in animal survivability between groups. A two way analysis of variance using Tukey's multiple comparison was performed where appropriate to evaluate differences in core temperature amongst the different groups. Differences in groups with equal sample size were evaluated by one way analysis of variance using Student Newman Keuls or Dunn's *post hoc* analysis for samples with unequal size. A one way ANOVA by ranks with Kruskal Wallis *post hoc* analysis was used for samples with non-normal distribution and a simple t-test was performed where appropriate using Sigmaplot 12 statistical software (Systat software Inc., San Jose, CA) to test for the presence of significant differences between groups. A probability value of *P < 0.05* was considered to be statistically significant.

Results

Characterization of CeO$_2$ nanoparticles

The mean hydrodynamic diameter of CeO$_2$ nanoparticles as estimated through dynamic light scattering experiments was found to be approximately 90nm (Figure 3-5, Panel A). TEM and STEM –HAAF analysis determined the size of individual nanoparticles to be approximately between 10-30nm (Figure 3-5, Panels B, C). The high resolution image of the CeO$_2$ nanoparticle shows that the atoms of CeO$_2$ are highly ordered (Figure 3-5, Panel D) and the EELS data (Figure 3-5, Panel F) confirms the existence of the Ce and O element. XRD was used to demonstrate cubic fluorite structure of CeO$_2$ nanoparticles as seen through typical peaks (Figure 3-5, Panel G).

Effect of CeO$_2$ nanoparticles on sepsis induced mortality and hypothermia

Nanoparticle treatment decreased sepsis mortality from 100% to ~25% (Figure 3-6, Panel A, *P < 0.05*). Improvement in survivability was associated with a significant improvement in core body temperature from 18h (Figure 3-6, Panel B, *P < 0.05*).

Nanoparticle treatment increased liver ceria and decreases systemic oxidative stress

Compared to untreated animals, liver ceria content was increased in the animals receiving nanoparticle injections (Table 3-2). Nanoparticle treatment decreased sepsis-induced increases in total serum ROS levels (Figure 3-6, Panel C, *P < 0.05*).

Nanoparticle treatment decreased sepsis induced hepatic damage, tissue ROS and iNOS levels

Histological analysis of the livers obtained from control animals revealed normal hepatic and sinusoidal morphology. Nanoparticle treatment diminished sepsis-induced sinusoidal dilatation and hepatocyte congestion (Figure 3-7, Panels A-D). Consistent with the histological findings, tissue superoxide levels were increased with sepsis and decreased with nanoparticle treatment (Figure 3-7, Panels E-I, $P < 0.05$). In a similar fashion, nanoparticle treatment also decreased sepsis induced increases in hepatic iNOS (Figure 3-8, Panel A, $P<0.05$) and protein nitrosylation at 3h (Figure 3-8, Panel B, $P < 0.05$).

Nanoparticle treatment modulated sepsis related cytokines, chemokines and growth factors

Nanoparticle treatment reversed sepsis-induced changes in several inflammatory chemokines (Table 3-3), cytokines (Table 3-4), and other inflammation related proteins (Table 3-5, $P < 0.05$). Consistent with these data, nanoparticle treatment also decreased sepsis induced increases in the hepatic damage markers GST-α and GST-Mu (Table 3-5, $P < 0.05$).

CeO$_2$ nanoparticles reduced cardiac Jak-Stat and endothelial cell activation

Sepsis increased and nanoparticle treatment decreased the phosphorylation of ERK 1/2 (Figure 3-9, Panel A, $P < 0.05$) and Stat-3 (Figure 3-9, Panel C, $P < 0.05$). These changes in protein phosphorylation were accompanied by treatment associated decreases in P-selectin (Figure 3-9, Panel E, $P < 0.05$) and VCAM-1 in the septic animals at 3 and 18 h respectively (Figure 3-9, Panel F, $P < 0.05$).

Nanoparticle treatment decreased indices of sepsis induced cardiac muscle damage

Sepsis increased and nanoparticle treatment decreased serum creatine kinase activity at 18 h (Figure 3-10, Panel A, $P < 0.05$). Similarly, nanoparticle treatment decreased sepsis induced increases in circulating myoglobin levels at 3 h and 18 h (Table 3-5, $P < 0.05$).

Cerium oxide nanoparticles modulated the inflammatory response in the peritoneum by decreasing the recruitment of immune cells

The number of circulating WBC, lymphocytes, monocytes and granulocytes was not changed with sepsis or nanoparticle treatment. Conversely, nanoparticle treatment reduced sepsis induced increases in the number of peritoneal lymphocytes and monocytes at 18 h (Table 3-6, $P < 0.05$).

Discussion

Despite decades of extensive research the sepsis induced mortality rate remains unacceptably high. The primary aim of this work was to evaluate whether CeO_2 nanoparticles are protective against sepsis induced organ damage and death in the Sprague Dawley rat. To test this possibility, we utilized a polymicrobial model of severe sepsis that is characterized by 100% mortality. Compared to the untreated controls, we found that a one-time CeO_2 nanoparticle intervention was able to decrease the mortality rate to only 25% (Figure 3-6).

To explore the mechanistic basis of this finding, we determined where the injected CeO_2 nanoparticles may accumulate. Previous work has shown that injected CeO_2 nanoparticles exhibit a proclivity to preferentially collect in the spleen, liver, kidneys, and lungs [17].

Consistent with these data, we found significantly higher amounts of CeO_2 nanoparticles in the liver compared to that observed in the heart (Table 3-2). To examine if the CeO_2 nanoparticles were able to protect the liver against a septic insult, we next examined if treatment was associated with improvements in liver morphology. Our histological studies demonstrated that sepsis caused hepatic sinusoidal dilatation and congestion of hepatocytes which appeared to be decreased with CeO_2 nanoparticle treatment (Figure 3-7). At the cellular level, sepsis is characterized by an increase in ROS and RNS which leads to cellular protein damage through carbonylation, nitrosylation or formation of AGE products [140-142]. Studies have shown that CeO_2 nanoparticles act as a free radical scavenger in addition to accelerating the decay of peroxynitrite which can induce protein nitrosylation [22, 143, 144]. As expected from our morphological analysis, we found that sepsis increased hepatic superoxide production, systemic ROS levels, and iNOS expression and importantly, that these changes were attenuated with nanoparticle treatment (Figures 3-7, 3-8).

Given that the liver is thought to be the primary source for the excessive inflammatory state seen with sepsis [145], we next sought to determine if the morphological changes we observed were also associated with modulation of the hepatic inflammatory response. Interestingly, we found that the CeO_2 nanoparticle treatment attenuated sepsis induced alterations in several different types of chemokines, cytokines, growth factors and inflammatory proteins (Tables 3-3, 3-4, 3-5). Of particular importance, we found that the CeO_2 nanoparticle treatment functioned to attenuate sepsis-induced increases in serum GST-α and GST-mu which are characteristic of hepatic damage (Table 3-5). In addition, we also found that

the levels of the inflammatory cytokine IL-6 and LIF were also significantly decreased with treatment (Table 3-4).

Previous studies have shown that IL-6 and LIF can bind to gp130 to cause activation of the Jak-Stat pathway [146, 147]. Other work has shown that the phosphorylation of ERK 1/2 can lead to activation of Stat-3 that can cause increased transcription of several inflammatory mediators [148, 149]. Here we show that sepsis led to activation of Jak-Stat pathway in the failing heart. Treatment with CeO_2 nanoparticles attenuated the activation of the ERK 1/2-Jak-Stat pathway (Figure 3-9). Moreover, studies have shown that Stat-3 signaling in the heart can cause increased microvascular permeability by increasing the expression of ICAM-1 and VCAM-1 [150]. It is thought that the increased expression of ICAM-1 and VCAM-1 on the endothelium is one of the priming events for recruitment of macrophages and neutrophils into myocardium which can lead to increased microvascular permeability [151]. Consistent with our finding of decreased ERK 1/2-Jak-Stat activation, we found that nanoparticle treatment attenuated sepsis induced increases in VCAM-1 expression (Figure 3-9). To explore the possibility that this treatment induced decrease in endothelial activation was associated with diminished vascular leakiness, we next examined the effect of the nanoparticle intervention on the accumulation of inflammatory cells in the peritoneal cavity. As expected, we found that the treated animals exhibited decreases in the number of peritoneal monocytes and lymphocytes (Table 3-6). Finally, we next asked if treatment might decrease sepsis induced cardiac muscle damage. Creatine kinase and myoglobin have been used in various pre-clinical and clinical studies as markers of cardiac damage [152-156]. We found that the treated animals had less serum creatine kinase activity and myoglobin levels than the untreated septic animals (Figure 3-10,

Table 3-5). While this study provides mechanistic insight into how the administration of CeO_2 nanoparticles might protect against a septic insult further experiments examining other organs and organ systems are likely warranted.

Conclusion

Our data suggest that a single dose of CeO_2 nanoparticles in the absence of antibiotics or fluid resuscitation, or other supportive treatment can significantly decrease animal mortality in a severe sepsis model. This increase in animal survivability was associated with modulation of the hepatic inflammatory response (Figure 3-11).

Acknowledgements

Figures

Figure 3-5. Characterization of CeO₂ nanoparticles.

CeO₂ nanoparticles are characterized by A) DLS B) TEM C) STEM-HAAF D&E) STEM F) EELS G) XRD
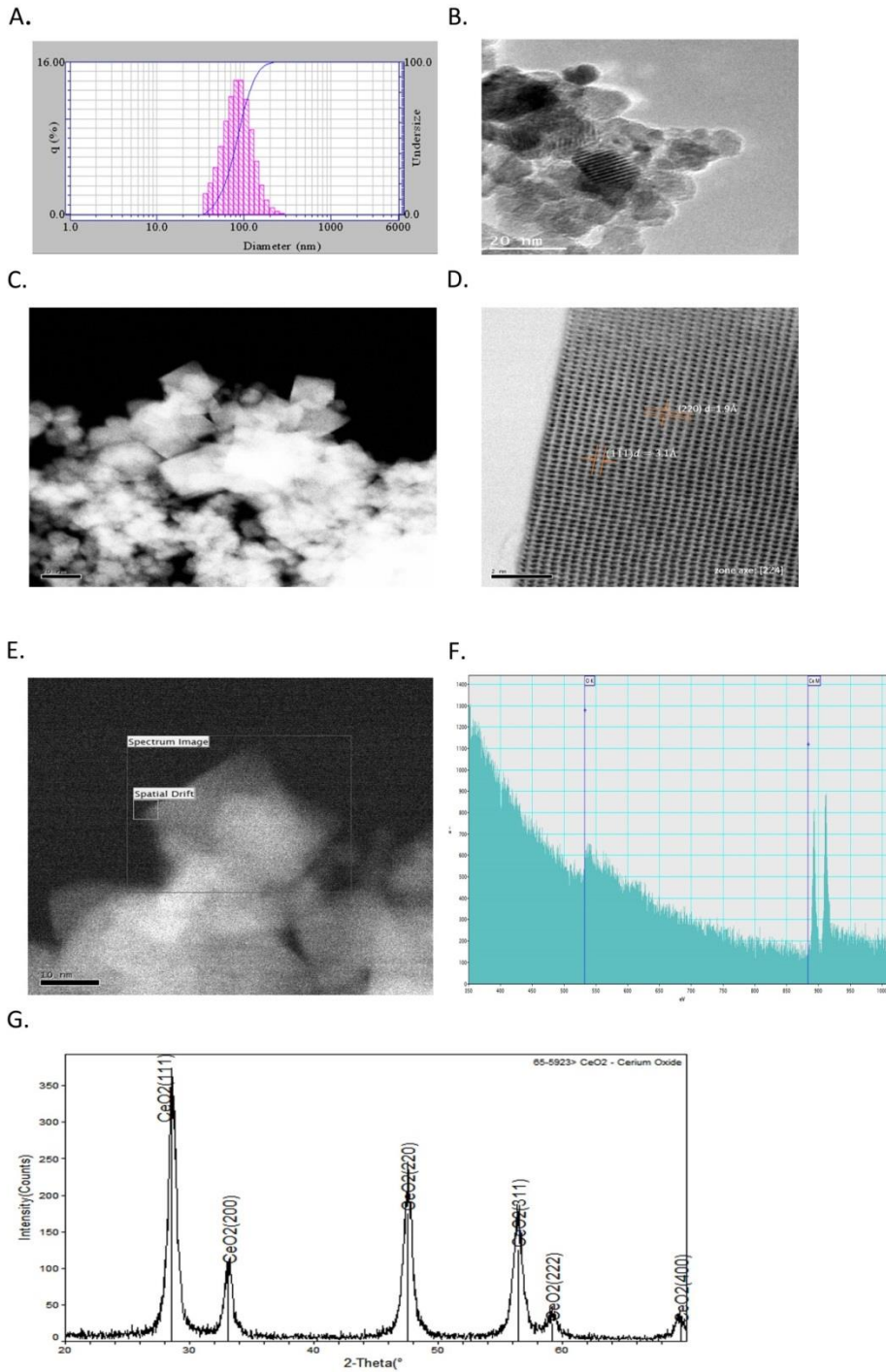
Figure 3-6. Nanoparticle treatment decreased sepsis induced mortality, animal hypothermia and total serum ROS levels.

A) Survival curve. B) Temperature. Control (n=8), CeO_2 (n=8), Sepsis (n=16), Sepsis+CeO_2 (n=16). *P<0.05 compared to control group, # P<0.05 compared to sepsis group and $ indicates that the last known temperature of dead sepsis animal has been used in consecutive time points for statistical purposes. C) Effect of nanoparticle treatment on total serum ROS levels (n=6/group). *P<0.05 compared to control group, $ P<0.05 compared to CeO_2 group and # P<0.05 compared to sepsis group

A.

B.

C.

18H
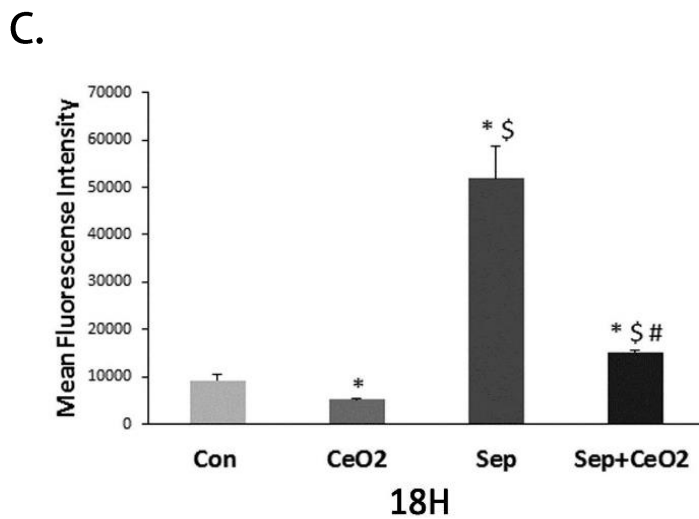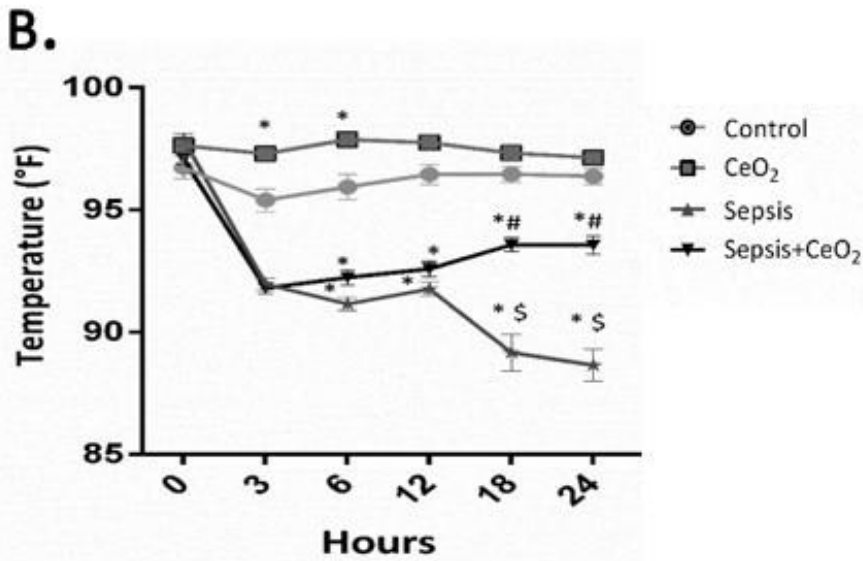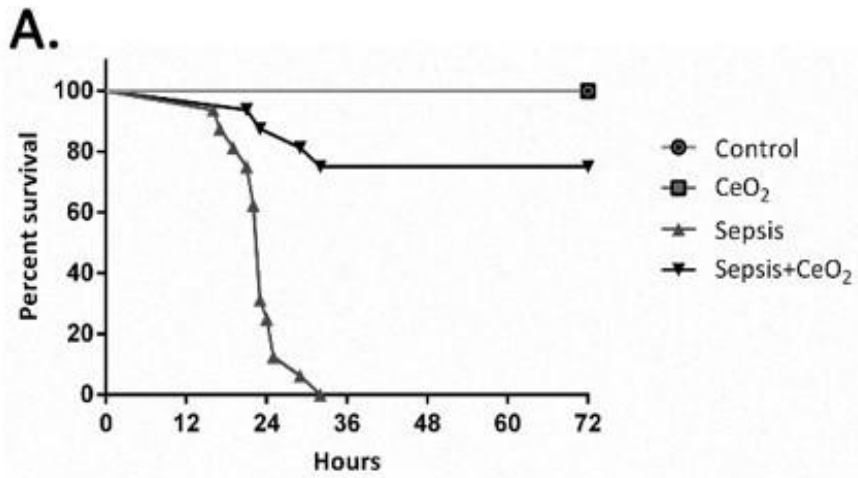
Figure 3-7. Effect of CeO$_2$ nanoparticles on sepsis induced hepatic inflammatory damage.

H&E staining of 18h time point liver sections imaged at 200X magnification A) Control B) CeO$_2$ C) Sepsis D) Sepsis+CeO$_2$. Dihydroethidium staining of 18h time point liver sections imaged at 200X magnification E) Control F) CeO$_2$ G) Sepsis H) Sepsis+CeO$_2$ and I) Quantification of superoxide levels in different groups. *P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=4/group).
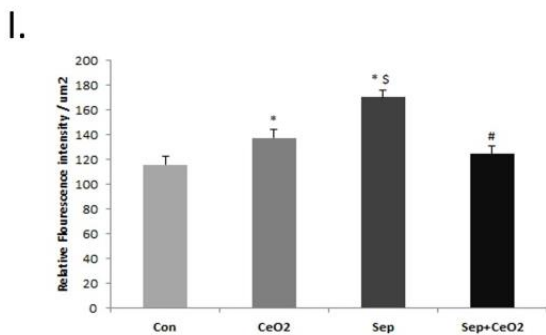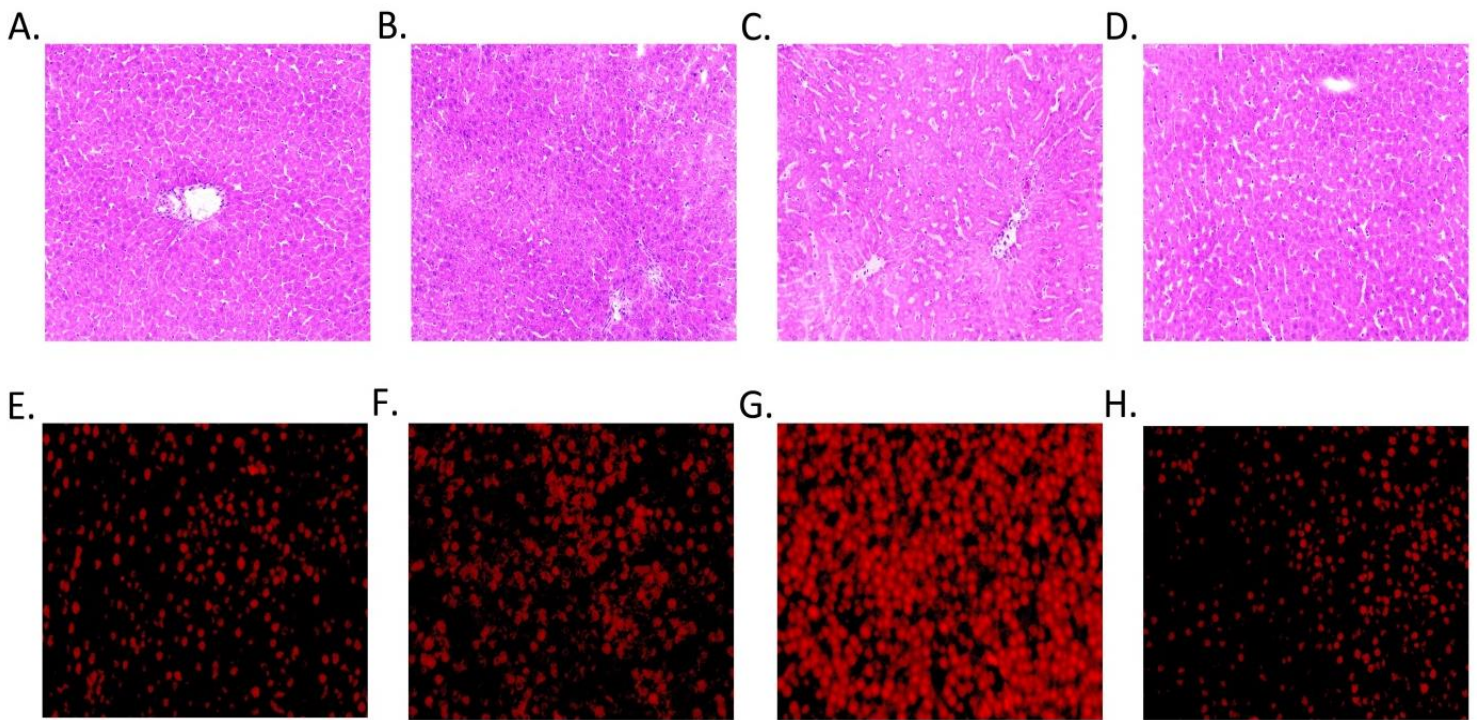
Figure 3-8. Effect of CeO$_2$ nanoparticles on sepsis induced hepatic nitrosative stress.

A) Levels of iNOS as determined by western blotting and normalized to GAPDH. B) Levels of nitrotyrosine as determined by western blotting and normalized to GAPDH. *P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=6/group).
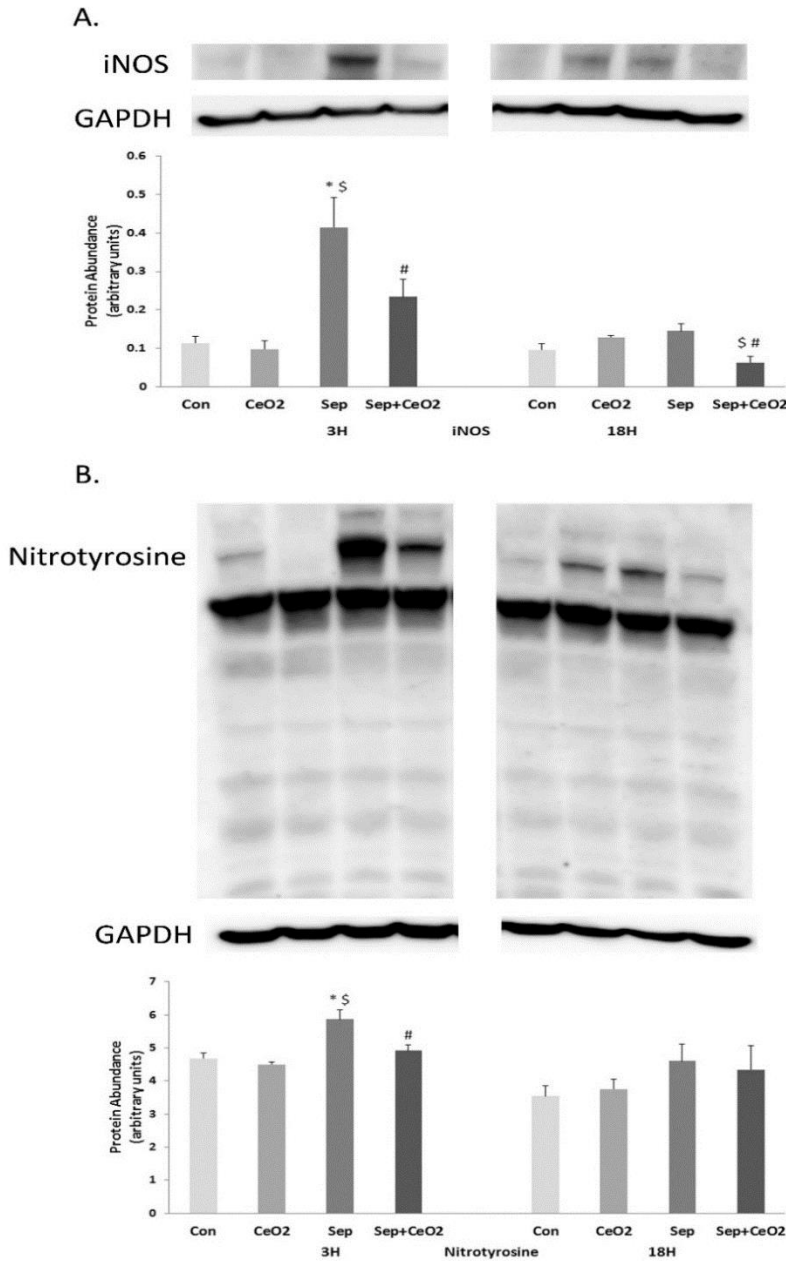
Figure 3-9. Effect of CeO$_2$ nanoparticles on sepsis induced cardiac inflammation.

A & B) Levels of phosphorylated and total ERK1/2 as determined by western blotting and normalized to GAPDH respectively. C & D) Levels of phosphorylated and total Stat-3 as determined by western blotting and normalized to GAPDH respectively. E) Levels of P selectin as determined by western blotting and normalized to GAPDH F) Levels of VCAM-1 as determined by western blotting and normalized to GAPDH. *P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=6/group).

62
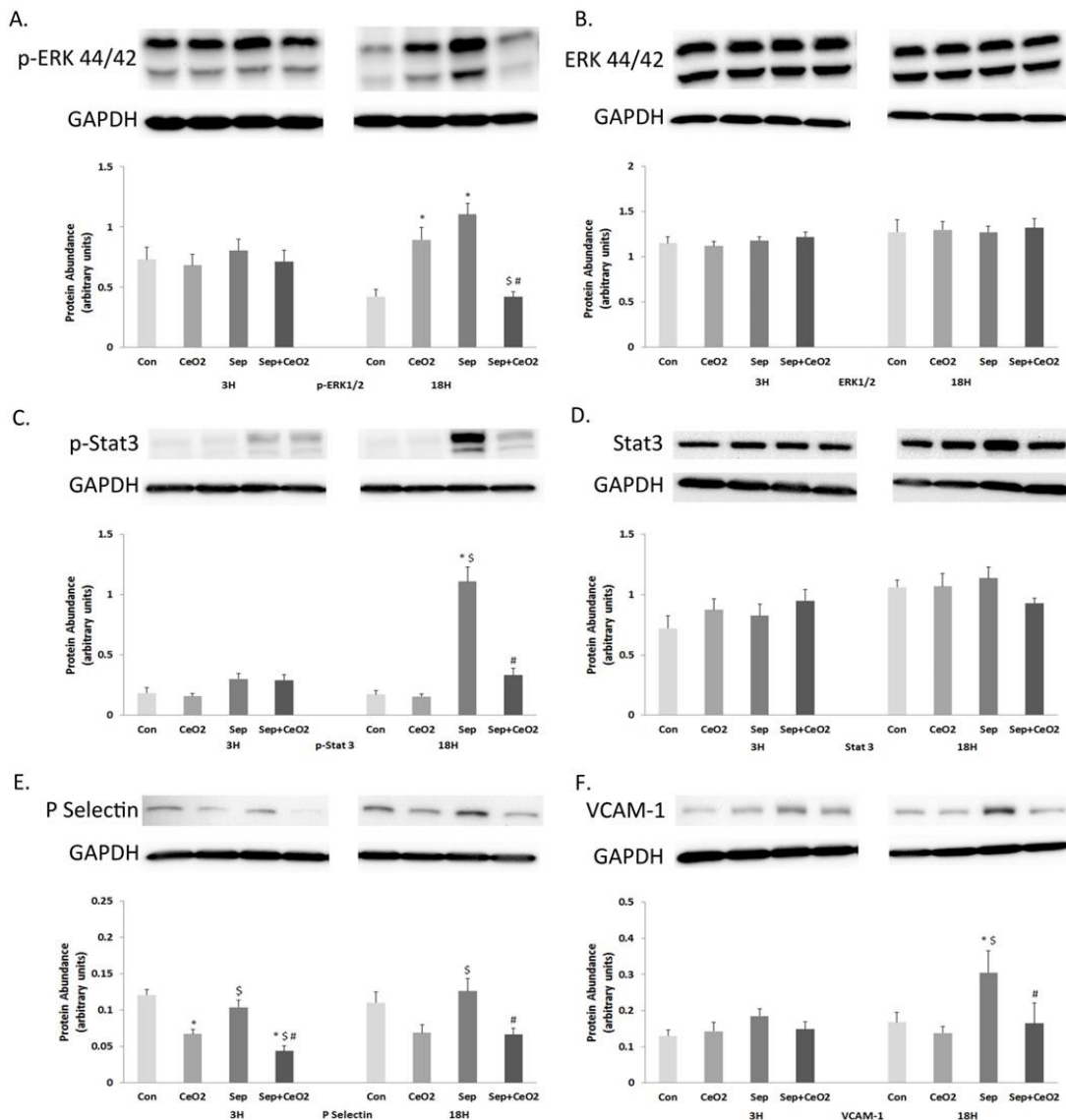
Figure 3-10. Cerium oxide nanoparticles attenuated sepsis induced serum creatine kinase activity.

*P≤0.05 compared to control group, $ P≤0.05 compared to CeO$_2$ group and # P≤0.05 compared to sepsis group. (n=6/group).

Figure 3-11. Schematic representation of mechanism of action of CeO_2 nanoparticles against polymicrobial sepsis induced MODS and death.

Tables

Table 3-2. Levels of ceria content in heart and liver as determined through ICP-MS.

Results are presented as mean ± SEM. N=3/group.

Organ	Control 3h	CeO$_2$ 3h	Sepsis 3h	Sepsis + CeO$_2$ 3h	Control 18h	Ceo$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h
Heart	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Liver	<LLOQ	12.00±0.76 ppm	<LLOQ	9.07±0.08 ppm	<LLOQ	10.67±0.17 ppm	<LLOQ	12.00±0.28 ppm

Table 3-3. Effect of CeO₂ nanoparticles on sepsis induced serum chemokines.

*P<0.05 compared to control group, $ P<0.05 compared to CeO₂ group and # P<0.05 compared

to sepsis group. (n=6/group).

| Analyte	Serum								Peritoneal fluid	
	Sham control 3h	CeO$_2$ 3h	Sepsis 3h	Sepsis + CeO$_2$ 3h	Sham control 18h	CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h
Eotaxin (pg/mL)	362.33±1.76	354.67±8.29	1273.33±28.48 [*$]	856.00±14.47 [*$#]	808.33±41.79	349.33±5.55 [*]	1866.67±26.03 [*$]	1020.00±5.77 [*$#]	2636.67±71.72	3716.67±131.70 [#]
Growth-Regulated Alpha Protein (ng/mL)	0.06±0.00	0.04±0.00 [*]	3.50±0.06 [*$]	2.57±0.03 [*$#]	Below LLOQ	Below LLOQ	5.23±0.07	2.20±0.06 [#]	23.33±0.33	16.67±0.33 [#]
Macrophage-Derived Chemokine (pg/mL)	938.00±18.56	995.67±27.42	1483.33±26.03 [*$]	1553.33±31.80 [*$]	1039.67±32.46	711.00±5.20 [*]	4410.00±55.68 [*$]	2500.00±60.00 [*$#]	24433.33±896.91	21633.33±1039.76
Macrophage Inflammatory Protein-1alpha (ng/mL)	Below LLOQ	Below LLOQ	4.20±0.20	6.37±0.17 [#]	Below LLOQ	Below LLOQ	20.67±0.67	6.70±0.29 [#]	1740.00±17.32	1413.33±71.26 [#]
Macrophage Inflammatory Protein-1 beta (pg/mL)	268.67±16.15	261.67±9.13	3003.33±92.07 [*$]	4766.67±93.87 [*$#]	199.00±30.83	128.33±7.97	8486.67±225.86 [*$]	5896.67±61.73 [*$#]	416000.00±13576.94	493666.67±25208.02
Macrophage Inflammatory Protein-2 (pg/mL)	25.67±1.20	20.67±1.33 [*]	318.33±7.26 [*$]	215.00±2.65 [*$#]	23.33±2.33	Below LLOQ	654.33±10.71 [*]	257.00±6.08 [*#]	43933.33±2760.64	32533.33±1502.59 [#]
Monocyte Chemotactic Protein 1 (pg/mL)	897.33±16.50	848.00±17.95	2270.00±65.06 [*$]	2240.00±75.06 [*$]	1483.33±42.56	973.00±19.66 [*]	2903.33±121.70 [*$]	2650.00±45.09 [*$#]	179333.33±6696.67	169000.00±9291.57
Monocyte Chemotactic Protein 3 (pg/mL)	723.00±21.00	655.67±17.05	2016.67±6.67 [*$]	1700.00±30.55 [*$#]	1009.00±23.97	597.67±12.55 [*]	3600.00±34.64 [*$]	3333.33±14.53 [*$#]	125333.33±3179.80	146000.00±5686.24 [#]
Monocyte Chemotactic Protein-5 (pg/mL)	Below LLOQ	Below LLOQ	3.70±0.17	1.98±0.42 [#]	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ
T-Cell-Specific Protein RANTES (pg/mL)	1.10±0.00	1.04±0.06	2.50±0.06 [*$]	2.10±0.06 [#]	1.43±0.03	0.74±0.01 [*]	2.30±0.00 [*$]	1.60±0.00 [*$#]	4.37±0.03	3.20±0.12 [#]

Table 3-4. Effect of CeO2 nanoparticles on sepsis induced serum cytokines.

*P<0.05 compared to control group, $ P<0.05 compared to CeO_2 group and # P<0.05 compared to sepsis group. (n=6/group).

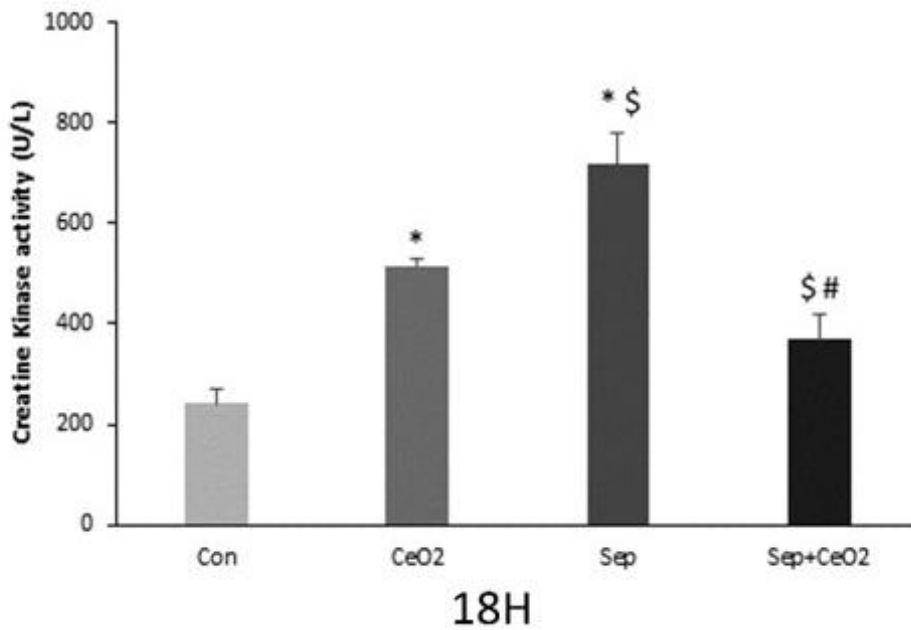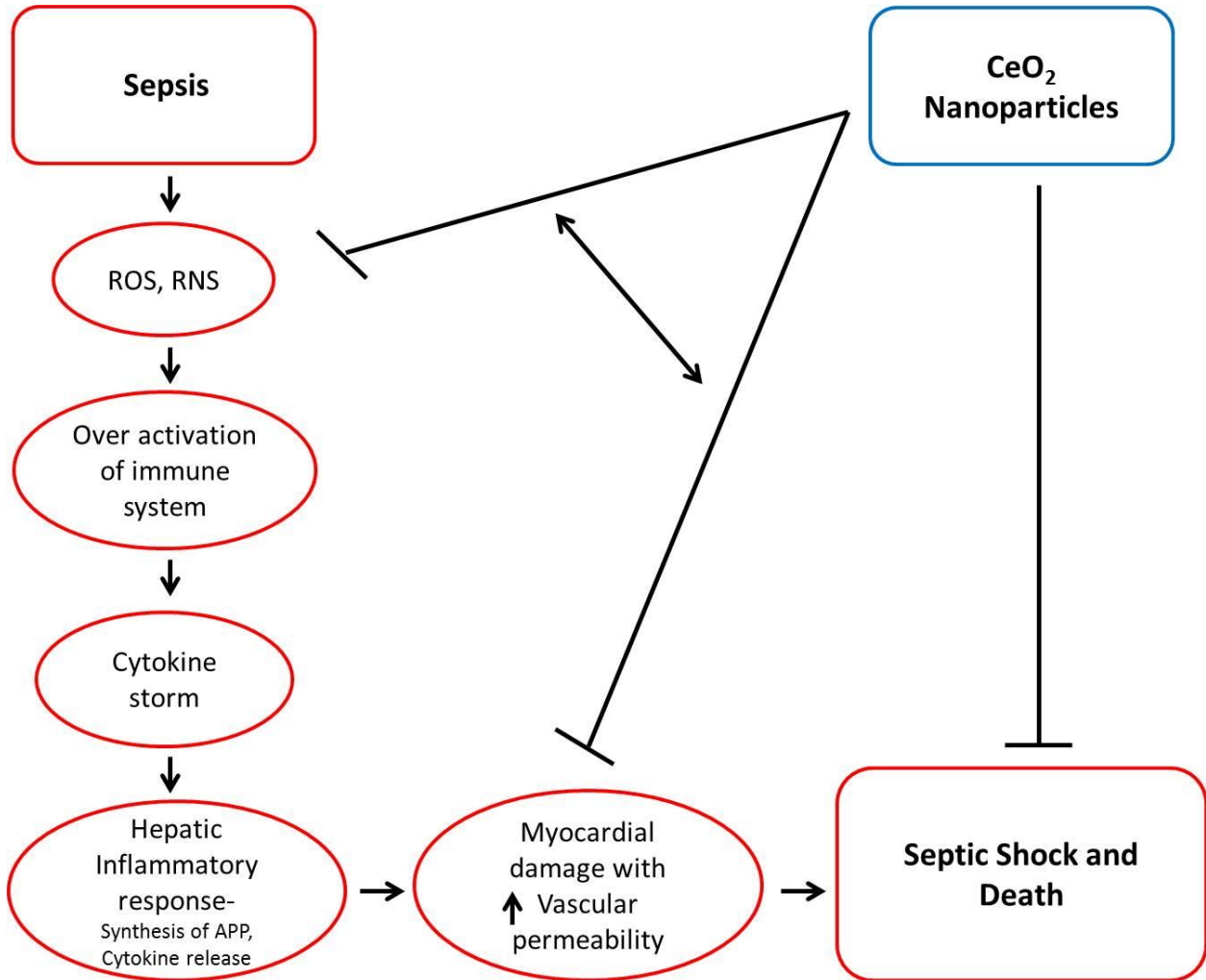| Analyte	Serum								Peritoneal fluid	
	Sham control 3h	CeO_2 3h	Sepsis 3h	Sepsis + CeO_2 3h	Sham control 18h	CeO_2 18h	Sepsis 18h	Sepsis + CeO_2 18h	Sepsis 18h	Sepsis + CeO_2 18h
Interferon gamma (IFN-gamma) (pg/mL)	Below LLOQ	Below LLOQ	102.33±5.78	67.00±8.00[#]	36.67±9.21	Below LLOQ	46.67±14.33	38.33±9.74	48.67±2.19	15.67±0.67[#]
Interferon gamma Induced Protein 10 (IP-10) (pg/mL)	42.33±2.60	40.67±2.85	192.33±4.98[*$]	153.33±5.55[*$#]	48.33±3.71	28.33±0.67[*]	117.00±1.73[*$]	91.67±2.67[*$#]	22.67±0.88	16.33±0.33[#]
Interleukin-6 (IL-6) (pg/mL)	Below LLOQ	Below LLOQ	7.50±1.00	4.97±0.27	Below LLOQ	Below LLOQ	5.27±0.67	Below LLOQ	36.33±1.76	13.67±0.33[#]
Interleukin-12 Subunit p70 (IL-12p70) (ng/mL)	0.13±0.03	0.12±0.02	0.28±0.02[*$]	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	0.09±0.01	0.06±0.01[#]
Leukemia Inhibitory Factor (LIF) (pg/mL)	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	1520.00±30.00	Below LLOQ	12000.00±321.46	7616.67±141.93[#]
Oncostatin-M (OSM) (ng/mL)	0.37±0.01	0.32±0.01[*]	0.81±0.05[*$]	0.66±0.03[*$#]	0.42±0.04	Below LLOQ	0.94±0.03[*]	0.73±0.02[*#]	19.33±1.76	27.67±1.33[#]
Tumor Necrosis Factor alpha (TNF-alpha) (ng/mL)	0.10±0.02	Below LLOQ	0.22±0.01[*]	0.13±0.01$[#]	Below LLOQ	Below LLOQ	0.14±0.01	0.14±0.01	0.39±0.01	0.34±0.01[#]

Table 3-5. Effect of CeO$_2$ nanoparticles on sepsis induced serum growth factors and other proteins related to inflammation.

*P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=6/group).

Analyte	Serum								Peritoneal fluid	
	Sham control 3h	CeO$_2$ 3h	Sepsis 3h	Sepsis + CeO$_2$ 3h	Sham control 18h	CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h
Fibroblast Growth Factor 9 (ng/mL)	3.82±1.27	3.87±0.38	10.20±0.42 *$	7.60±0.26 *$#	5.37±0.47	2.52±0.54 *	11.67±0.33 *$	9.43±0.38 *$#	2.90±0.12	1.83±0.20#
Leptin (ng/mL)	0.44±0.02	0.43±0.02	0.75±0.01 *$	0.68±0.02 *$#	0.27±0.01	0.25±0.01	2.97±0.15 *$	1.83±0.07#	10.00±0.00	10.00±0.00
Myeloperoxidase (ng/mL)	11.00±0.58	11.33±0.33	30.67±0.88 *$	24.00±0.58 *$#	25.67±0.33	25.00±0.58	39.33±0.67 *$	34.00±0.00 *$#	16433.33±970.11	11600.00±305.51#
Myoglobin (ng/mL)	1153.33±12.02	1373.33±31.80 *	3083.33±23.33 *$	1546.67±20.28 *$#	1086.67±31.80	1186.67±17.64	6220.00±200.75 *$	3246.67±52.39 *$#	1004.33±22.41	756.67±15.45#
Glutathione S-Transferase alpha (ng/mL)	54.00±2.00	30.33±1.67*	69.33±2.19*$	42.67±3.93*$#	62.67±2.60	35.00±1.53*	2126.67±56.67*$	147.00±1.53*$#	213.33±11.78	138.00±6.56#
Glutathione S-Transferase Mu (ng/mL)	376.33±20.20	Below LLOQ	228.33±37.44*	173.33±25.43*	232.00±0.00	Below LLOQ	14133.33±328.30*	764.67±40.53#	10666.67±272.84	5830±245.02#
Vascular Endothelial Growth Factor A (pg/mL)	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	459.00±12.70	296.67±31.33#	5563.33±149.48	5763.33±79.65

Table 3-6. Effect of CeO$_2$ nanoparticles on sepsis induced changes in immune cells.

$ P<0.05 compared to CeO$_2$ group, # P<0.05 compared to sepsis group. (n=5-9/group).

Cell Type	Whole Blood								Peritoneal Fluid	
	Control 3h	CeO$_2$ 3h	Sepsis 3h	Sepsis + CeO$_2$ 3h	Control 18h	CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h
WBC (10^9/L)	2.156±0.689	1.410±0.331	1.406±0.235	2.158±0.652	2.821±0.385	2.124±0.330	2.106±0.439	2.612±0.486	27.045±2.357	24.506±6.031
Lymphocytes (10^9/L)	0.946±0.321	0.887±0.362	0.646±0.094	1.292±0.356	1.194±0.253	0.959±0.245	1.266±0.314	1.514±0.335	3.191±0.441	1.756±0.382[#]
Monocytes (10^9/L)	0.060±0.0193	0.0117±0.003	0.120±0.031	0.092±0.055	0.126±0.041	0.191±0.034	0.0256±0.010[$]	0.191±0.075	2.766±0.385	1.440±0.513[#]
Granulocytes (10^9/L)	1.153±0.418	0.540±0.159	0.640±0.148	0.778±0.314	1.504±0.349	0.973±0.090	0.025±0.09	0.910±0.142	21.091±1.981	19.124±4.318

Paper-3

The following paper corresponds to specific aim-3 and deals with the hypothesis whether CeO$_2$

nanoparticles can attenuate severe sepsis-induced inflammatory damage to the kidney during a

septic insult in male Sprague Dawley rats.

Cerium oxide nanoparticles attenuate polymicrobial sepsis induced acute kidney injury

Running title- Treatment of sepsis induced AKI with cerium oxide nanoparticles

Key Words- Cerium oxide nanoparticles, sepsis, kidney injury molecule-1, cystatin-C, oxidative stress

Abstract

Sepsis is the leading cause of death in non-cardiac intensive care units. Sepsis-induced acute kidney injury (AKI) is a complicating morbidity that is associated with poor prognosis. Herein, we examined whether treatment with cerium oxide (CeO_2) nanoparticles can reduce AKI in a severe polymicrobial sepsis model that causes severe microvascular leak that leads to deficit in circulatory volume and subsequent development of AKI. Administration of a single dose of CeO_2 nanoparticles (0.5mg/kg) to male Sprague Dawley rats attenuated sepsis-induced tubular dilatation and the loss of brush border. These changes in renal structure were accompanied by decreases in sepsis-induced renal oxidative stress, stat-3 phosphorylation, and caspase-3 cleavage which are suggestive of diminished kidney inflammation and apoptosis. At the systemic level, CeO_2 nanoparticle treatment reduced sepsis-induced increases in serum levels of kidney injury molecule-1 (KIM-1), cystatin-C, osteopontin, β-2 microglobulin, vascular endothelial growth factor-A (VEGF-A), blood urea nitrogen (BUN), potassium, sodium, and circulating glucose levels. Taken together, these data suggest that CeO_2 nanoparticles may help to prevent the development of AKI during severe sepsis.

Introduction

Severe sepsis is characterized by an overwhelming immune response that is often associated with acute kidney injury (AKI). Sepsis-induced AKI is thought to be primarily caused by the increased levels of reactive oxygen species (ROS) that are typically seen during the systemic inflammatory response syndrome (SIRS) that proceeds the development of frank sepsis [157, 158]. AKI is associated with damage to renal tubular cells, cellular apoptosis, increased tubular permeability, the loss of brush border [159] and increased tubular dilatation [72]. AKI is a serious complication which is associated with increased morbidity and poor prognosis [133]. Despite improvements in medical technology, current treatment modalities are largely supportive in nature and do little to address underlying causes.

Recent advances in nanotechnology have allowed the development of new compounds that can effectively scavenge ROS [160-162]. One such compound is cerium oxide (CeO_2) which when formulated in nanoparticle form, may be useful for the treatment of inflammation [163], diabetes [93, 164], cancer [165, 166], and ischemic stroke [103]. Herein, we examined the therapeutic efficacy of using CeO_2 nanoparticles for the treatment of sepsis-induced AKI. On the basis of previous data demonstrating that CeO_2 nanoparticles can function to scavenge ROS, we hypothesized that CeO_2 nanoparticle treatment would be associated with decreased renal oxidative stress and sepsis-induced damage.

Materials and Methods

Characterization of CeO$_2$ nanoparticles

CeO$_2$ nanoparticles were commercially purchased from US Research Nanomaterials, Inc. (Houston, TX). Atomic force microscopy was performed to estimate the mean particle size. Briefly, 20 µl of CeO2 nanoparticles was placed on freshly cleaved mica (V1 mica, SPI Inc, West Chester, PA) incubated for 15 minutes, rinsed with deionized water and dried under nitrogen. Nanoparticles were imaged in noncontact mode at a frequency of 319 kHz and a scan speed of 0.5 Hz using a Nano-R microscope (Pacific Nanotechnology Inc., Santa Clara, CA) equipped with a TM300A noncontact probe (SensaProbes, Inc., Santa Clara, CA). The size of the CeO$_2$ particles was characterized by a JEOL JEM-2010 transmission electron microscope.

Polymicrobial sepsis induction and CeO$_2$ nanoparticle treatment

Seventy two male Sprague Dawley rats aged 10 weeks were purchased from Hill-Top laboratories and allowed to acclimatize for 2 weeks prior to experimentation. All surgical procedures were performed in accordance with the guidelines provided by the Marshall University Institutional Animal Care and Use Committee (IACUC), and The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Briefly, animals were anesthetized under isoflurane and a small mid ventral incision of 0.5 cm was made. Sham controls and CeO$_2$ only groups were injected with 5ml/kg of 5% sterile dextrose solution intraperitoneally (*i.p.*) while sepsis and sepsis+CeO$_2$ groups received cecal inoculum of 600mg/kg BW in 5ml/kg BW of 5% sterile dextrose solution *i.p*. Cecal material was obtained

from healthy rats that served as donors. Sham control and sepsis groups were injected with 200 μl of sterile distilled water intravenously (*i.v.*) while the CeO_2 and sepsis+CeO_2 groups received CeO_2 nanoparticles (0.5mg/kg) in 200 μl of sterile distilled water *i.v.* via the tail vein at the time of sham surgery or sepsis induction.

Tissue collection

Animals from different sets were humanely sacrificed under isoflurane anesthesia at 3h and 18h after sepsis induction and the kidneys were excised, the capsule removed and were washed in Krebs–Ringer bicarbonate buffer (KRB) to remove any blood. Kidneys were frozen in liquid nitrogen and stored at -80°C for further analysis. Serum was obtained from whole blood through cardiac puncture by centrifugation at 5,000 x g for 10 min at room temperature.

Renal histology and staining for F-actin

Frozen kidneys were sectioned (4μm) with a Leica CM1950 cryostat and transferred to poly-L-lysine coated slides. Hematoxylin and eosin staining was performed using a Histoperfect kit (BBC biochemical, Seattle WA) to assess kidney morphology and imaged using an Evos XL microscope (Life Technologies, Grand Island, NY). Renal sections were also stained for F-actin using rhodamine phalloidin (Life Technologies, Grand Island, NY) as detailed elsewhere [167]. Briefly, frozen sections were washed with PBS and then fixed in 4% methanol free formaldehyde for 10 min. After a PBS wash (3 x 5 min), sections were permeabilized with 0.3% Triton X-100 in PBS for 20 min, washed with PBS (3 x 5 min) and then blocked with 1% BSA for 30 min. Actin was stained by incubation of the tissue sections with 0.165 μM rhodamine

phalloidin for 20 min in the dark. After washing (3 x 5 min in PBS), tissues were mounted and imaged using an Evos FL microscope (Life Technologies, Grand Island, NY) before quantifying the fluorescence intensity using Image J analysis software.

Estimation of renal superoxide levels

Levels of superoxide in renal sections were estimated using dihydroethidium staining [168]. Superoxide indicator dihydroethidium exhibits blue fluorescence under normal conditions, but exhibits a bright red fluorescence when oxidized and intercalates with DNA. Briefly, sections were washed with PBS and incubated with 5mM dihydroethidium for 1 h at room temperature in the dark. Sections were washed with PBS (3 x 5 min) and imaged with an Evos FL microscope (Life Technologies, Grand Island, NY). Mean fluorescence intensity was determined using Image J analysis software.

SDS-PAGE and immunoblotting

Approximately 100 mg of frozen kidney was pulverized to a fine powder and then homogenized in 900ul of T-PER (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors. Homogenates were centrifuged at 5,000 g x 10 min at 4°C to collect the supernatant. The 660nm protein assay (Pierce, Rockford, IL, USA) was used to determine the amount of protein in each sample. Samples were equally diluted with 4x Laemlli buffer, separated using SDS-PAGE on 10% PAGEr Gold Precast gels (Lonza, Rockland, ME) and then transferred to nitrocellulose membranes as detailed elsewhere [139]. Membranes were blocked with 5% milk in TBST for 1 h before being probed with antibodies against p-Stat-3 (Tyr 705), Stat-3, cleaved

caspase 3 and caspase 3 (Cell Signaling Technology, Danvers, MA). After washing (3 x 5 min TBST), membranes were incubated with secondary anti-rabbit antibody (Cell Signaling Technology, Danvers, MA). Immunoreactivity was visualized using Supersignal West Pico Chemiluminiscent substrate (Pierce, Rockford, IL, USA) before quantification using Fluorchem 9900 software (Protein Simple, Santa Clara, CA).

Multiplex immunoassay and serum biochemical analysis

Serum samples from different animals in each group were pooled and sent to Myriad RBM (Austin, TX) for the analysis of KIM-1, cystatin-C, osteopontin, β-2 microglobulin and VEGF-A using rodent kidney multiplex immunoassay (MAP). Pooled samples were run in triplicate for statistical analysis. Levels of glucose, BUN, sodium and potassium were determined in serum using an Abaxis VetScan® analyzer (Abaxis, Union City, CA, USA).

Statistical analysis

Results are presented in the form of mean ± standard error of mean. Differences between groups were determined using a one way analysis of variance (ANOVA) with Student Newman Keul's post hoc analysis, an ANOVA on ranks with Student Newman Keul's post hoc analysis for non-normally distributed samples or the Students t-test where applicable. A probability value of $P < 0.05$ was accepted as statistically significant.

Results

Characterization of nanoceria

CeO$_2$ nanoparticle size as determined using AFM was approximately 30 nm (Figure 3-12, Panel A). Analysis by scanning electron microscopy and transmission electron microscopy demonstrated a particle size of 10 – 40 nm (Figure 3-12, Panels B, C). Energy dispersive X-ray spectroscopy analysis of ceria and oxygen content was 80.4% and 16.36%, respectively (Figure 3-12, Panel D).

Nanoparticle treatment decreased serum indices of renal failure during sepsis

Compared to the control animals, sepsis increased the levels of β-2 microglobulin at 3 h and 18 h. Similarly, sepsis was also associated with increased levels of KIM-1, cystatin-C, osteopontin, and VEGF-A at 18h. CeO$_2$ nanoparticle treatment attenuated sepsis-induced increases in KIM-1 cystatin-C, osteopontin, β-2 microglobulin, and VEGF-A at the 18 h time point (Table 3-7, *P < 0.05*). In a similar fashion, treatment also decreased sepsis-induced increases in blood glucose, BUN, and potassium (Table 3-8, $P < 0.05$).

Cerium oxide nanoparticles attenuated sepsis induced renal damage and breakdown of tubular F-actin

Sepsis-induced AKI was characterized by renal tubular dilatation, a loss of the brush border and damage to the glomerular capillary network. These alterations in renal structure were attenuated with treatment (Figure 3-13, Panels A-H). In addition to changes in gross morphology, sepsis was also associated with a marked loss of F-actin in the proximal tubular

cells (Figure 3-14, Panel C) which was attenuated with nanoparticle treatment (Figure 3-14 Panels D, E, *P < 0.05*).

Cerium oxide nanoparticles attenuated sepsis induced oxidative stress, prevented Stat-3 activation and cleavage of caspase 3

Nanoparticle treatment decreased sepsis-induced increases in renal superoxide levels (Figure 3-15, *P < 0.05*), Stat-3 activation (Figure 3-16 Panel A, *P < 0.05*) and the cleavage of caspase-3 at 18h (Figure 3-16, Panel B, *P < 0.05*).

Discussion

Although increases in serum levels of creatinine have been considered to be a traditional biomarker for AKI, recent data has suggested that sepsis-induced AKI may exhibit a distinct pathophysiology from that seen in non-septic AKI [169]. One of the distinguishing features between non-septic AKI vs septic AKI is the presence of non-hemodynamic mechanisms of cellular injury as result of immunological, toxic and inflammatory factors in sepsis. On the basis of these data, and others demonstrating that sepsis-associated decreases in creatinine production may limit the use of this molecule as a marker for AKI [170], we chose to examine how sepsis may affect the regulation of KIM-1, β-2 microglobulin, cystatin-C, and osteopontin. Kim-1 was chosen as it is thought to be a biomarker for proximal tubular injury [171] while β-2 microglobulin was selected as it has been shown to be a reliable indicator of marker for functional status of proximal tubular cells [172]. Similarly, we also chose to examine cystatin-C as it has been shown to be a reliable surrogate for the assessment of glomerular

filtration rate [173] while osteopontin is thought to be a marker of macrophage and neutrophil infiltration [174]. Consistent with our histopathological findings, we found that sepsis-induced increases in the levels of β-2 microglobulin, KIM-1, cystatin-C and osteopontin were attenuated with CeO_2 nanoparticle treatment (Table 3-7).

In addition to changes in renal structure, sepsis has also been shown to affect the serum content of several renal metabolites and ions. Increases in blood urea nitrogen levels are a byproduct of protein metabolism with subsequent decrease in renal function. With sepsis, BUN levels have been shown to be increased due to decrease in circulatory volume as a result of marked vasodilation and microvascular leak. Consistent with this, we also found that sepsis increased serum BUN levels and importantly that these increases were diminished with nanoparticle treatment (Table 3-8). Similarly, sepsis has also been found to cause elevations in serum glucose levels [175]. Although not fully understood, it has been postulated that the development of hyperglycemia is associated with increased insulin clearance along with elevation in plasma levels of cortisol and glucagon that promote hepatic gluconeogenesis [176]. While elevation in glucose levels is an adaptive response to meet metabolic demands of the body such as wound healing, it is also shown that hyperglycemia causes predisposition to multiple complications such as neuropathy and MODS [177]. Supporting our analysis of serum BUN, we also found significant increases in serum glucose in the septic animals and that treatment appeared to attenuate the serum glucose levels. In addition to alterations in serum metabolites it is known that sepsis can also affect the concentration of serum Na+ / K+ ions. Indeed, hyponatremia and hyperkalemia are generally found in acute renal failure with decreased renal tubular flow rate [135]. Similar to previous studies we found that sepsis caused

81

a decrease in serum levels of sodium [178] and increase in potassium, which were attenuated with CeO_2 nanoparticle treatment (Table 3-8).

Sepsis-induced AKI is characterized by increases in tissue ROS which are thought to be the cause of the renal inflammation, tubular dilatation, vacuolization, and loss of brush border that characterize this disorder [179, 180]. We observed similar findings in the present study and importantly, also found that CeO_2 nanoparticle treatment appeared to attenuate these changes in the kidney. Specifically, we found that CeO_2 nanoparticles attenuated sepsis induced loss of brush border in proximal tubular cells and also attenuated tubular dilatation. Moreover, treatment with CeO_2 nanoparticles also improved renal glomerular integrity when compared to sepsis group (Figure 3-13).

To extend upon these findings, we next sought whether CeO_2 nanoparticles can protect the kidney against sepsis-induced decreases in F-actin. AKI is characterized by loss of F-actin that leads to disruption in cytoskeleton network and impairs renal structural and functional integrity [167]. Consistent with our other assessments of renal function and morphology, we found that sepsis caused a significant decrease in loss of F-actin and importantly that nanoparticle treatment appeared to attenuate these changes (Figure 3-14). Taken together with our analysis of renal function, these data suggest that the CeO_2 nanoparticle treatment functioned to improve both kidney structure and function.

We next sought to understand how the CeO_2 nanoparticles may have prevented sepsis-induced changes at the molecular level. Sepsis is characterized by increases in oxidative stress that result in uncontrolled SIRS and multi-organ failure [181]. Studies have shown that CeO_2 nanoparticles are potent ROS scavengers and that they can function as catalase and SOD

mimetics [17]. To investigate this possibility, we next examined how sepsis and nanoparticle treatment affected renal superoxide levels. Consistent with the effects of treatment on renal structure and function, we found that CeO_2 nanoparticle administration significantly decreased sepsis-induced elevations in renal superoxide oxide levels (Figure 3-15).

Given the role that inflammation plays in the pathogenesis of sepsis, we next evaluated the Janus kinase signal transduction (Jak-Stat) pathway. The Jak-Stat pathway is one of the principal signaling mechanisms for a wide variety of cytokines and growth factors that are thought to be important in regulating the renal remodeling and fibrosis [182-185]. Consistent with these data, we found that sepsis was associated with the activation of Stat-3 in the kidney at 18hand importantly, that this activation was attenuated with nanoparticle treatment (Figure 3-16). Whether this decrease in Stat-3 phosphorylation is due to treatment induced decreases in the amount of circulating cytokines or increased activation of intracellular phosphatases is currently unclear and will require further investigation.

In addition to inflammation, a growing body of evidence suggests that the apoptosis of renal tubular cells is a major causes for AKI in sepsis [186]. Studies have shown that the activation of Jak-Stat pathway in renal tubular cells results in tubular cell death through caspase 3 activation [183]. Other studies have shown that cleavage of caspase-3 is also responsible for the activation of gelsolin which has F-actin severing properties [187]. It is thought that the loss of F-actin is associated with cytoskeletal derangement that can lead to apoptotic cell death and development of AKI [167]. Supporting our F-actin data, we observed that sepsis was associated with evidence of increased caspase-3 cleavage at 18h. Similar to our other data, this finding appeared to be abrogated following nanoparticle treatment (Figure 3-16).

Conclusion

Taken together, the results of the current study demonstrate that a single dose of CeO_2 nanoparticles confers protection against severe sepsis induced acute kidney injury. Although not fully understood, our data suggest that the protective effect of the CeO_2 nanoparticles appears to be related to their ability to scavenge reactive oxygen species which prevents caspase-3 mediated loss of F-actin and renal tubular cell damage. Further investigation to better understand the pharmacological potential of CeO_2 nanoparticles for the treatment of sepsis induced AKI may be warranted.

Acknowledgements

Figures

Figure 3-12. Characterization of CeO$_2$ nanoparticles.

CeO$_2$ nanoparticles are characterized by A) AFM, B) SEM, C) TEM, D) EDS
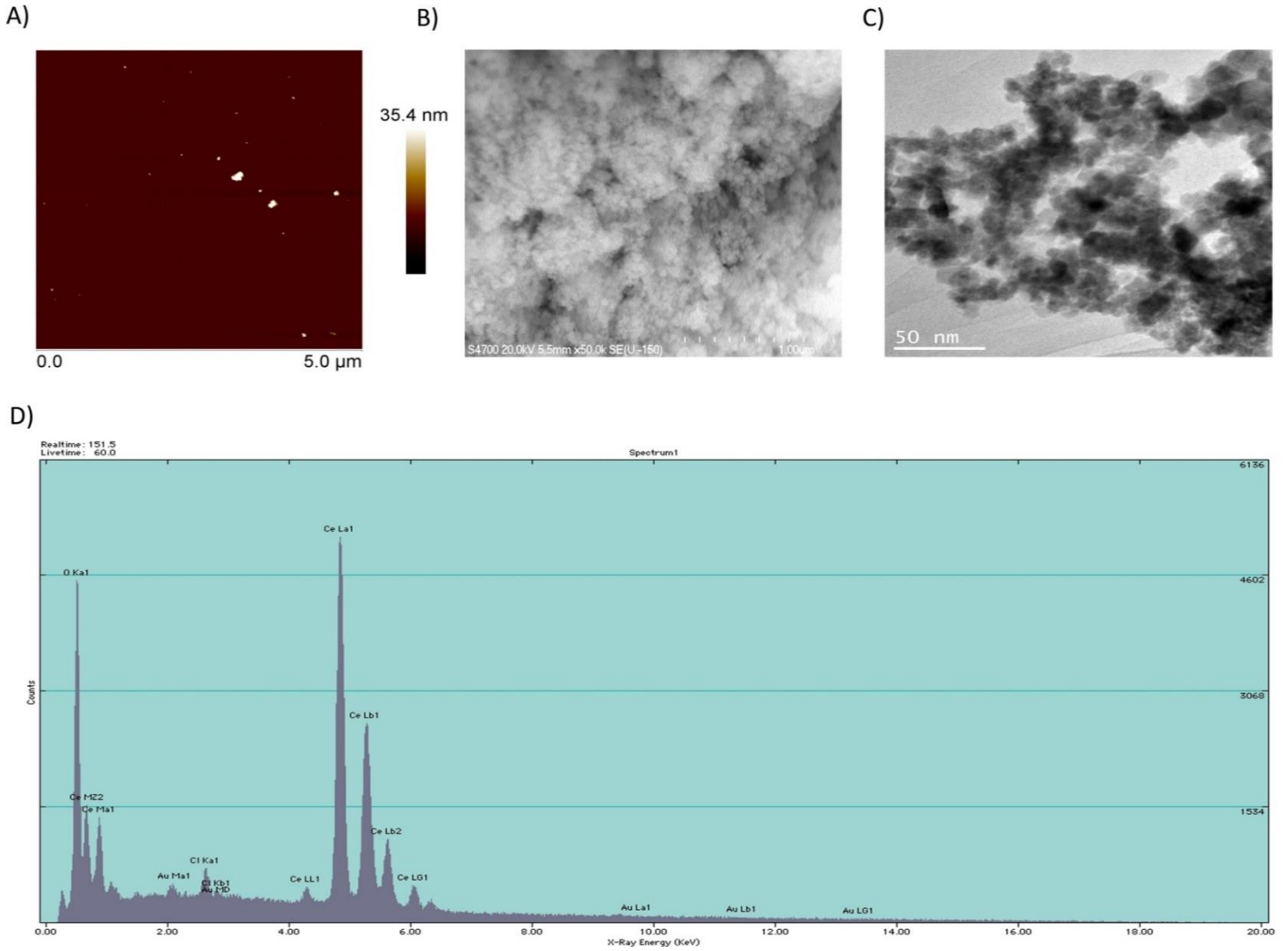
Figure 3-13. CeO$_2$ nanoparticles attenuated sepsis induced renal damage.

Hematoxylin and eosin staining of 18h time point kidney sections A) Control B) CeO$_2$ C) Sepsis D) Sepsis+CeO$_2$ (imaged at 200X magnification) E) Control F) CeO$_2$ G) Sepsis H) Sepsis+CeO$_2$ (imaged at 400X magnification) (n=4/group).
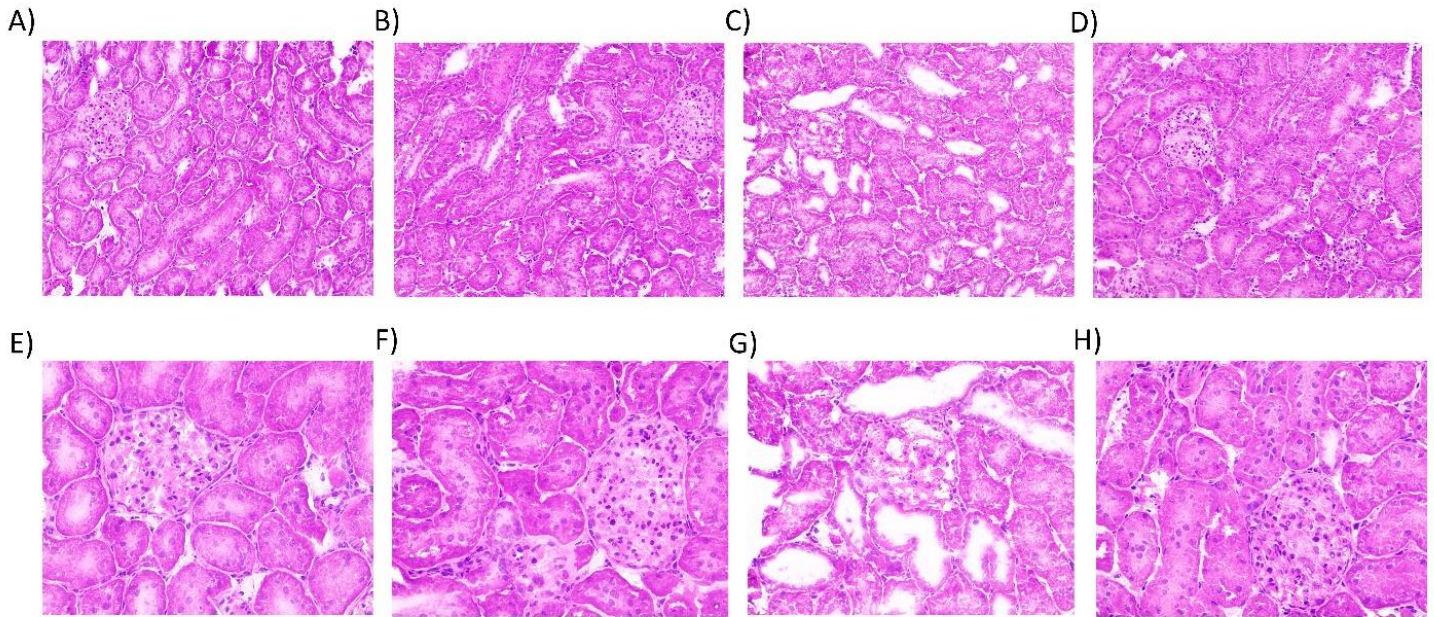
Figure 3-14. CeO$_2$ nanoparticles attenuated sepsis induced loss of F-actin.

Rhodamine phallodin staining for F-actin of 18h time point kidney sections imaged at 200X magnification A) Control B) CeO$_2$ C) Sepsis D) Sepsis+CeO$_2$ and E) Relative fluorescence intensity as a measure of F-actin. *P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=3/group).
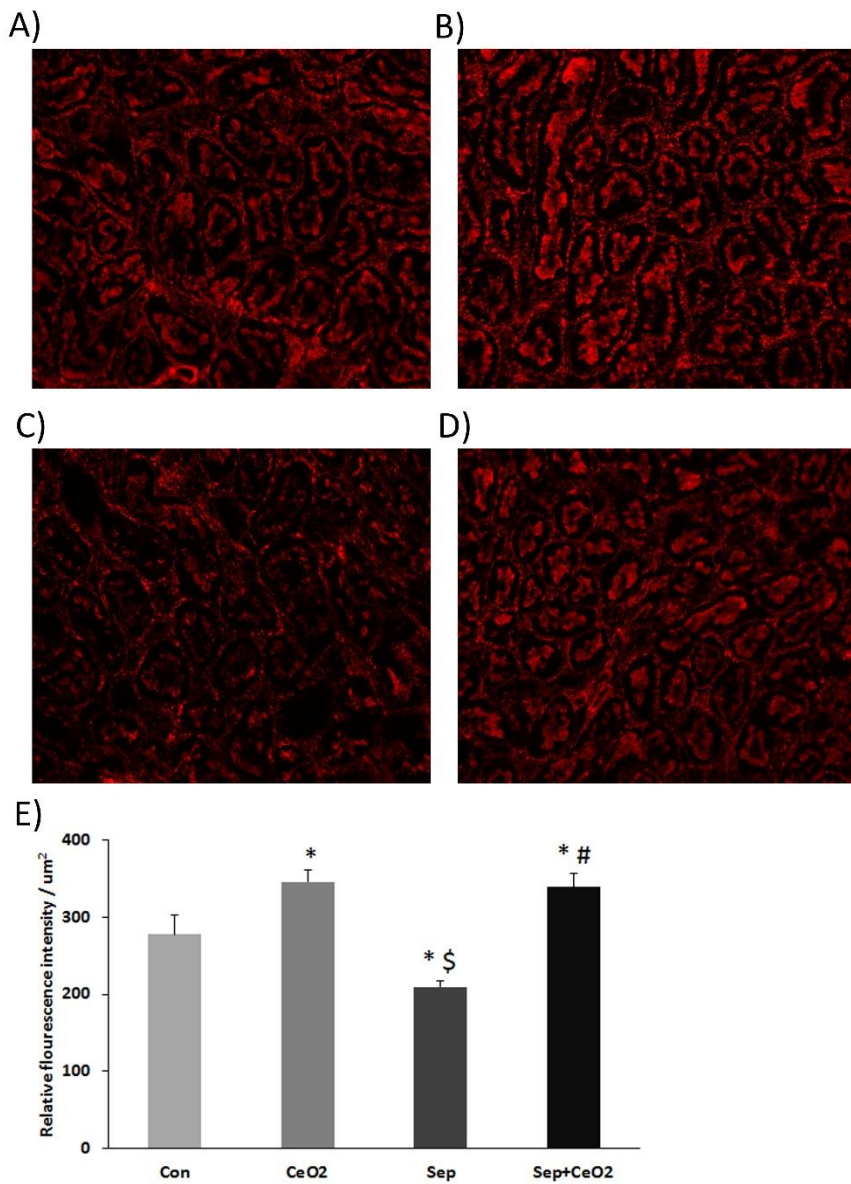
Figure 3-15. CeO$_2$ nanoparticles attenuated sepsis induced renal superoxide levels.

Dihydroethidium staining of 18h time point kidney sections imaged at 200X magnification A) Control B) CeO$_2$ C) Sepsis D) Sepsis+CeO$_2$ and E) Quantification of superoxide levels in different groups. *P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=4/group).
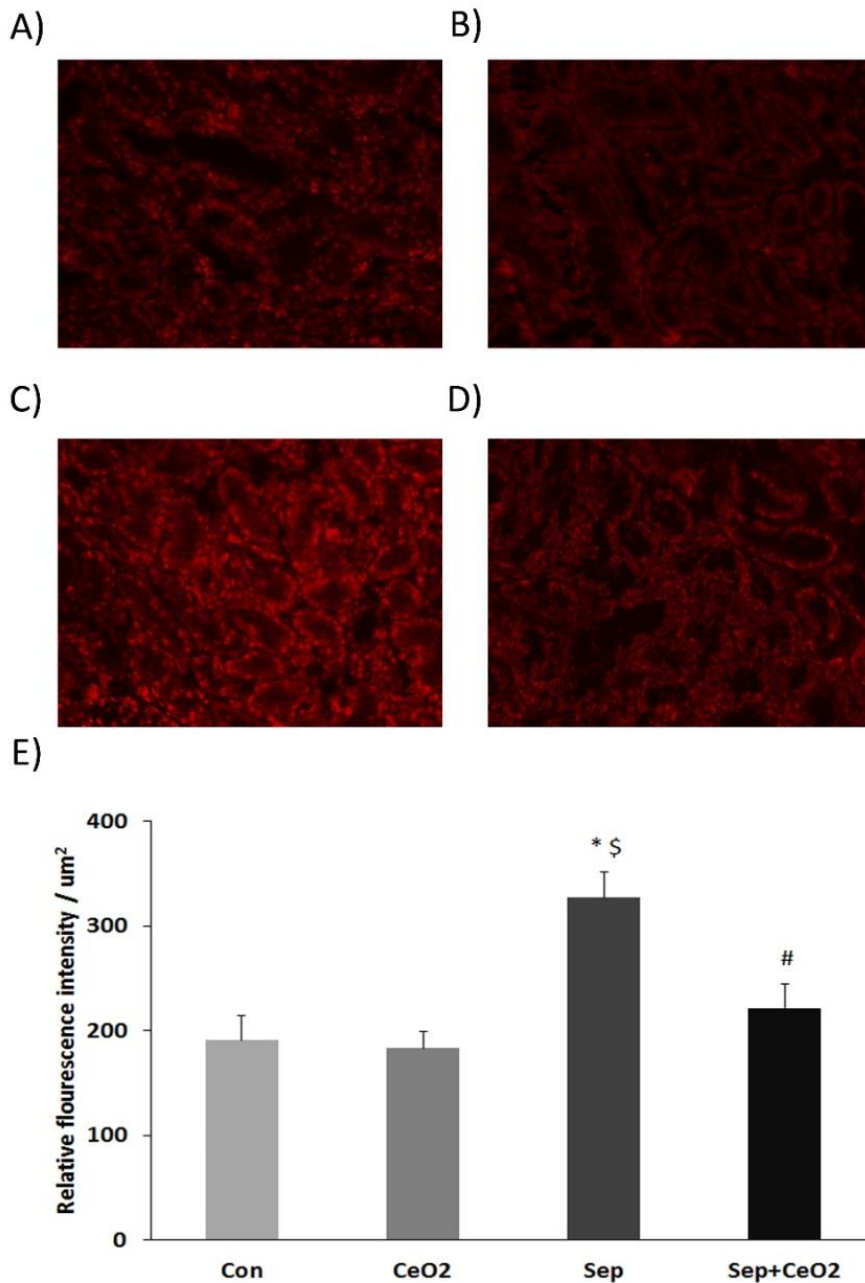
Figure 3-16. CeO$_2$ nanoparticles attenuated sepsis induced renal inflammation and apoptosis.

A) Levels of phosphorylated to total Stat-3 as determined by western blotting. B) Levels of cleaved to total caspase 3 as determined by western blotting. *P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=6/group).
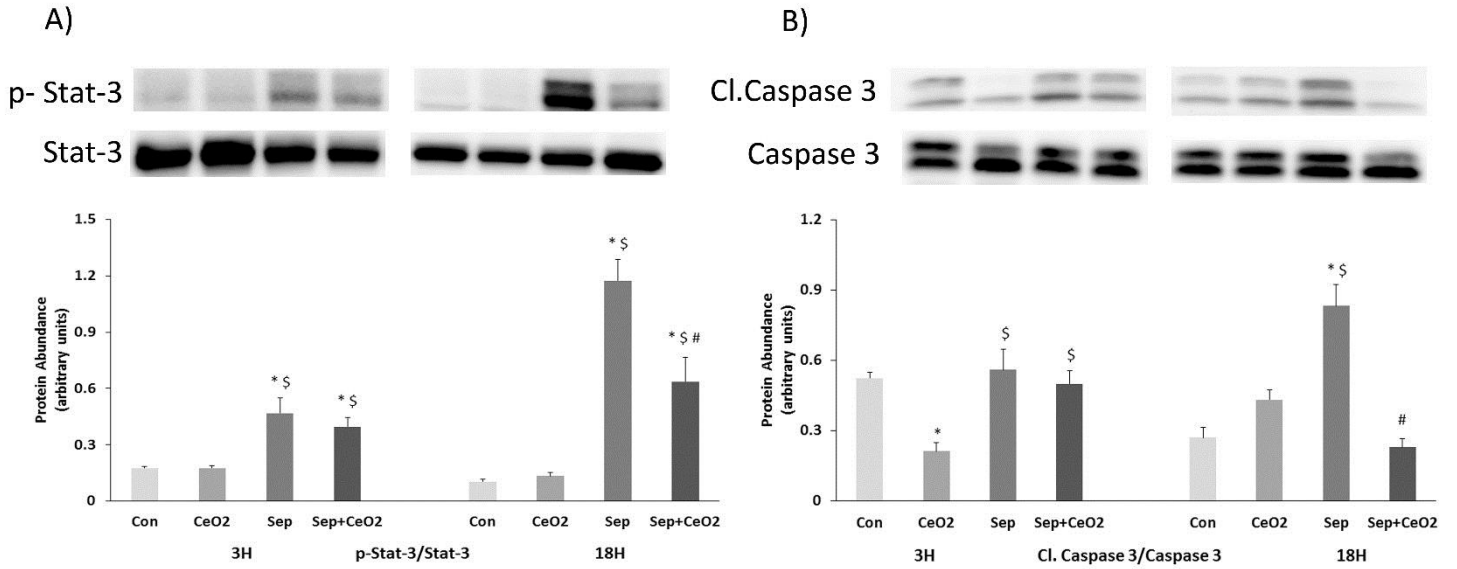
Tables

Table 3-7. CeO$_2$ nanoparticles attenuated sepsis induced increases in biomarkers of AKI.

*P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=6/group).

Analyte	Serum							
	Sham control 3h	CeO$_2$ 3h	Sepsis 3h	Sepsis + CeO$_2$ 3h	Sham control 18h	CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h
Beta-2-Microglobulin (μg/mL)	54.0 ± 3.0	49.7 ± 2.4	76.0 ± 1.5$^{*\$}$	77.3 ± 1.8$^{*\$}$	52.0 ± 3.5	32.7 ± 1.5*	128.0 ± 5.9$^{*\$}$	78.3 ± 7.2$^{*\$\#}$
Cystatin-C (ng/mL)	578.7 ± 14.3	633.0 ±30.6	686.0 ± 14.5	690.3 ± 42.1	699.7 ± 52.7	555.3 ± 41.6	1890.0 ± 62.5$^{*\$}$	1093.3 ± 37.6$^{*\$\#}$
KIM-1 (ng/mL)	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	3.3 ±0.13	2.5 ± 0.1$^{\#}$
Osteopontin (ng/mL)	10.8 ± 1.2	11.0 ± 0.6	11.0 ± 0.6	12.3 ± 0.3	9.7 ± 0.0	7.9 ± 0.2*	72.3 ± 4.8$^{*\$}$	36.3 ± 1.8$^{*\$\#}$
VEGF-A (pg/mL)	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	Below LLOQ	459.0 ± 12.7	296.7 ± 31.3$^{\#}$

Table 3-8. CeO$_2$ nanoparticles attenuated sepsis induced alterations in serum biochemical parameters.

*P<0.05 compared to control group, $ P<0.05 compared to CeO$_2$ group and # P<0.05 compared to sepsis group. (n=6/group).

Analyte	Serum							
	Sham control 3h	CeO$_2$ 3h	Sepsis 3h	Sepsis + CeO$_2$ 3h	Sham control 18h	CeO$_2$ 18h	Sepsis 18h	Sepsis + CeO$_2$ 18h
Glucose	284.0 ± 8.1	262.3 ± 10.1	426.9 ± 19.7$^{*\$}$	308.6 ± 22.6$^{\#}$	245.3 ± 13.3	290.6 ± 17.3*	105.5 ± 8.5$^{*\$}$	125.3 ± 5.1$^{*\$}$
Blood urea nitrogen	22.0 ± 1.1	21.0 ± 1.0	29.9 ± 2.0$^{*\$}$	24.71±1.17$^{\#}$	19.2 ± 0.5	18.5 ± 0.9	71.3 ± 7.2$^{*\$}$	57.4 ± 3.1$^{\$}$
Sodium	142.4 ± 0.7	142.9 ± 0.9	136.8 ± 1.4$^{*\$}$	140.1 ± 0.5$^{\#}$	142.0 ± 1.2	141.6 ± 0.9	138.8 ± 0.8	140.6 ± 0.5
Potassium	5.9 ± 0.4	5.4 ± 0.1	6.4 ± 0.3	6.3 ± 0.2	5.6 ± 0.2	5.8 ± 0.3	7.8 ± 0.2$^{*\$}$	7.1 ± 0.2$^{*\$\#}$

Chapter 4

Discussion

Sepsis is the leading cause of death globally in spite of intense research efforts and recent advance in medical care [188]. The complex pathophysiology of sepsis in conjunction with growing antibiotic resistance is likely to further worsen the prognosis of septic patients. Although not fully understood, it is thought that the uncontrolled systemic inflammatory response that characterizes the transition from sepsis to multiple organ dysfunction syndrome (MODS) is the primary cause of septic associated death [181].

Over the past decade, the field of nanotechnology has seen immense growth and it has played a vital role in the continued growth of several different manufacturing sectors [84, 189]. More recently, nanotechnology has also begun to be used for biomedical applications where the unique properties of nanomaterials can be exploited for diagnostic and even therapeutic uses [190, 191]. As an example, and of particular relevance to this research, CeO_2 nanoparticles are currently being tested for the detection of lactate levels and chronic inflammation [192], the treatment of radiation-induced damage [193], ovarian cancer [166] and stroke [194].

The main objective of the current study is to evaluate whether CeO_2 nanoparticles can be used to prevent sepsis induced MODS and death. To accomplish this objective, this study was divided into two different sub-projects. In the first, we examined whether CeO_2 nanoparticles could be used to protect Sprague Dawley rats from moderate sepsis induced SIRS. Once completed, we then undertook the second aspect of this study where we examined if CeO_2 nanoparticles could be used to prevent against severe sepsis induced MODS. Our findings are summarized as follows:

Effect of CeO₂ nanoparticles on sepsis induced hypothermia and mortality

Sepsis is characterized by high mortality rates that are closely associated with decreased core body temperature [195]. Previous studies have shown that the sepsis-induced cytokine response is associated with increased levels of TNF-α, IL-1β and prostaglandins which function to alter the hypothalamic set point causing hypothermia [196]. Coincident with the release of cytokines, other work has shown that sepsis also causes the release of the potent vasodilator nitric oxide which can cause the loss of heat from the peripheral circulation [197]. In the current study, we found that the induction of moderate sepsis in male Sprague Dawley rats results in mortality rate of 75% while severe sepsis results in 100% mortality rate. We also observed that decreases in core body temperature in the severely septic animals were closely associated with the death of the animal. Treatment with the CeO₂ nanoparticles reduced the mortality rate to ~17% in animals with moderate sepsis and to 25% in animals with severe sepsis. Treatment induced increases in survivability were found to be associated with the restoration of core body temperature towards baseline.

Effect of CeO₂ nanoparticles on sepsis induced systemic inflammatory response

It was previously thought that the sepsis induced mortality was a result of direct microbial insult. More recently, additional data has suggested that sepsis-induced mortality is more likely to be caused by the uncontrolled immune response that characterizes the development of MODS [198]. Similar to previous studies, we found that both moderate and severe sepsis resulted in increased levels of serum and peritoneal fluid inflammatory cytokines, chemokines and growth factors. These increases in systemic inflammation were attenuated

with CeO$_2$ nanoparticle treatment. Consistent with these data, other work has also suggested that CeO$_2$ nanoparticles can also modulate cellular ROS levels / inflammation [199]. How treatment with CeO$_2$ nanoparticles might function to decrease cellular ROS levels / inflammation *in vivo* is currently unclear and will require further study.

Effect of CeO$_2$ nanoparticles on sepsis induced multi-organ dysfunction syndrome

Previous studies have shown that the sepsis-induced inflammatory cytokine storm leads to MODS and death [200]. It is thought that much of the circulating cytokines seen during the cytokine storm are released by the resident macrophages, Kuppfer cells, of the liver [201]. Similar to other studies, we found that the intravenous injection of CeO$_2$ nanoparticles lead to their highest accumulation in liver but not in other organs [17]. We next sought to evaluate whether CeO$_2$ nanoparticles can modulate oxidative and nitrosative stress. As expected, CeO$_2$ nanoparticles attenuated sepsis-induced hepatic superoxide levels along with protein nitrosylation. In addition, CeO$_2$ nanoparticles also attenuated GST-α and GST-mu which are hallmarks of hepatic damage. We next examined whether CeO$_2$ nanoparticles can prevent sepsis induced cardiac failure and found that CeO$_2$ nanoparticles attenuated serum myoglobin levels and creatine kinase activity along with attenuation in cardiac ERK1/2-Jak-Stat pathway and decreased expression of VCAM-1. In addition, we also found that CeO$_2$ nanoparticles attenuated sepsis-induced increases in renal damage markers BUN, KIM-1, cystatin C, osteopontin and β-2 microglobulin. Histologically, CeO$_2$ nanoparticles attenuated sepsis-induced tubular dilatation, the loss of brush border, and improved F-actin levels in the kidney. These changes were associated with decrease in Stat-3 activation and cleavage of caspase 3.

Taken together, the data from the current study suggest that CeO_2 nanoparticles protect against sepsis-induced organ damage by modulating the hepatic inflammatory response.

Summary

1. CeO_2 nanoparticles significantly improve survivability in male Sprague Dawley rats against moderate and severe septic insults.

2. Improvement in survival rate is associated with the restoration of core body temperature towards baseline.

3. CeO_2 nanoparticles specifically accumulate in the liver and modulate the hepatic inflammatory response to septic insult.

4. CeO_2 nanoparticles attenuate hepatic damage by decreasing sepsis-induced oxidative and nitrosative stress which is associated with improvements in hepatic morphology. Modulation of the hepatic inflammatory response by CeO_2 nanoparticles is associated with decreased levels of systemic inflammatory mediators.

5. CeO_2 nanoparticles in conjunction with decreased cytokine release from the liver appear to protect the heart during sepsis by attenuating the activation of the inflammatory ERK 1/2-Jak-Stat pathway.

6. Nanoparticle treatment is associated with evidence of decreased sepsis-induced cardiac damage and microvascular leak.

7. CeO_2 nanoparticles attenuate sepsis-induced alterations in serum myoglobin and creatine kinase activity levels.

8. CeO_2 nanoparticles attenuate sepsis-induced AKI by preserving the structural integrity and F-actin.

9. CeO$_2$ nanoparticles attenuate sepsis-induced increases in renal oxidative stress and apoptosis by inhibition of Stat-3 and cleavage of caspase 3.

Figure 4-1. Schematic representation of mechanism of action of CeO$_2$ nanoparticles against severe sepsis induced MODS and death.
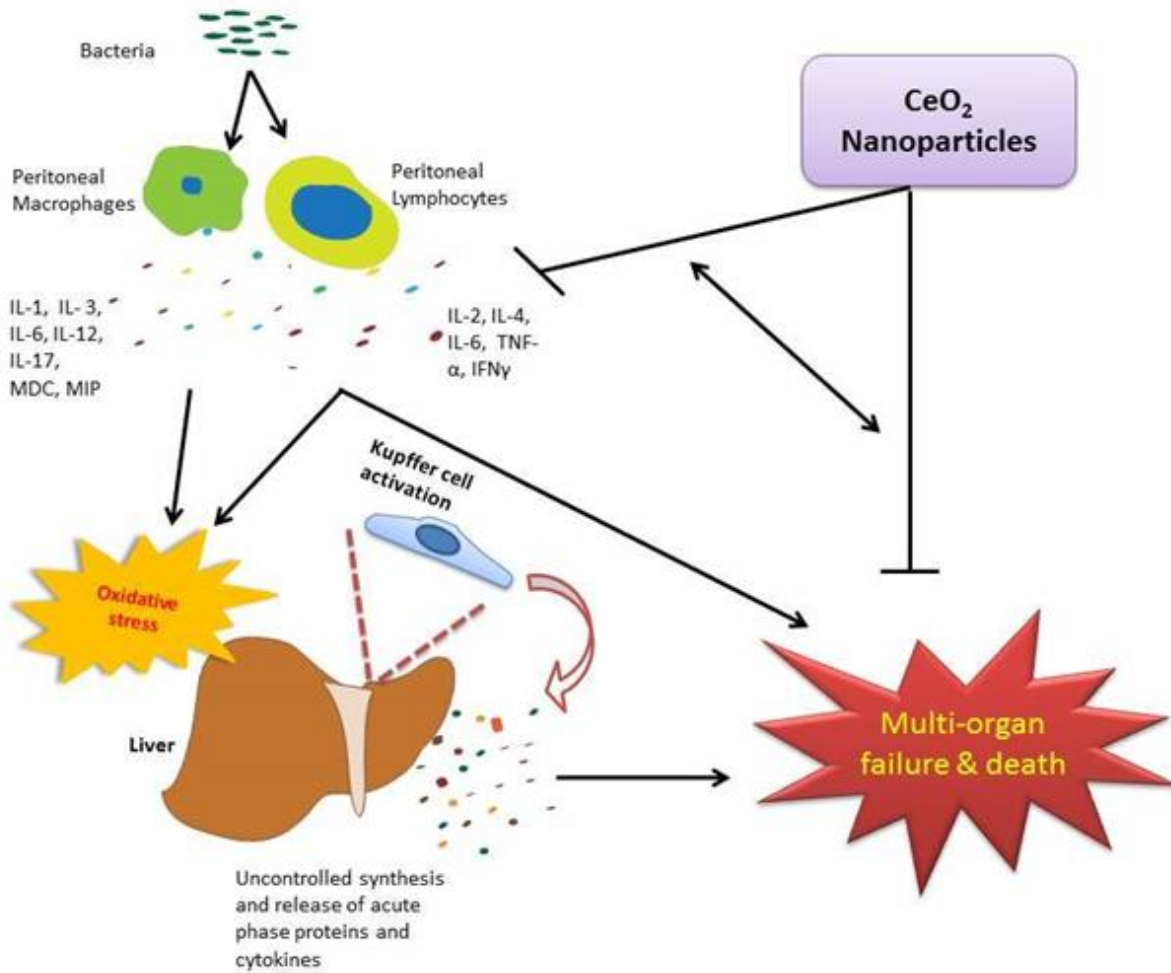
Future directions

The present study provides a first glimpse detailing the potential protective effects of CeO_2 nanoparticles against sepsis induced MODS and death. While the study is novel, the limitation of the study is that we tested the effects of the co-administration of CeO_2 nanoparticles at the time of sepsis induction. It is unlikely that this situation models a real-world clinical situation where sepsis is diagnosed prior to the initiation of treatment. Similarly, the animals used in this study did not suffer from comorbidities which is often seen in clinical settings. To address these shortcomings, further studies based on the following specific aims may be warranted.

Specific Aim I:

Severe sepsis is a complex pathophysiological process in which the host immune system becomes deregulated leading to organ failure. Early stages of systemic septic insult are characterized by the activation of circulating macrophages and neutrophils that cause the release of pro-inflammatory mediators such as TNF- α and IL-1β [202]. These cytokines then activate the resident macrophages such as Kupffer cells in liver, mesangial cells in kidney and dust cells in lungs. Because the liver is the primary organ for the synthesis of acute phase proteins and it also contains the largest number of resident macrophages, it may be useful to determine whether CeO_2 nanoparticles can prevent cytokine induced activation of Kupffer cells. While the current study found that CeO_2 nanoparticles accumulate in the liver, it is still unclear whether CeO_2 nanoparticles modulate Kupffer cell activation. To specifically address this question, Kupffer cells from livers of animals injected with CeO_2 nanoparticles could be isolated and analyzed for the presence of ceria content through ICPMS and electron microscopy. Finally

to determine whether CeO_2 nanoparticles can inhibit Kupffer cell activation, the determination of mRNA expression for various inflammatory cytokines in Kupffer cells across different groups may be warranted.

Specific Aim II:

The sepsis mortality rate is higher in neonatal children and the elderly due to an underdeveloped or compromised immune system [203]. The current study was performed in adult male Sprague Dawley rats where their innate and adaptive immune system has completely developed. Whether CeO_2 nanoparticles are efficacious for the treatment of the very young or the aged has not been investigated.

Previous studies in conjunction with this current study have demonstrated that CeO_2 nanoparticles exhibit the ability to scavenger ROS [11]. Unlike traditional anti-oxidants or anti-inflammatory agents that are rapidly metabolized and eliminated, CeO_2 nanoparticles do not undergo any metabolic change and may persist in the body for a long period of time [204]. In addition, the autocatalytic property of CeO_2 nanoparticles is thought to allow repeated cycling of Ce^{+4} to Ce^{+3} which may allow the particles to scavenge ROS indefinitely [118]. As such, studies investigating a second septic insult weeks after the initial one is resolved in the absence of subsequent treatments may be useful to better understand the true potential of using CeO_2 nanoparticles to treat sepsis.

Specific Aim III:

Findings from the current study demonstrate that CeO_2 nanoparticles protect male Sprague Dawley rats against severe sepsis when administered at the time of sepsis induction. However, most cases of deaths are seen in people that are admitted to the hospital at late or

final stages of sepsis where most of the irreparable damage has already been done. As such, studies investigating the therapeutic potential of CeO_2 nanoparticles by post-administration of nanoceria several hours after sepsis induction may be warranted.

Finally, another major drawback to the current therapeutic regime aimed for the treatment of sepsis is the presence of comorbidity. Studies have shown that patients with preexisting conditions such as chronic renal failure or heart failure have a poorer prognosis following sepsis induction than those that do not [205]. As such, it may be of use to explore if post-administration of CeO_2 nanoparticles can prevent sepsis induced MODS and mortality in individuals that have pre-existing diseases.

References

1. Angus, D.C. and T. van der Poll, *Severe sepsis and septic shock.* N Engl J Med, 2013. **369**(9): p. 840-51.
2. Marshall, J.C., *Sepsis: rethinking the approach to clinical research.* J Leukoc Biol, 2008. **83**(3): p. 471-82.
3. Mayr, F.B., S. Yende, and D.C. Angus, *Epidemiology of severe sepsis.* Virulence, 2014. **5**(1): p. 4-11.
4. Mayr, F.B., S. Yende, and D.C. Angus, *Epidemiology of severe sepsis.* Virulence, 2013. **5**(1).
5. Basu, R.K., et al., *Identification of candidate serum biomarkers for severe septic shock-associated kidney injury via microarray.* Crit Care, 2011. **15**(6): p. R273.
6. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clin Microbiol Rev, 2009. **22**(2): p. 240-73, Table of Contents.
7. Blackwell, T.S. and J.W. Christman, *Sepsis and cytokines: current status.* Br J Anaesth, 1996. **77**(1): p. 110-7.
8. Boisrame-Helms, J., et al., *Endothelial dysfunction in sepsis.* Curr Vasc Pharmacol, 2013. **11**(2): p. 150-60.
9. Lush, C.W. and P.R. Kvietys, *Microvascular dysfunction in sepsis.* Microcirculation, 2000. **7**(2): p. 83-101.
10. Wang, Z., Z. Quan, and J. Lin, *Remarkable changes in the optical properties of CeO(2) nanocrystals induced by lanthanide ions doping.* Inorg Chem, 2007. **46**(13): p. 5237-42.
11. Lee, S.S., et al., *Antioxidant properties of cerium oxide nanocrystals as a function of nanocrystal diameter and surface coating.* ACS Nano, 2013. **7**(11): p. 9693-703.
12. Yabe, S. and T. Sato, *Cerium oxide for sunscreen cosmetics.* Journal of Solid State Chemistry. **171**(1–2): p. 7-11.
13. Pearman, B.P., et al., *The degradation mitigation effect of cerium oxide in polymer electrolyte membranes in extended fuel cell durability tests.* Journal of Power Sources, 2013. **225**(0): p. 75-83.
14. Hedrick, J.B. and S.P. Sinha, *Cerium-based polishing compounds: discovery to manufacture.* Journal of Alloys and Compounds, 1994. **207–208**(0): p. 377-382.
15. Pagliari, F., et al., *Cerium oxide nanoparticles protect cardiac progenitor cells from oxidative stress.* ACS Nano, 2012. **6**(5): p. 3767-75.
16. Tarnuzzer, R.W., et al., *Vacancy engineered ceria nanostructures for protection from radiation-induced cellular damage.* Nano Lett, 2005. **5**(12): p. 2573-7.
17. Hirst, S.M., et al., *Bio-distribution and in vivo antioxidant effects of cerium oxide nanoparticles in mice.* Environ Toxicol, 2013. **28**(2): p. 107-18.
18. Thill, A., et al., *Cytotoxicity of CeO2 nanoparticles for Escherichia coli. Physico-chemical insight of the cytotoxicity mechanism.* Environ Sci Technol, 2006. **40**(19): p. 6151-6.
19. Babenko, L.P., et al., *Antibacterial activity of cerium colloids against opportunistic microorganisms in vitro.* Mikrobiol Z, 2012. **74**(3): p. 54-62.
20. Horst, A.M., et al., *An assessment of fluorescence- and absorbance-based assays to study metal-oxide nanoparticle ROS production and effects on bacterial membranes.* Small, 2013. **9**(9-10): p. 1753-64.
21. Hirst, S.M., et al., *Anti-inflammatory properties of cerium oxide nanoparticles.* Small, 2009. **5**(24): p. 2848-56.
22. Ciofani, G., et al., *Transcriptional profile of genes involved in oxidative stress and antioxidant defense in PC12 cells following treatment with cerium oxide nanoparticles.* Biochim Biophys Acta, 2014. **1840**(1): p. 495-506.

23. Lever, A. and I. Mackenzie, *Sepsis: definition, epidemiology, and diagnosis.* BMJ, 2007. **335**(7625): p. 879-83.

24. Nystrom, P.O., *The systemic inflammatory response syndrome: definitions and aetiology.* J Antimicrob Chemother, 1998. **41 Suppl A**: p. 1-7.

25. Funk, D.J., J.E. Parrillo, and A. Kumar, *Sepsis and septic shock: a history.* Crit Care Clin, 2009. **25**(1): p. 83-101, viii.

26. Melamed, A. and F.J. Sorvillo, *The burden of sepsis-associated mortality in the United States from 1999 to 2005: an analysis of multiple-cause-of-death data.* Crit Care, 2009. **13**(1): p. R28.

27. Rhee, C., S. Gohil, and M. Klompas, *Regulatory mandates for sepsis care--reasons for caution.* N Engl J Med, 2014. **370**(18): p. 1673-6.

28. Reinhart, K., et al., *The burden of sepsis-a call to action in support of World Sepsis Day 2013.* J Crit Care, 2013. **28**(4): p. 526-8.

29. Lynn, W.A., *Anti-endotoxin therapeutic options for the treatment of sepsis.* J Antimicrob Chemother, 1998. **41 Suppl A**: p. 71-80.

30. Alexander, C. and E.T. Rietschel, *Bacterial lipopolysaccharides and innate immunity.* J Endotoxin Res, 2001. **7**(3): p. 167-202.

31. Copeland, S., et al., *Acute inflammatory response to endotoxin in mice and humans.* Clin Diagn Lab Immunol, 2005. **12**(1): p. 60-7.

32. Mestas, J. and C.C. Hughes, *Of mice and not men: differences between mouse and human immunology.* J Immunol, 2004. **172**(5): p. 2731-8.

33. Esmon, C.T., *Why do animal models (sometimes) fail to mimic human sepsis?* Crit Care Med, 2004. **32**(5 Suppl): p. S219-22.

34. Dejager, L., et al., *Cecal ligation and puncture: the gold standard model for polymicrobial sepsis?* Trends Microbiol, 2011. **19**(4): p. 198-208.

35. Toscano, M.G., D. Ganea, and A.M. Gamero, *Cecal ligation puncture procedure.* J Vis Exp, 2011(51).

36. Merx, M.W., et al., *Statin treatment after onset of sepsis in a murine model improves survival.* Circulation, 2005. **112**(1): p. 117-24.

37. Zantl, N., et al., *Essential role of gamma interferon in survival of colon ascendens stent peritonitis, a novel murine model of abdominal sepsis.* Infect Immun, 1998. **66**(5): p. 2300-9.

38. Chopra, M., et al., *Modulation of myocardial mitochondrial mechanisms during severe polymicrobial sepsis in the rat.* PLoS One, 2011. **6**(6): p. e21285.

39. Fenton, M.J. and D.T. Golenbock, *LPS-binding proteins and receptors.* J Leukoc Biol, 1998. **64**(1): p. 25-32.

40. Schumann, R.R., *Function of lipopolysaccharide (LPS)-binding protein (LBP) and CD14, the receptor for LPS/LBP complexes: a short review.* Res Immunol, 1992. **143**(1): p. 11-5.

41. Fitzgerald, K.A., D.C. Rowe, and D.T. Golenbock, *Endotoxin recognition and signal transduction by the TLR4/MD2-complex.* Microbes Infect, 2004. **6**(15): p. 1361-7.

42. Saitoh, S., et al., *Ligand-dependent Toll-like receptor 4 (TLR4)-oligomerization is directly linked with TLR4-signaling.* J Endotoxin Res, 2004. **10**(4): p. 257-60.

43. Wesche, H., et al., *MyD88: an adapter that recruits IRAK to the IL-1 receptor complex.* Immunity, 1997. **7**(6): p. 837-47.

44. Adhikari, A., M. Xu, and Z.J. Chen, *Ubiquitin-mediated activation of TAK1 and IKK.* Oncogene, 2007. **26**(22): p. 3214-26.

45. Oeckinghaus, A. and S. Ghosh, *The NF-kappaB family of transcription factors and its regulation.* Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a000034.

46. Carlson, D., et al., *Antioxidant vitamin therapy alters sepsis-related apoptotic myocardial activity and inflammatory responses.* Am J Physiol Heart Circ Physiol, 2006. **291**(6): p. H2779-89.

47. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. **301**(5633): p. 640-3.

48. Yamamoto, M., et al., *TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway.* Nat Immunol, 2003. **4**(11): p. 1144-50.

49. Honda, K. and T. Taniguchi, *IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors.* Nat Rev Immunol, 2006. **6**(9): p. 644-58.

50. Pietras, E.M., S.K. Saha, and G. Cheng, *The interferon response to bacterial and viral infections.* J Endotoxin Res, 2006. **12**(4): p. 246-50.

51. Meydani, S.N., et al., *Antioxidant modulation of cytokines and their biologic function in the aged.* Z Ernahrungswiss, 1998. **37 Suppl 1**: p. 35-42.

52. Yang, D., et al., *Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells.* Exp Eye Res, 2007. **85**(4): p. 462-72.

53. Adams, V., et al., *Induction of iNOS expression in skeletal muscle by IL-1beta and NFkappaB activation: an in vitro and in vivo study.* Cardiovasc Res, 2002. **54**(1): p. 95-104.

54. Sheng, W., et al., *Pro-inflammatory cytokines and lipopolysaccharide induce changes in cell morphology, and upregulation of ERK1/2, iNOS and sPLA(2)-IIA expression in astrocytes and microglia.* J Neuroinflammation, 2011. **8**: p. 121.

55. Natarajan, V., et al., *Reactive oxygen species signaling through regulation of protein tyrosine phosphorylation in endothelial cells.* Environ Health Perspect, 1998. **106 Suppl 5**: p. 1205-12.

56. Goldman, E.H., L. Chen, and H. Fu, *Activation of apoptosis signal-regulating kinase 1 by reactive oxygen species through dephosphorylation at serine 967 and 14-3-3 dissociation.* J Biol Chem, 2004. **279**(11): p. 10442-9.

57. Ramasamy, R., et al., *Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation.* Glycobiology, 2005. **15**(7): p. 16R-28R.

58. Mylonas, C. and D. Kouretas, *Lipid peroxidation and tissue damage.* In Vivo, 1999. **13**(3): p. 295-309.

59. Beckman, J.S. and W.H. Koppenol, *Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly.* Am J Physiol, 1996. **271**(5 Pt 1): p. C1424-37.

60. Coffey, M.J., S.M. Phare, and M. Peters-Golden, *Peroxynitrite-induced nitrotyrosination of proteins is blocked by direct 5-lipoxygenase inhibitor zileuton.* J Pharmacol Exp Ther, 2001. **299**(1): p. 198-203.

61. Hollenberg, S.M., et al., *Increased microvascular reactivity and improved mortality in septic mice lacking inducible nitric oxide synthase.* Circ Res, 2000. **86**(7): p. 774-8.

62. Castellheim, A., et al., *Innate immune responses to danger signals in systemic inflammatory response syndrome and sepsis.* Scand J Immunol, 2009. **69**(6): p. 479-91.

63. Gruys, E., et al., *Acute phase reaction and acute phase proteins.* J Zhejiang Univ Sci B, 2005. **6**(11): p. 1045-56.

64. Ikarashi, M., et al., *Distinct development and functions of resident and recruited liver Kupffer cells/macrophages.* J Leukoc Biol, 2013. **94**(6): p. 1325-36.

65. Yu, W.K., et al., *Influence of acute hyperglycemia in human sepsis on inflammatory cytokine and counterregulatory hormone concentrations.* World J Gastroenterol, 2003. **9**(8): p. 1824-7.

66. Nicholson, T.E. and K.W. Renton, *Role of cytokines in the lipopolysaccharide-evoked depression of cytochrome P450 in the brain and liver.* Biochem Pharmacol, 2001. **62**(12): p. 1709-17.

67. Koskinas, J., et al., *Liver histology in ICU patients dying from sepsis: a clinico-pathological study.* World J Gastroenterol, 2008. **14**(9): p. 1389-93.

68. Nesseler, N., et al., *Clinical review: The liver in sepsis.* Crit Care, 2012. **16**(5): p. 235.

69. Legrand, M., et al., *Renal hypoxia and dysoxia after reperfusion of the ischemic kidney.* Mol Med, 2008. **14**(7-8): p. 502-16.

70.   Heyman, S.N., et al., *Cellular adaptive changes in AKI: mitigating renal hypoxic injury.* Nephrol Dial Transplant, 2012. **27**(5): p. 1721-8.

71.   de Geus, H.R., M.G. Betjes, and J. Bakker, *Biomarkers for the prediction of acute kidney injury: a narrative review on current status and future challenges.* Clin Kidney J, 2012. **5**(2): p. 102-108.

72.   Doi, K., et al., *Animal models of sepsis and sepsis-induced kidney injury.* J Clin Invest, 2009. **119**(10): p. 2868-78.

73.   The National Heart, L. and A.C.T.N. Blood Institute, *Rosuvastatin for Sepsis-Associated Acute Respiratory Distress Syndrome.* N Engl J Med, 2014.

74.   Matthay, M.A., L.B. Ware, and G.A. Zimmerman, *The acute respiratory distress syndrome.* J Clin Invest, 2012. **122**(8): p. 2731-40.

75.   Broermann, A., et al., *Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo.* J Exp Med, 2011. **208**(12): p. 2393-401.

76.   Antonucci, E., et al., *Myocardial depression in sepsis: From pathogenesis to clinical manifestations and treatment.* J Crit Care, 2014.

77.   Merx, M.W. and C. Weber, *Sepsis and the heart.* Circulation, 2007. **116**(7): p. 793-802.

78.   Hunter, J.D. and M. Doddi, *Sepsis and the heart.* Br J Anaesth, 2010. **104**(1): p. 3-11.

79.   Fernandes, C.J., Jr. and M.S. de Assuncao, *Myocardial dysfunction in sepsis: a large, unsolved puzzle.* Crit Care Res Pract, 2012. **2012**: p. 896430.

80.   Miller, J., *Surviving sepsis: a review of the latest guidelines.* Nursing, 2014. **44**(4): p. 24-30; quiz 30-1.

81.   Castranova, V., *Overview of current toxicological knowledge of engineered nanoparticles.* J Occup Environ Med, 2011. **53**(6 Suppl): p. S14-7.

82.   Webster, T.J., *IJN's second year is now a part of nanomedicine history!* Int J Nanomedicine, 2007. **2**(1): p. 1-2.

83.   Horodecki, M. and J. Oppenheim, *Fundamental limitations for quantum and nanoscale thermodynamics.* Nat Commun, 2013. **4**: p. 2059.

84.   Raj, S., et al., *Nanotechnology in cosmetics: Opportunities and challenges.* J Pharm Bioallied Sci, 2012. **4**(3): p. 186-93.

85.   Seaton, A., et al., *Nanoparticles, human health hazard and regulation.* J R Soc Interface, 2010. **7 Suppl 1**: p. S119-29.

86.   Kato, K., *[Development trend of nanomedicines].* Yakugaku Zasshi, 2013. **133**(1): p. 43-51.

87.   Azzopardi, E.A., E.L. Ferguson, and D.W. Thomas, *The enhanced permeability retention effect: a new paradigm for drug targeting in infection.* J Antimicrob Chemother, 2013. **68**(2): p. 257-74.

88.   Psarros, C., et al., *Nanomedicine for the prevention, treatment and imaging of atherosclerosis.* Nanomedicine, 2012. **8 Suppl 1**: p. S59-68.

89.   Ngwa, W., G.M. Makrigiorgos, and R.I. Berbeco, *Gold nanoparticle enhancement of stereotactic radiosurgery for neovascular age-related macular degeneration.* Phys Med Biol, 2012. **57**(20): p. 6371-80.

90.   Meddahi-Pelle, A., et al., *Organ Repair, Hemostasis, and In Vivo Bonding of Medical Devices by Aqueous Solutions of Nanoparticles.* Angew Chem Int Ed Engl, 2014.

91.   Kodiha, M., et al., *Gold nanoparticles induce nuclear damage in breast cancer cells, which is further amplified by hyperthermia.* Cell Mol Life Sci, 2014.

92.   Haggstrom, J., et al., *Virucidal properties of metal oxide nanoparticles and their halogen adducts.* Nanoscale, 2010. **2**(4): p. 529-34.

93.   Hosseini, A., et al., *Antiapoptotic effects of cerium oxide and yttrium oxide nanoparticles in isolated rat pancreatic islets.* Hum Exp Toxicol, 2013. **32**(5): p. 544-53.

94.   Vivek, R., et al., *Multifunctional HER2-Antibody Conjugated Polymeric Nanocarrier-Based Drug Delivery System for Multi-Drug-Resistant Breast Cancer Therapy.* ACS Appl Mater Interfaces, 2014. **6**(9): p. 6469-80.

95.   Han, Y., et al., *Co-delivery of plasmid DNA and doxorubicin by solid lipid nanoparticles for lung cancer therapy.* Int J Mol Med, 2014. **34**(1): p. 191-6.

96.   Chen, Z.Y., et al., *Advance of molecular imaging technology and targeted imaging agent in imaging and therapy.* Biomed Res Int, 2014. **2014**: p. 819324.

97.   Xu, C. and X. Qu, *Cerium oxide nanoparticle: a remarkably versatile rare earth nanomaterial for biological applications.* NPG Asia Materials, 2014. **6**(3): p. e90.

98.   Cassee, F.R., et al., *Exposure, health and ecological effects review of engineered nanoscale cerium and cerium oxide associated with its use as a fuel additive.* Crit Rev Toxicol, 2011. **41**(3): p. 213-29.

99.   Chen, H.T., et al., *Identifying the O2 diffusion and reduction mechanisms on CeO2 electrolyte in solid oxide fuel cells: a DFT + U study.* J Comput Chem, 2009. **30**(15): p. 2433-42.

100.   Chen, S., et al., *Cerium oxide nanoparticles protect endothelial cells from apoptosis induced by oxidative stress.* Biol Trace Elem Res, 2013. **154**(1): p. 156-66.

101.   Asati, A., et al., *pH-tunable oxidase-like activity of cerium oxide nanoparticles achieving sensitive fluorigenic detection of cancer biomarkers at neutral pH.* Anal Chem, 2011. **83**(7): p. 2547-53.

102.   Arya, A., et al., *Cerium oxide nanoparticles prevent apoptosis in primary cortical culture by stabilizing mitochondrial membrane potential.* Free Radic Res, 2014.

103.   Estevez, A.Y., et al., *Neuroprotective mechanisms of cerium oxide nanoparticles in a mouse hippocampal brain slice model of ischemia.* Free Radic Biol Med, 2011. **51**(6): p. 1155-63.

104.   Arya, A., et al., *Cerium oxide nanoparticles protect rodent lungs from hypobaric hypoxia-induced oxidative stress and inflammation.* Int J Nanomedicine, 2013. **8**: p. 4507-20.

105.   Pelletier, D.A., et al., *Effects of engineered cerium oxide nanoparticles on bacterial growth and viability.* Appl Environ Microbiol, 2010. **76**(24): p. 7981-9.

106.   Zholobak, N.M., et al., *[Antiviral effect of cerium dioxide nanoparticles stabilized by low-molecular polyacrylic acid].* Mikrobiol Z, 2010. **72**(3): p. 42-7.

107.   Celardo, I., et al., *Ce(3)+ ions determine redox-dependent anti-apoptotic effect of cerium oxide nanoparticles.* ACS Nano, 2011. **5**(6): p. 4537-49.

108.   Wang, Q., J.M. Perez, and T.J. Webster, *Inhibited growth of Pseudomonas aeruginosa by dextran- and polyacrylic acid-coated ceria nanoparticles.* Int J Nanomedicine, 2013. **8**: p. 3395-9.

109.   Dahaba, A.A., et al., *Procalcitonin for early prediction of survival outcome in postoperative critically ill patients with severe sepsis.* Br J Anaesth, 2006. **97**(4): p. 503-8.

110.   Martin, G.S., *Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes.* Expert Rev Anti Infect Ther, 2012. **10**(6): p. 701-6.

111.   Claessens, Y.E. and J.F. Dhainaut, *Diagnosis and treatment of severe sepsis.* Crit Care, 2007. **11 Suppl 5**: p. S2.

112.   Chang, Y.C., et al., *The therapeutic potential and mechanisms of action of quercetin in relation to lipopolysaccharide-induced sepsis in vitro and in vivo.* PLoS One, 2013. **8**(11): p. e80744.

113.   Kim, T.H., S.J. Yoon, and S.M. Lee, *Genipin attenuates sepsis by inhibiting Toll-like receptor signaling.* Mol Med, 2012. **18**: p. 455-65.

114.   Villa, P. and P. Ghezzi, *Effect of N-acetyl-L-cysteine on sepsis in mice.* Eur J Pharmacol, 1995. **292**(3-4): p. 341-4.

115.   Zapelini, P.H., et al., *Antioxidant treatment reverses mitochondrial dysfunction in a sepsis animal model.* Mitochondrion, 2008. **8**(3): p. 211-8.

116.   Rahman, I., *Pharmacological antioxidant strategies as therapeutic interventions for COPD.* Biochim Biophys Acta, 2012. **1822**(5): p. 714-28.

117. Berger, M.M. and R.L. Chiolero, *Antioxidant supplementation in sepsis and systemic inflammatory response syndrome.* Crit Care Med, 2007. **35**(9 Suppl): p. S584-90.

118. Niu, J., K. Wang, and P.E. Kolattukudy, *Cerium oxide nanoparticles inhibit oxidative stress and nuclear factor-kappaB activation in H9c2 cardiomyocytes exposed to cigarette smoke extract.* J Pharmacol Exp Ther, 2011. **338**(1): p. 53-61.

119. Zholobak, N.M., et al., *UV-shielding property, photocatalytic activity and photocytotoxicity of ceria colloid solutions.* J Photochem Photobiol B, 2011. **102**(1): p. 32-8.

120. Karakoti, A.S., et al., *Nanoceria as Antioxidant: Synthesis and Biomedical Applications.* JOM (1989), 2008. **60**(3): p. 33-37.

121. Colon, J., et al., *Protection from radiation-induced pneumonitis using cerium oxide nanoparticles.* Nanomedicine, 2009. **5**(2): p. 225-31.

122. Colon, J., et al., *Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2.* Nanomedicine, 2010. **6**(5): p. 698-705.

123. Das, M., et al., *Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons.* Biomaterials, 2007. **28**(10): p. 1918-25.

124. Baulin, N.A., et al., *[Selection of the method of plastic surgery of the abdominal wall in ventral hernia].* Khirurgiia (Mosk), 1990(7): p. 102-5.

125. Nalabotu, S.K., et al., *Intratracheal instillation of cerium oxide nanoparticles induces hepatic toxicity in male Sprague-Dawley rats.* Int J Nanomedicine, 2011. **6**: p. 2327-35.

126. Dombrovskiy, V.Y., et al., *Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003.* Crit Care Med, 2007. **35**(5): p. 1244-50.

127. Singer, M., *The role of mitochondrial dysfunction in sepsis-induced multi-organ failure.* Virulence, 2014. **5**(1): p. 66-72.

128. Antonelli, M., et al., *High IL-6 serum levels are associated with septic shock and mortality in septic patients with severe leukopenia due to hematological malignancies.* Scand J Infect Dis, 1995. **27**(4): p. 381-4.

129. Oda, S., et al., *Sequential measurement of IL-6 blood levels in patients with systemic inflammatory response syndrome (SIRS)/sepsis.* Cytokine, 2005. **29**(4): p. 169-75.

130. Xu, M., et al., *Protective effects of the combination of sodium ferulate and oxymatrine on cecal ligation and puncture-induced sepsis in mice.* Exp Ther Med, 2014. **7**(5): p. 1297-1304.

131. Punyadeera, C., et al., *A biomarker panel to discriminate between systemic inflammatory response syndrome and sepsis and sepsis severity.* J Emerg Trauma Shock, 2010. **3**(1): p. 26-35.

132. Wong, L.L. and J.F. McGinnis, *Nanoceria as bona fide catalytic antioxidants in medicine: what we know and what we want to know.* Adv Exp Med Biol, 2014. **801**: p. 821-8.

133. Majumdar, A., *Sepsis-induced acute kidney injury.* Indian J Crit Care Med, 2010. **14**(1): p. 14-21.

134. Yasuda, H., et al., *Simvastatin improves sepsis-induced mortality and acute kidney injury via renal vascular effects.* Kidney Int, 2006. **69**(9): p. 1535-42.

135. Doi, K., et al., *Pre-existing renal disease promotes sepsis-induced acute kidney injury and worsens outcome.* Kidney Int, 2008. **74**(8): p. 1017-25.

136. De Kock, I., C. Van Daele, and J. Poelaert, *Sepsis and septic shock: pathophysiological and cardiovascular background as basis for therapy.* Acta Clin Belg, 2010. **65**(5): p. 323-9.

137. Miller, A.M. and J.X. Zhang, *Altered endothelin-1 signaling in production of thromboxane A2 in kupffer cells from bile duct ligated rats.* Cell Mol Immunol, 2009. **6**(6): p. 441-52.

138. Heckert, E.G., et al., *The role of cerium redox state in the SOD mimetic activity of nanoceria.* Biomaterials, 2008. **29**(18): p. 2705-9.

139. Manne, N.D., et al., *Altered cardiac muscle mTOR regulation during the progression of cancer cachexia in the ApcMin/+ mouse.* Int J Oncol, 2013. **42**(6): p. 2134-40.

140.   Dalle-Donne, I., et al., *Protein carbonylation in human diseases.* Trends Mol Med, 2003. **9**(4): p. 169-76.

141.   Seija, M., et al., *Role of peroxynitrite in sepsis-induced acute kidney injury in an experimental model of sepsis in rats.* Shock, 2012. **38**(4): p. 403-10.

142.   Baumann, M., *Advanced glycation endproducts in sepsis and mechanical ventilation: extra or leading man?* Crit Care, 2009. **13**(4): p. 164.

143.   Dowding, J.M., S. Seal, and W.T. Self, *Cerium oxide nanoparticles accelerate the decay of peroxynitrite (ONOO).* Drug Deliv Transl Res, 2013. **3**(4): p. 375-379.

144.   Dowding, J.M., et al., *Cerium oxide nanoparticles protect against Abeta-induced mitochondrial fragmentation and neuronal cell death.* Cell Death Differ, 2014.

145.   Bauer, M., A.T. Press, and M. Trauner, *The liver in sepsis: patterns of response and injury.* Curr Opin Crit Care, 2013. **19**(2): p. 123-7.

146.   Greenhill, C.J., et al., *IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3.* J Immunol, 2011. **186**(2): p. 1199-208.

147.   Zouein, F.A., M. Kurdi, and G.W. Booz, *LIF and the heart: just another brick in the wall?* Eur Cytokine Netw, 2013. **24**(1): p. 11-9.

148.   Bueno, O.F. and J.D. Molkentin, *Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death.* Circ Res, 2002. **91**(9): p. 776-81.

149.   Xu, Q., et al., *Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways.* Oncogene, 2005. **24**(36): p. 5552-60.

150.   Lee, J.E., et al., *Janex-1, a JAK3 inhibitor, ameliorates tumor necrosis factor-alpha-induced expression of cell adhesion molecules and improves myocardial vascular permeability in endotoxemic mice.* Int J Mol Med, 2012. **29**(5): p. 864-70.

151.   Gao, M., et al., *Toll-like receptor 3 plays a central role in cardiac dysfunction during polymicrobial sepsis.* Crit Care Med, 2012. **40**(8): p. 2390-9.

152.   Ahmed, L.A., *Protective effects of magnesium supplementation on metabolic energy derangements in lipopolysaccharide-induced cardiotoxicity in mice.* Eur J Pharmacol, 2012. **694**(1-3): p. 75-81.

153.   Lodha, R., et al., *Myocardial cell injury is common in children with septic shock.* Acta Paediatr, 2009. **98**(3): p. 478-81.

154.   Iwaki, T., et al., *A cardioprotective role for the endothelial protein C receptor in lipopolysaccharide-induced endotoxemia in the mouse.* Blood, 2005. **105**(6): p. 2364-71.

155.   Fukui, M., et al., *Cell damage and liberation of nitric oxide synthase in rat heart induced by endotoxin administration.* Nihon Ika Daigaku Zasshi, 1995. **62**(5): p. 469-81.

156.   Arlati, S., et al., *Myocardial necrosis in ICU patients with acute non-cardiac disease: a prospective study.* Intensive Care Med, 2000. **26**(1): p. 31-7.

157.   Schrier, R.W. and W. Wang, *Acute renal failure and sepsis.* N Engl J Med, 2004. **351**(2): p. 159-69.

158.   Wang, Z., et al., *Development of oxidative stress in the peritubular capillary microenvironment mediates sepsis-induced renal microcirculatory failure and acute kidney injury.* Am J Pathol, 2012. **180**(2): p. 505-16.

159.   Xu, C., et al., *TNF-mediated damage to glomerular endothelium is an important determinant of acute kidney injury in sepsis.* Kidney Int, 2014. **85**(1): p. 72-81.

160.   Katsumi, H., et al., *Pharmacokinetics and preventive effects of platinum nanoparticles as reactive oxygen species scavengers on hepatic ischemia/reperfusion injury in mice.* Metallomics, 2014. **6**(5): p. 1050-6.

161.   Yang, X., et al., *Antioxidative nanofullerol prevents intervertebral disk degeneration.* Int J Nanomedicine, 2014. **9**: p. 2419-30.

162. Chistyakov, V.A., et al., *Possible mechanisms of fullerene C(6)(0) antioxidant action.* Biomed Res Int, 2013. **2013**: p. 821498.

163. Chaudhury, K., et al., *Mitigation of endometriosis using regenerative cerium oxide nanoparticles.* Nanomedicine, 2013. **9**(3): p. 439-48.

164. Pourkhalili, N., et al., *Biochemical and cellular evidence of the benefit of a combination of cerium oxide nanoparticles and selenium to diabetic rats.* World J Diabetes, 2011. **2**(11): p. 204-10.

165. Gao, Y., et al., *Cerium oxide nanoparticles in cancer.* Onco Targets Ther, 2014. **7**: p. 835-840.

166. Alili, L., et al., *Downregulation of tumor growth and invasion by redox-active nanoparticles.* Antioxid Redox Signal, 2013. **19**(8): p. 765-78.

167. Park, S.W., et al., *Human heat shock protein 27-overexpressing mice are protected against acute kidney injury after hepatic ischemia and reperfusion.* Am J Physiol Renal Physiol, 2009. **297**(4): p. F885-94.

168. Wang, C., et al., *Metabolic syndrome-induced tubulointerstitial injury: role of oxidative stress and preventive effects of acetaminophen.* Free Radic Biol Med, 2013. **65**: p. 1417-26.

169. Zarjou, A. and A. Agarwal, *Sepsis and acute kidney injury.* J Am Soc Nephrol, 2011. **22**(6): p. 999-1006.

170. Doi, K., et al., *Reduced production of creatinine limits its use as marker of kidney injury in sepsis.* J Am Soc Nephrol, 2009. **20**(6): p. 1217-21.

171. Lim, A.I., et al., *Kidney injury molecule-1: more than just an injury marker of tubular epithelial cells?* J Cell Physiol, 2013. **228**(5): p. 917-24.

172. Astor, B.C., et al., *Serum beta2-microglobulin at discharge predicts mortality and graft loss following kidney transplantation.* Kidney Int, 2013. **84**(4): p. 810-7.

173. Delanaye, P., et al., *Detection of decreased glomerular filtration rate in intensive care units: serum cystatin C versus serum creatinine.* BMC Nephrol, 2014. **15**(1): p. 9.

174. Vaidya, V.S., M.A. Ferguson, and J.V. Bonventre, *Biomarkers of acute kidney injury.* Annu Rev Pharmacol Toxicol, 2008. **48**: p. 463-93.

175. Maitra, S.R., M.M. Wojnar, and C.H. Lang, *Alterations in tissue glucose uptake during the hyperglycemic and hypoglycemic phases of sepsis.* Shock, 2000. **13**(5): p. 379-85.

176. Marik, P.E. and M. Raghavan, *Stress-hyperglycemia, insulin and immunomodulation in sepsis.* Intensive Care Med, 2004. **30**(5): p. 748-56.

177. Brierre, S., R. Kumari, and B.P. Deboisblanc, *The endocrine system during sepsis.* Am J Med Sci, 2004. **328**(4): p. 238-47.

178. Hannon, R.J. and V.E. Boston, *Hyponatraemia and intracellular water in sepsis: an experimental comparison of the effect of fluid replacement with either 0.9% saline or 5% dextrose.* J Pediatr Surg, 1990. **25**(4): p. 422-5.

179. Pinto, C.F., et al., *[The sepsis as cause of acute kidney injury: an experimental model].* Rev Esc Enferm USP, 2012. **46 Spec No**: p. 86-90.

180. Wu, L., N. Gokden, and P.R. Mayeux, *Evidence for the role of reactive nitrogen species in polymicrobial sepsis-induced renal peritubular capillary dysfunction and tubular injury.* J Am Soc Nephrol, 2007. **18**(6): p. 1807-15.

181. Bosmann, M. and P.A. Ward, *The inflammatory response in sepsis.* Trends Immunol, 2013. **34**(3): p. 129-36.

182. Chuang, P.Y. and J.C. He, *JAK/STAT signaling in renal diseases.* Kidney Int, 2010. **78**(3): p. 231-4.

183. Yang, N., et al., *Blockage of JAK/STAT signalling attenuates renal ischaemia-reperfusion injury in rat.* Nephrol Dial Transplant, 2008. **23**(1): p. 91-100.

184. Rawlings, J.S., K.M. Rosler, and D.A. Harrison, *The JAK/STAT signaling pathway.* J Cell Sci, 2004. **117**(Pt 8): p. 1281-3.

185.   Hoeben, A., et al., *Vascular endothelial growth factor and angiogenesis.* Pharmacol Rev, 2004. **56**(4): p. 549-80.

186.   Kockara, A. and M. Kayatas, *Renal cell apoptosis and new treatment options in sepsis-induced acute kidney injury.* Ren Fail, 2013. **35**(2): p. 291-4.

187.   Kothakota, S., et al., *Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis.* Science, 1997. **278**(5336): p. 294-8.

188.   Riley, C. and D.S. Wheeler, *Prevention of sepsis in children: a new paradigm for public policy.* Crit Care Res Pract, 2012. **2012**: p. 437139.

189.   in *Implications of Nanotechnology for Environmental Health Research*, L. Goldman and C. Coussens, Editors. 2005: Washington (DC).

190.   Zhao, Z.X., et al., *Cancer therapy improvement with mesoporous silica nanoparticles combining photodynamic and photothermal therapy.* Nanotechnology, 2014. **25**(28): p. 285701.

191.   Zhu, J., et al., *Targeted cancer theranostics using alpha-tocopheryl succinate-conjugated multifunctional dendrimer-entrapped gold nanoparticles.* Biomaterials, 2014. **35**(26): p. 7635-46.

192.   Nesakumar, N., et al., *Fabrication of lactate biosensor based on lactate dehydrogenase immobilized on cerium oxide nanoparticles.* J Colloid Interface Sci, 2013. **410**: p. 158-64.

193.   Wason, M.S., et al., *Sensitization of pancreatic cancer cells to radiation by cerium oxide nanoparticle-induced ROS production.* Nanomedicine, 2013. **9**(4): p. 558-69.

194.   Kyle, S. and S. Saha, *Nanotechnology for the Detection and Therapy of Stroke.* Adv Healthc Mater, 2014.

195.   Tiruvoipati, R., et al., *Hypothermia predicts mortality in critically ill elderly patients with sepsis.* BMC Geriatr, 2010. **10**: p. 70.

196.   Steiner, A.A., et al., *The hypothermic response to bacterial lipopolysaccharide critically depends on brain CB1, but not CB2 or TRPV1, receptors.* J Physiol, 2011. **589**(Pt 9): p. 2415-31.

197.   Hiller, S., et al., *alpha-Lipoic acid protects mitochondrial enzymes and attenuates lipopolysaccharide-induced hypothermia in mice.* Free Radic Biol Med, 2014. **71**: p. 362-7.

198.   Reinhart, K., et al., *New approaches to sepsis: molecular diagnostics and biomarkers.* Clin Microbiol Rev, 2012. **25**(4): p. 609-34.

199.   Schanen, B.C., et al., *Immunomodulation and T helper TH(1)/TH(2) response polarization by CeO(2) and TiO(2) nanoparticles.* PLoS One, 2013. **8**(5): p. e62816.

200.   Fry, D.E., *Sepsis, systemic inflammatory response, and multiple organ dysfunction: the mystery continues.* Am Surg, 2012. **78**(1): p. 1-8.

201.   Koo, D.J., I.H. Chaudry, and P. Wang, *Kupffer cells are responsible for producing inflammatory cytokines and hepatocellular dysfunction during early sepsis.* J Surg Res, 1999. **83**(2): p. 151-7.

202.   Schulte, W., J. Bernhagen, and R. Bucala, *Cytokines in sepsis: potent immunoregulators and potential therapeutic targets--an updated view.* Mediators Inflamm, 2013. **2013**: p. 165974.

203.   Satar, M. and F. Ozlu, *Neonatal sepsis: a continuing disease burden.* Turk J Pediatr, 2012. **54**(5): p. 449-57.

204.   Yokel, R.A., et al., *Distribution, elimination, and biopersistence to 90 days of a systemically introduced 30 nm ceria-engineered nanomaterial in rats.* Toxicol Sci, 2012. **127**(1): p. 256-68.

205.   Nasa, P., D. Juneja, and O. Singh, *Severe sepsis and septic shock in the elderly: An overview.* World J Crit Care Med, 2012. **1**(1): p. 23-30.

Appendix

Letter from Institutional Research board

MARSHALL UNIVERSITY
www.marshall.edu

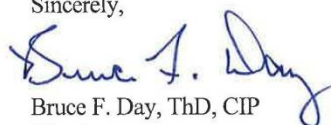Office of Research Integrity

July 9, 2014

Nandini Manne, PhD Candidate
Center for Diagnostic Nanosystems
Joan C. Edwards School of Medicine
Marshall University

Dear Mr. Manne:

This letter is in response to the submitted dissertation abstract entitled *"Therapeutic Efficacy of Cerium Oxide Nanoparticles Against Sepsis Induced Multi-Organ Dysfunction Syndrome and Death in Sprague Dawley Rats."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocols #494 and #540. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity

WE ARE... MARSHALL ™
401 11th Street, Suite 1300 • Huntington, West Virginia 25701 • Tel 304/696-7320
A State University of West Virginia • An Affirmative Action/Equal Opportunity Employer

Nandini D.P.K. Manne

Curriculum Vitae

_____

manne@marshall.edu
Ph.No: 304-730-9976

Summary:

- ✓ **Research experience of 4 ½ years involving scientific design of projects and their implementation.**
- ✓ **Highly motivated and enthusiastic to work on different research projects which gave me a broad range of knowledge on different subjects.**
- ✓ **Skilled veterinarian with good experience in animal handling and sound knowledge in pathology, pharmacology and physiology of lab and small animals.**
- ✓ **Designed and implemented a project involving treatment of sepsis using cerium oxide nanoparticles which is a novel idea.**

Career Objective

To seek the position of an investigative pharmacologist in a pharmaceutical industry where I can utilize my skills and knowledge to perform pre-clinical drug testing.

Education

July 2011- Aug 2014	Ph.D. Biomedical Sciences (Toxicology and Environmental Health Sciences) Joan C Edwards School of Medicine, Marshall University, Huntington, WV
Jan 2010-Jun 2011	MS, Biological Sciences, College of Science, Marshall University, Huntington, WV-25755
Sep 2008-Dec2009	Research assistant in Department of Epidemiology and Preventive Medicine at Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, INDIA
Aug 2003-Aug 2008	B.V.Sc& A.H College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, INDIA

Professional Experience

2010-Present   Graduate Research Assistant, Center for Diagnostic Nano systems, Marshall University, Huntington, WV

- ✓ Devised and implemented research projects.
- ✓ Designed and implemented academic research project to investigate the therapeutic activity of cerium oxide nanoparticles to treat polymicrobial sepsis.
- ✓ Investigated the cancer cachectic mechanisms in the hearts of Apc^{Min+} mice which can be useful to design therapeutic drugs in preventing muscle loss.
- ✓ Worked in other research projects involving therapeutic efficacy of acetaminophen and saxagliptin in treating diabetes.
- ✓ Worked in project involving skeletal muscle ablation in Obese Zucker rats to understand the molecular mechanisms for diminished hypertrophy in diabetic animals.
- ✓ Investigated the molecular mechanisms regarding regulation of iron related molecules in rat hippocampus.
- ✓ Significant contribution to studies concerning toxicity as well as therapeutic properties of nanoceria.
- ✓ Critical analysis of the data and publishing the manuscript in peer reviewed journals
- ✓ Trained graduate and undergraduate students
- ✓ Made successful collaboration with a research laboratory at University of South Carolina.

Sep 2008-Dec2009   Research assistant in Department of Epidemiology and Preventive Medicine at Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, INDIA
- ✓ Professional work involved projects concerning molecular techniques for diagnosis and screening of Brucellosis and Leptospirosis in domestic animals.
- ✓ Training undergraduates in various laboratory techniques.
- ✓ Laboratory maintenance

Feb 2008-Aug 2008   Intern Veterinary Doctor, Veterinary Poly Clinic, Visakhapatnam, AP, India
- ✓ Responsibilities include treating small and large animals for various diseases. Organized campaigns for public awareness about various zoonotic diseases. Maintenance of drug and patient records.

Skills and Techniques

- ✓ Lab animal handling and maintenance
- ✓ Lab animal surgeries- Induction of sepsis in animals (survival surgery) and induction of brain ischemia through bilateral ligation of common carotid artery.
- ✓ Small animal internal medicine and surgery
- ✓ Biochemical and physiological monitoring of lab animals
- ✓ Histopathology
- ✓ Immunohistochemistry
- ✓ Fluorescent microscopy
- ✓ Immunoblotting
- ✓ Cell culture
- ✓ Nanomaterial handling and maintenance along with addressing safety issues.
- ✓ Fabrication of microfluidic devices for studying the impact of nanomaterial toxicity in *C.elegans.*
- ✓ Photolithography
- ✓ Elisa
- ✓ RT-PCR

Familiar with
- ✓ Echocardiography and electrocardiography of rodents
- ✓ HPLC
- ✓ MALDI-TOF Mass Spectrometry
- ✓ FTIR Spectrometry
- ✓ Confocal microscopy

Patents
1. Methods for treating sepsis (ref no-61978337) (pending).

Publications

1. ***Manne, N. D.,*** *M. Lima, R. T. Enos, P. Wehner, J. A. Carson and E. Blough (2013). "Altered cardiac muscle mTOR regulation during the progression of cancer cachexia in the ApcMin/+ mouse." Int J Oncol 42(6): 2134-2140.*

2. Wang, C., E. R. Blough, R. Arvapalli, X. Dai, S. Paturi, **N. Manne**, H. Addagarla, W. E. Triest, O. Olajide and M. Wu (2013). "Metabolic syndrome-induced tubulointerstitial injury: role of oxidative stress and preventive effects of acetaminophen." <u>Free Radic Biol Med</u> 65: 1417-1426.

3. *Katta, A., S. Thulluri,* **N. D. Manne**, *H. S. Addagarla, R. Arvapalli, S. K. Nalabotu, M. Gadde, K. M. Rice and E. R. Blough (2013)."Overload induced heat shock proteins (HSPs), MAPK and miRNA (miR-1 and miR133a) response in insulin-resistant skeletal muscle." Cell Physiol Biochem 31(2-3): 219-229.*

4. Katta A, Kakarla SK, **Manne ND**, Wu M, Kundla S, Kolli M, Nalabotu SK, Blough ER. *Diminished muscle growth in the obese Zucker rat following overload is associated with hyperphosphorylation of AMPK and dsRNA-dependent protein kinase.* J Appl Physiol, 2012

5. Siva K. Nalabotu, Madhukar B. Kolli, William E. Triest, Jane Y. Ma, **Manne ND**, Anjaiah Katta, Hari S. Addagarla, Kevin M. Rice, and Eric R. Blough. *Intratracheal instillation of cerium oxide nanoparticles induces hepatic toxicity in male Sprague dawley rats.* Int J Nanomedicine. 2011;6:2327-35

6. Thulluri S, Wu M, Blough ER, **Manne ND**, Litchfield AB, Wang B.*Regulation of iron-related molecules in the rat hippocampus: sex- and age-associated differences.* Ann Clin Lab Sci, 2012. 42(2): p. 145-51.

In Preparation

1. Therapeutic applications of *cerium oxide nanoparticles in treatment of polymicrobial sepsis.* **Nandini D.P.K. Manne**, *Selvaraj Vellaisamy and Eric.R. Blough*

2. Therapeutic applications of anti-oxidant and anti-inflammatory properties of cerium oxide nanoparticles in treatment of sepsis induced multi-organ failure. **Nandini D.P.K. Manne** and Eric.R. Blough

3. Cerium oxide nanoparticles prevent sepsis induced renal failure by improving renal function and structural integrity in Sprague Dawley rats. **Nandini D.P.K. Manne** and Eric.R. Blough.

4. Cerium oxide nanoparticles attenuate monocrotaline induced right ventricular hypertrophy following pulmonary arterial hypertension. *Madhukar B. Kolli,* **Nandini D.P.K. Manne** and Eric R. Blough.

Poster Presentations

1. **Nandini Manne**, Ravikumar Arvapalli, Niraj Nepal, Geeta Nandyala, Kevin Rice, Asano Shinichi, and Eric Blough. Therapeutic efficacy of cerium oxide nanoparticles in

treatment of sepsis induced renal failure. Present at Experimental Biology conference, San-Diego, 2014.

2. **Nandini Manne,** Eric Blough. Therapeutic potential of cerium oxide nanoparticles for the treatment of sepsis induced cardiac dysfunction. Presented at 3rd International conference on Naotek & Expo, Las Vegas, 2013 **(Received award for best poster)**.

3. **Nandini Manne**, Nalabotu SK, J. A. Carson, Srinivas Thulluri, Kevin M. Rice and Eric R. Blough. The regulation of mTOR signaling in cardiac muscle during the progression of cancer cachexia in the ApcMin/+ mouse. Presented at Marshall University Research day Huntington, WV, 2012.

4. Wu M, Arvapalli R, Wang C, Paturi S**, Manne ND**, Dornon L, Wehner P, and Blough E. Acetaminophen reduces lipid accumulation and improves cardiac function in the obese Zucker rat. Presented at the American Association of Colleges of Pharmacy (AACP) 2012 Annual Meeting; Kissimmee, FL. July, 2012.

5. Wang C, Blough E, Arvapalli R, Paturi S, **Manne ND,** and. Wu M . Protective Effect of Acetaminophen on Renal Dysfunction in the Obese Zucker Rat. Presented at the American Association of Colleges of Pharmacy (AACP) 2012 Annual Meeting; Kissimmee, FL. July, 2012.

6. Akhtar O, Blough E, Yaqub A, Arvapalli R, Paturi S, **Manne ND**, Herndon P, and Wu M. Effect of dimethyl sulfoxide on bone hemostasis with obesity. Presented at the American Association of Clinical Endocrinologists (AACE) 2012 Annual Meeting; Philadelphia, PA. May 2012.

References

1. **Dr. Eric R. Blough, Ph.D.**
   Professor,
   Dept. of Pharmaceutical Science & Research
   School of Pharmacy, Marshall University,
   Huntington, WV.
   Phone: (304)696-2708
   E-mail: blough@marshall.edu

2. **Dr. Monica Valentovic**
   Professor
   Department of Pharmacology, Physiology, and Toxicology
   Joan C. Edwards School of Medicine, Marshall University,
   Huntington, WV.
   Phone: (304) 696-7332
   E-mail: valentov@marshall.edu

3. **Dr. Todd L. Green**
   Associate Professor and Director of Graduate Studies, BMS Graduate Program
   Department of Pharmacology, Physiology and Toxicology
   Joan C. Edwards School of Medicine, Marshall University,
   Huntington, WV.
   Phone: (304) 696-3531
   E-mail: green@marshall.edu