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Cerium Oxide Nanoparticles Attenuate Polymicrobial Sepsis Induced Splenic Damage in male Sprague Dawley Rats

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CERIUM OXIDE NANOPARTICLES ATTENUATE POLYMICROBIAL SEPSIS INDUCED
SPLENIC DAMAGE IN MALE SPRAGUE DAWLEY RATS

A thesis submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Master of Science
in
Biological Sciences
by

Venkata Vinay Kumar Bandarupalli

Approved by

Dr. Eric R. Blough, Committee Chairperson

Dr. David S. Mallory

Dr. Guo-Zhang Zhu

Marshall University
December 2015

APPROVAL OF THESIS/DISSERTATION

We, the faculty supervising the work of Venkata Vinay Kumar Bandarupalli, affirm that the thesis, Cerium Oxide Nanoparticles Attenuate Polymicrobial Sepsis Induced Splenic Damage in Male Sprague Dawley Rats, meets the high academic standards for original scholarship and creative work established by the Biological Sciences and the Marshall University. This work also conforms to the editorial standards of our discipline and the graduate college of Marshall University. With our signatures, we approve the manuscript for publication.

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ABBREVIATIONS

AAALAC	Association for assessment and accreditation of laboratory animal care
ACCP	American College of Chest Physicians
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CARS	Compensatory anti-inflammatory response syndrome
CeO ₂	Cerium oxide
EDS	Energy-dispersive X-ray spectroscopy
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H ₂ O ₂	Hydrogen peroxide
HMGB1	High mobility group box protein 1
IL	Interleukin
IRAK4	Interleukin-1 receptor associated kinase 4
i.p.	Intra peritoneal
i.v.	Intravenous
JNK	Jun-N-terminal kinase
KRB	Krebs-Ringers Buffer Solution
LPS	Lipopolysaccharide
LPS-BP	Lipopolysaccharide binding protein
MAPK	Mitogen activated protein kinase
MODS	Multi-organ dysfunction syndrome
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PMN	Polymorphonuclear

ROS	Reactive oxygen species
RNS	Reactive nitrogen species
s.c.	Subcutaneous
SCCM	Society of Critical Care Medicine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SIRS	Systemic inflammatory response syndrome
TBS	Tris buffered saline
TRAF	TNF receptor associated factor
TBST	Tris buffered saline with 0.5% tween
TEM	Transmission electron microscope
TNF- α	Tumor necrosis factor alpha
WBC	White blood cells

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ABSTRACT

Sepsis is a serious life threatening medical emergency which, if not treated properly, oftentimes results in organ failure and death. Current sepsis treatment protocols are largely centered on the use of antibiotics and supportive care. Recent studies have suggested that antibiotics fail to be effective for sepsis treatment when administered during hypo-dynamic phase of sepsis that is usually characterized by the presence of a cytokine storm. As such, there is an urgent need to develop novel therapeutic drugs that target the inflammatory cytokines that are secreted as a result of increased reactive oxygen species. Cerium oxide nanoparticles (CeO_2) have been shown to act as anti-inflammatory and anti-oxidant agent. More recently, they have been shown to attenuate polymicrobial insult-induced mortality in Sprague Dawley rats. Here, we investigated whether CeO_2 nanoparticles can attenuate splenic damage in this animal model of sepsis. A single intravenous dose (0.5 mg/kg) of CeO_2 nanoparticles attenuated the sepsis-induced loss in splenic cell structural integrity. These improvements in splenic structure were accompanied by a decrease in expression of late phase pro-inflammatory cytokine high mobility group box 1 (HMGB1) along with reduced bacterial load in the blood and peritoneal fluid of septic animals. Taken together these findings suggest that CeO_2 nanoparticles can be used to attenuate polymicrobial insult-induced splenic damage in Sprague dawley rats.

CHAPTER 1

INTRODUCTION

The term sepsis was first coined by Hippocrates who described sepsis as a process by which flesh rots and foul air develops near swamps (Marshall 2008). Scientists like Robert Koch, Louis Pasteur, and others postulated that microbial invasion was the prime cause of sepsis but they were not able to determine the type of pathogen responsible for the disease with any certainty at that time (Wilmot, Wongtawan et al. 2011). In non-coronary intensive care units, sepsis is the most common cause of mortality (Mayr, Yende et al. 2013). Sepsis is a serious medical condition whose incidence is increasing by approximately 13% each year (Messori, Fadda et al. 2014). This multifactorial disorder is characterized by widespread systemic inflammation that can lead to organ failure and death if not properly controlled. Sepsis is thought to occur as a result of infection by bacteria (gram positive and gram negative), fungi, and viruses that can rapidly progress in the aged and immunocompromised patients (Nasa, Juneja et al. 2012). How sepsis causes organ failure is not well understood, but it is thought that the systemic inflammation caused by uncontrolled immune response results in release of several cytokines and inflammatory mediators that affect the vasculature. These inflammatory mediators disrupt the endothelial barrier and increase the vascular permeability which can lead to the development of hypotension and blood clots in the periphery. Each year approximately 700,000 cases of septic patients are admitted to the hospitals and nearly \$16 billion is spent to treat the hospitalized patients (Garber, Gibney et al. 2002).

Sepsis usually starts with the invasion of an infectious agent at the local or systemic level and gradually results in development of the systemic inflammatory response syndrome (SIRS) if the infectious agent overwhelms the initial immune response. Although SIRS is seen in other

conditions such as pancreatitis, trauma and burns, it is differentiated in sepsis by the presence of an infectious agent (Balk 2014). SIRS/sepsis is characterized by marked increase in inflammatory cytokine levels including tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) in the earlier stages and by high mobility group box 1 (HMGB1) during the later phase (Schulte, Bernhagen et al. 2013). While sepsis is generally thought to be pro-inflammatory in nature, it has been recently proposed that the uncontrolled hyper-inflammatory state is usually followed or counteracted by compensatory anti-inflammatory response syndrome (CARS) that tries to protect the host from excessive inflammatory insult (Ward, Casserly et al. 2008). If the infection is not checked, SIRS can overwhelm the CARS response which can lead to the development of septic shock and multiple organ dysfunctions. Although previous studies have focused on detecting different microorganisms responsible for sepsis, the recent approach has centered on identifying the relation between sepsis and the immune response. The SIRS response is caused by an excess production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that adversely affect the normal cellular processes (Biswal and Remick 2007). While it remains unclear whether mitochondrial dysfunction is the initiating cause of the excess ROS formation or whether increased ROS amplifies ROS production cycle in the mitochondria (Galley 2011). Given the role that ROS is thought to play in sepsis progression, it has been hypothesized that anti-oxidants may be a beneficial strategy to treat sepsis. The current treatment regime of sepsis largely resides on the use of antibiotics and intravenous fluids which are largely ineffective in the final stages of sepsis (Daniels 2011).

In recent years the study of nanotechnology has given rise to several anti-oxidants that exhibit auto-regenerative properties. The cerium oxide nanoparticle (CeO₂) is one of those

compounds that is currently used as a polishing agent in industry and as a catalyst to improve fuel combustion (Dunnick, Pillai et al. 2015). In the biomedical field, CeO₂ nanoparticles have been shown to treat cancer (Pesic, Podolski-Renic et al. 2015), stroke (Estevez, Pritchard et al. 2011) and diabetes (Rocca, Moscato et al. 2015). Recent studies have also shown that these nanoparticles are capable of preventing cell damage and death induced by ROS because of their anti-oxidant properties (Arya, Sethy et al. 2014). Whether nanocerium can be used to treat splenic damage seen in sepsis is not well understood.

Specific Aim 1

To determine whether CeO₂ nanoparticles can attenuate sepsis induced splenic damage.

Hypothesis

CeO₂ nanoparticles will attenuate sepsis induced structural damage in spleen and also attenuate expression of HMGB1 in spleen.

Specific Aim 2

To determine whether CeO₂ nanoparticles can attenuate polymicrobial insult associated increase in microbial load in blood and peritoneal fluid.

Hypothesis

CeO₂ nanoparticles will attenuate the sepsis associated increase in bacterial number in blood and peritoneal fluid.

CHAPTER 2

REVIEW OF LITERATURE

The current chapter provides a review of literature regarding sepsis as well as the potential applications of nanotechnology in the treatment of inflammatory diseases. The following research topics are discussed: 1. Etiology and pathophysiology of sepsis, 2. Splenic dysfunction in sepsis, 3. Current research on sepsis and its management, and 4. Nanomedicine and applications of CeO₂ nanoparticles for the treatment of inflammatory diseases.

Etiology and Pathophysiology of Sepsis

Sepsis is a serious pathological condition that is on constant rise and ranks among the top ten mortality causing diseases in United States (Mayr, Yende et al. 2013). Each year approximately 700,000 cases are registered in the United States and nearly 200,000 cases result in death (Burchardi and Schneider 2004). The major causes of sepsis are bacterial infection followed by fungi, viruses, and parasites (Tupchong, Koyfman et al. 2015). The process of sepsis normally begins with the entry of an infectious agent into the host. When the invading pathogen is highly infectious with a very short incubation period, an uncontrolled immune response can arise. This response is characterized by the release of several inflammatory cytokines such as TNF- α , IL-1 β and IL-6 that further drive sepsis progression (Tupchong, Koyfman et al. 2015). The resulting “cytokine storm” is known as systemic inflammatory response syndrome (SIRS) and is characterized by increase in body temperature greater than 38 °C or less than 36 °C, increase in heart rate above 90 beats/minute, increase in respiratory rate above 20 breaths/minute along with change in white blood cell count with greater than 12,000/ μ L or less than 4,000/ μ L (Comstedt, Storgaard et al. 2009). Usually, the inflammatory insult can be successfully brought under control with appropriate medical intervention in a timely manner. However, without

intervention, SIRS can damage the vascular endothelial barrier resulting in the leakage of plasma into the extracellular space (Jacobson and Garcia 2007). A loss in circulating volume, in turn, can result in the development of hypotension and decreased supply of nutrients to the metabolically active cells. These sequences of events result in septic shock and can progress to multiple organ dysfunctions. Failure of four or more organs usually account for 65% of the deaths in hospitalized patients (Grozdanovski, Milenkovic et al. 2012). Thus far, clinical trials to improve sepsis survival have been for the most part ineffective. Although not fully understood, it is thought that differences in sepsis severity and sepsis classification have complicated clinical progress. According to the American College of Chest Physicians/Society of Critical Care Medicine consensus, sepsis is defined as the presence of at least two SIRS criteria in the presence of an infectious agent while severe sepsis is characterized by sepsis plus marked hypotension/hypo perfusion and multiple organ failure (Martin 2012).

At the cellular level, the immune response starts with the interaction between the lipopolysaccharide (LPS) portion of gram negative bacteria and the cluster of differentiation 14 (CD14) co-receptor (Sagy, Al-Qaqa et al. 2013). CD14 is a glycosylphosphatidylinositol anchored protein that occurs in a receptor form and as a soluble protein in the circulating blood stream. The LPS-CD14 complex binds to the toll-like receptor 4 (TLR-4) which undergoes oligomerization and initiates downstream intracellular signaling (Van Amersfoort, Van Berkel et al. 2003). Activation of TLR-4 receptors results in propagation of signals in one of two downstream effector pathways, the myeloid differentiation primary response gene 88 (MYD-88) dependent and independent pathways (Van Amersfoort, Van Berkel et al. 2003).

The MYD-88 is a signaling protein with unknown function and contains a C-terminal toll/interleukin receptor (TIR) domain and an N-terminal death domain (Deguine and Barton

2014). This signaling adaptor protein recruits interleukin-1 receptor associated kinase 4 (IRAK4) and upon activation causes activation of downstream protein tumor necrosis receptor-associated factors (TRAF) (Deguine and Barton 2014). The TRAF 6, a member of the TRAF protein family, then activates transforming growth factor beta-activated kinase (TAK1), a serine-threonine kinase that further propagates the signal through the nuclear factor kappa B (NF- κ B) and mitogen activated protein kinase (MAPK) pathways (Akira and Takeda 2004). The NF- κ B is a transcription factor that is responsible for the synthesis and release of several inflammatory mediators.

The MYD-88 independent pathway requires adaptor molecules TIR-domain-containing adapter-inducing interferon- β (TRIF) and thyroid receptor activator molecule (TRAM) for TLR-3 and TLR-4 signaling (Bagchi, Herrup et al. 2007). TRAF3 recruited by TRIF is involved in the activation of downstream interferon regulatory factor 3 (IRF3) which is necessary for NF- κ B late phase activation (Bagchi, Herrup et al. 2007). IRF3 and NF- κ B activation in turn leads to production of interferons that are responsible for protecting the host against invading bacteria, viruses, and fungi.

The inflammatory process is involved in the release of chemical mediators such as histamines, cytokines, and prostaglandins from macrophages, neutrophils, and lymphocytes. These inflammatory mediators act in both an autocrine and paracrine manner on various cell types. The activation of immune cells also results in production and release of nitric oxide (NO). NO is an anti-microbial agent and at the same time, a potent vasodilator (De Cruz, Kenyon et al. 2009). NO in conjunction with inflammatory cytokines lead to vasodilation and disruption of endothelial barrier that can result in plasma leakage, subsequent hypo perfusion, and multi-organ failure (Hauser, Matejovic et al. 2008).

Splenic Dysfunction in Sepsis

The spleen, the body's largest lymphoid organ, plays an important role in protecting the host against pathological agents and also acts as filter for circulating blood (Altamura, Caradonna et al. 2001). One of the major functions of spleen is the modulation of the immune response and maintenance of peripheral tolerance (Bronte and Pittet 2013). The spleen is also required for the activation and differentiation of T and B lymphocytes upon invasion by pathogenic microorganisms (Liu 1997). As such, diminished or impaired splenic function in patients is associated with high risk of severe sepsis.

Studies have shown that the removal of the spleen is associated with a 10-20 fold higher risk of sepsis and sepsis-induced mortality (Tarantino, Scalera et al. 2013). The depletion of functionally matured T-helper cells directly affects the ability to generate immune responses against invading pathogens. Previous studies in animal models of sepsis have shown that apoptosis of lymphocytes is mediated by both death receptor and mitochondrial pathways (Hotchkiss, Swanson et al. 1999). Overexpression of the B cell lymphoma-2 (Bcl-2) anti-apoptotic protein increased survival in septic animals by preventing mitochondrial damage (Hotchkiss, Swanson et al. 1999, Iwata, Stevenson et al. 2003). Similarly, others have reported that blockage of the apoptosis antigen 1 (Fas) death receptor is also associated with improved survival following a septic insult (Ayala, Chung et al. 1999). More recently, it has been shown that levels of HMGB1, a late phase inflammatory cytokine is associated with splenomegaly. In addition, the administration of anit-HMGB1 antibodies attenuated splenomegaly (Valdes-Ferrer, Rosas-Ballina et al. 2013) suggesting that therapeutic approaches to target HMGB1 could attenuate sepsis associated splenic dysfunction and lymphocyte apoptosis.

Current Research on Sepsis and its Management

Understanding the pathogenesis of sepsis is of paramount importance to developing optimal therapeutic strategies. Because of the difficulties associated with performing invasive studies in humans, most of the initial sepsis research is done in animals. Commonly used sepsis animal models include: a) the lipopolysaccharide model (LPS), b) inoculation of live bacteria, c) cecal ligation and puncture (CLP), d) colon ascendens stent peritonitis (CASP), and e) polymicrobial sepsis (fecal peritonitis/ cecal inoculum) (Buras, Holzmann et al. 2005). Each model of sepsis has unique advantages and disadvantages. The LPS model of sepsis is quick in onset and more aptly describes the cytokine storm seen in human sepsis (Doi, Leelahavanichkul et al. 2009). In comparison, studies that apply live bacteria usually involve only type of bacteria (Gram-negative or gram-positive) and do not mimic the polymicrobial nature of human sepsis (Doi, Leelahavanichkul et al. 2009). To overcome these deficiencies, researchers have developed a fecal peritonitis of sepsis model that is polymicrobial in nature. However, even this animal model is not error free, as the sudden onset of microbial colonization typically does not mimic the gradual progression of sepsis seen in humans. Nonetheless, the amount of bacterial load can be controlled in this model and as such the variability associated is low (Garrido, Figueiredo et al. 2004). The CLP model of sepsis is currently considered as the gold standard model of sepsis (Dejager, Pinheiro et al. 2011). This model involves ligation of and puncture of cecum through which the cecal material extrudes over time into peritoneal space (Nemzek, Hugunin et al. 2008). A major disadvantage of this model is the variability in response seen from one animal to other due to the differences in the amount of cecal content that extrudes into the peritoneal space (Dejager, Pinheiro et al. 2011). As such, the choice of model used is typically based on the hypothesis of the study.

The initial studies regarding sepsis treatment were, for the most part, based on identifying the type of bacteria and treating them with suitable antibiotics. Recent studies suggest that antibiotics play an important role in the early stages of sepsis where they tend to prevent their multiplication giving the host immune system time to clear the infection from the circulation (Daniels 2011). However, it has also been shown that administration of antibiotics at later phases of sepsis is of little benefit as it is the cytokine storm that causes the multi-organ failure and septic shock (Ferrer, Martin-Loeches et al. 2014). With increasing antibiotic resistance and delay in administration, research regarding sepsis treatment has shifted towards the development of anti-oxidants that could scavenge ROS and prevent the cytokine storm associated organ failure (Andrades, Morina et al. 2011).

Oxidative stress occurs when there is an imbalance between production of ROS and the total antioxidant capacity of the cell (Galley 2011). Oxidative stress has been shown to initiate activation of the redox sensitive transcription factor nuclear factor-kappa-B (NF- κ B) in septic patients (Macdonald, Galley et al. 2003). It is thought that this transcription factor is responsible for the synthesis of the inflammatory mediators which initiate the development of the SIRS response (Arnalich, Garcia-Palomero et al. 2000). What causes the ROS that initially activates NF- κ B is currently unknown. While the mitochondria are the major ROS producing organelles of the cell, it is unclear whether mitochondrial dysfunction precipitates the oxidative stress or conversely, whether oxidative stress contributes to mitochondrial failure and the generation of ROS (Galley 2011). Lowes and colleagues, have shown that intravenous administration of potent antioxidants such as MitoQ and MitoE that specifically accumulate in mitochondria can attenuate LPS-induced increases in IL-6 levels and increase animal survivability (Lowes, Webster et al. 2013). In another study, Zapelini and co-workers, demonstrated that the administration of NAC

several hours after the induction of sepsis by CLP attenuated cytochrome-c release from mitochondria along with mitochondrial swelling (Zapellini, Rezin et al. 2008). While these studies were targeted at observing the effects of anti-oxidants in sepsis on mitochondria, it should be noted that small levels of anti-oxidants were also present in cytoplasm. As such, it is difficult to specifically determine the role of mitochondrial dysfunction might play in sepsis progression. Nonetheless, other studies using different anti-oxidants including vitamin E (Yao, Carlson et al. 2015), vitamin C (Kim, Ha et al. 2015), and melatonin (Kleber, Kubulus et al. 2014) have also shown survival benefit in animal models of sepsis when given in combination with conventional antibiotic and intravenous fluid therapy.

Nanomedicine and Applications of CeO₂ Nanoparticles for the Treatment of Inflammatory Diseases

Nanotechnology is characterized by the study and application of matter at the atomic and molecular level (Patil, Mehta et al. 2008). Nanomedicine is one particular area of nanotechnology of increasing importance. At outgrowth of this area is the development of nanoparticles or nano-devices for the treatment of several different pathologies including cancer (Paulmurugan, Bhethanabotla et al. 2015), stroke (Panagiotou and Saha 2015), and diabetes (Sharma, Sharma et al. 2015). Nanoparticles are particles between 1 and 100 nm in size. As particle size decreases, the surface to volume ratio greatly increases which can lead to increased therapeutic efficacy (Jena, Mishra et al. 2013). Of particular interest, researchers are now trying to employ the use of nanoparticles in the treatment of pathological diseases that are characterized by increased ROS. Elevated levels of ROS are seen in stroke, sepsis, diabetes, and other cardiovascular pathologies. Currently, conventional anti-oxidants such as edaravone or N-acetyl cysteine are being used to treat stroke and Parkinson's disease (Lapchak 2010). While effective

in action, one of the major drawbacks with this treatment is the need for repetitive administration over long periods of time. In contrast nanotechnology has given rise to several nanoparticles that act as catalytic ROS scavengers that can function for long periods of time in the body. One such compound are the SOD-conjugated poly (D, L-lactic-co-glycolic acid) (PLGA) nanoparticles developed by Reddy and coworkers to treat cerebral ischemia reperfusion injury in rats (Reddy and Labhasetwar 2009). Similarly platinum nanoparticles have also been shown to possess catalase mimetic activity that can be used to scavenge superoxide and even increase life span in *C. elegans* (Kim, Shirasawa et al. 2010).

Cerium oxide (CeO_2) nanoparticles are perhaps the most widely used anti-oxidant nanoparticles in the biomedical field. Cerium belongs to the lanthanide series of transition metals and it exhibits that capability to cycle between Ce^{+3} and Ce^{+4} depending on the surrounding environment (Pulido-Reyes, Rodea-Palomares et al. 2015). This unique property of nanoceria allows it to scavenge ROS for an extended period of time. Furthermore, in therapeutic doses these nanoparticles have been shown to be largely non-toxic (Hirst, Karakoti et al. 2013). CeO_2 nanoparticles have been shown to inhibit the progression of ovarian cancer (Giri, Karakoti et al. 2013) and decrease insulin resistance (Rocca, Moscato et al. 2015) in animal models. Kolli and coworkers demonstrated that these nanoparticles can also inhibit the development of monocrotaline induced right ventricular hypertrophy by scavenging ROS (Kolli, Manne et al. 2014). Similarly, Oro and coworkers, have shown that CeO_2 nanoparticles can attenuate carbon tetrachloride-induced liver fibrosis by attenuating the expression of the EPX, NCF1 and NCF2 proteins that are involved in oxidative stress (Oro, Yudina et al. 2015). In addition, CeO_2 nanoparticles have also been shown to possess anti-bacterial activity (Shah, Shah et al. 2012). A study by Babu and coworkers, demonstrated that gold supported CeO_2 nanoparticles exhibited a

strong inhibitory effect on *Bacillus subtilis*, *Salmonella enteritidis*, *Escherichia coli*, and *Staphylococcus aureus* (Babu, Anandkumar et al. 2014). In an *in vivo* model, CeO₂ nanoparticles have been shown to reduce sepsis induced mortality by scavenging reactive oxygen species in the liver (Manne, Arvapalli et al. 2015) and kidneys (Selvaraj, Nepal et al. 2015). Whether, CeO₂ nanoparticles provide protection against sepsis induced splenic damage is not known.

Nanotechnology is a recent scientific discipline that has its branches rooted in several fields including medicine. Recently, nanotechnology has given rise to new devices that can effectively clear bacteria from the circulation (Kang, Super et al. 2014) along with various nanoparticles that are anti-microbial (Ramalingam, Rajaram et al. 2014) and anti-inflammatory (Lai, Shieh et al. 2015) in nature. Cerium oxide (CeO₂) nanoparticles exhibit potent anti-oxidant activity (Caputo, De Nicola et al. 2015) and can exist in both oxidized and reduced states (Walkey, Das et al. 2015). This latter ability provides CeO₂ nanoparticles with the capacity to absorb free radicles. Recent studies have suggested that these particles may be useful for treating several pathologies including cancer (Pesic, Podolski-Renic et al. 2015), cardiovascular disease (Kolli, Manne et al. 2014), and diabetes (Pourkhalili, Hosseini et al. 2011). Previous work has also shown that CeO₂ nanoparticles can act as a superoxide dismutase mimetic (Korsvik, Patil et al. 2007) and this property has been exploited for the treatment of right ventricular hypertrophy (Kolli, Manne et al. 2014), hypoxia (Arya, Sethy et al. 2013), endometriosis (Chaudhury, Babu et al. 2013), wound healing (Chigurupati, Mughal et al. 2013) and autoimmune encephalitis (Eitan, Hutchison et al. 2015). In addition, other investigation has shown that CeO₂ nanoparticles can inhibit the growth of pathogenic bacteria such as *E. coli* and *B. subtilis* by adsorbing on to their membranes (Pelletier, Suresh et al. 2010).

Previous work by our laboratory has demonstrated that CeO₂ nanoparticles can prevent polymicrobial insult-induced mortality, decreased systemic cytokine levels and attenuated liver and renal failure (Manne, Arvapalli et al. 2015). The action of CeO₂ nanoparticles on a spleen following polymicrobial insult is not known. Our findings suggest that a single dose treatment with CeO₂ nanoparticles can be used to attenuate splenic damage, reduce the levels of the pro-inflammatory cytokine high mobility box group 1(HMGB1) and that these findings were also associated with decreased bacterial load in the circulation.

CHAPTER 3

MATERIALS AND METHODS

CeO₂ Nanoparticle Characterization

Nanoparticles were purchased from US Research Nanomaterials Inc. (Houston, TX) and characterized. Structural characterization of the CeO₂ nanoparticles was performed using a JEOL JSM-6320F field emission scanning electron microscope (FESEM). The particle size of the CeO₂ nanoparticles was determined by transmission electron microscopy using a JEOL JEM-2010 transmission electron microscope (TEM) operated at 300 Kev. Energy dispersive X-ray spectroscopy was performed to determine the purity of sample.

Animal Model of Polymicrobial Sepsis and Therapeutic Intervention

Ten week old male Sprague Dawley rats were purchased from Hill-Top laboratories and housed two-per cage at $22 \pm 2^\circ$ C with a 12:12 light-dark cycle. Animals were allowed to acclimate to their surroundings for two weeks before any experimentation. Animals were fed with standard rodent chow and had access to food and water *ad libitum* during the entire study. 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 deeply anesthetized under isoflurane and a small mid ventral incision of 0.5 cm was made. Sham controls and CeO₂ nanoparticles only groups were injected with 5 ml/kg of 5% sterile dextrose solution intraperitoneally. The incision was closed with 3-0 silk sutures as described elsewhere. For sepsis and sepsis+CeO₂ nanoparticles groups, animals received a cecal inoculum of 600 mg/kg BW in 5 ml/kg BW of 5% sterile dextrose solution intraperitoneally. Cecal material was collected from the caecum of healthy rats following sacrifice under isoflurane

anesthesia. Two hundred microliters of sterile distilled water was injected intravenously via the tail vein to animals in the sham control and sepsis groups while the CeO₂ and sepsis + CeO₂ groups received CeO₂ nanoparticles (0.5 mg/kg) in 200 µl of sterile distilled water as described previously (Manne, Arvapalli et al. 2015). Animals were randomly assigned to one of the four groups and were sacrificed at 3 h and 18 h after sepsis induction to study the molecular events during early and late phases of sepsis.

Collection and Preservation of Spleen

Animals from different groups were sacrificed under anesthesia at 3h and 18h after sepsis induction. Peritoneal fluid was collected with a sterile syringe while whole blood was collected through cardiac puncture. Spleen was collected and washed with Krebs–Ringer bicarbonate buffer (KRB) to remove any blood, blot dried and frozen in liquid nitrogen. Spleens were stored at -80°C for further experiments.

Histology

Frozen spleens were sectioned (8µm) using Leica CM1950 cryostat onto poly-L-lysine coated slides at -18°C and then stored at -80°C. Hematoxylin and eosin staining was performed using BBC Biochemical H & E staining kit (Cat No. NC9960321, Fisher Scientific, Pittsburgh, PA) on frozen spleen sections to visualize the change in red pulp and white pulp morphology with sepsis and CeO₂ nanoparticle treatment. Briefly, frozen sections were washed with running tap water and stained with Harris hematoxylin for 5 min. Slides were then washed with water for 1 min and then with acid wash for 45 s. This was followed by washing and incubation with blueing solution for 15 s. Slides were washed again, incubated with 70% alcohol for 30 s and Eosin for 1 min. After alcohol dehydration and xylene clearing the splenic morphology was visualized using an Evos XL microscope (Life technologies, Grand Island, NY).

SDS-PAGE and Immunoblotting

Approximately 50 mg of frozen spleen tissue was measured and pulverized in liquid nitrogen. Four hundred and fifty microliters of T-PER (Pierce, Rockford, IL, USA) containing 1% protease and phosphatase inhibitors (P8340 and P5726, Sigma- Aldrich, St. Louis, MO, USA) was added to the sample, the samples homogenized and then centrifuged at 5,000 x g for 15 min at 4°C to collect the supernatant. The 660 nm assay (Pierce, Rockford, IL, USA) was performed to determine the protein content in the supernatant and the samples were normalized with T-PER and 4x Laemlli buffer to obtain equal protein concentration across all samples. Forty micrograms of protein were loaded in 10% PAGEr Gold Precast gel (Lonza, Rockland, ME) and transferred to nitrocellulose membranes as described elsewhere (Selvaraj, Nepal et al. 2015). Nitrocellulose membranes were then blocked with 5% milk in Tris-Buffered saline with 0.05% Tween-20 (TBST) for 1h at room temperature, washed three times with TBST and probed with antibodies specific for Bax, cleaved caspase 3, caspase 3, HMGB1, and GAPDH (Cell Signaling Technology, Danvers, MA). Membranes were incubated with primary antibody for 16 h at 4° C and washed three times with TBST for 5 min each. After incubation with a secondary anti-rabbit antibody (Cell Signaling Technology, Danvers, MA) for 1 h at room temperature, immunoreactivity was visualized using Super signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) and quantified using Fluorchem 9900 software (Protein Simple, Santa Clara, CA). Protein expression relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize protein expression.

Bacterial Cultures

Whole blood was collected via cardiac puncture and peritoneal lavage fluid was obtained using a sterile syringe. Blood and peritoneal lavage samples were serially diluted in sterile saline

and plated on sheep's blood agar/ nutrient agar plates. Plates were incubated overnight at 37° C and colony counts were determined 24 hours later. Colony counts were expressed as CFU/ml of fluid and then converted to a logarithmic scale for statistical analysis.

Statistical Analysis

Results are presented as mean \pm standard error of mean. One way analysis of variance (ANOVA) was performed to detect significant differences in means. *Post hoc* comparisons were done using a Student Newman Keul's test for normally distributed samples or a Kruskal-Wallis one-way ANOVA by ranks for samples exhibiting a non-normal distribution. A probability value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Nanoceria Characterization

Field emission scanning electron microscopy demonstrated that the nanoparticles were round to spherical in shape (Figure 1A). Transmission electron microscopy demonstrated the typical lattice structure of the nanoparticles with an approximate diameter of 10-20 nanometers (Figure 1B).

Cerium Oxide Nanoparticles Attenuate the Splenic Damage Induced by Polymicrobial Sepsis

Compared to that observed in the controls, polymicrobial sepsis did not result in extensive splenic damage/necrosis in early stages (3h) of sepsis (Figure 2, A-D). Conversely, in the latter stages of sepsis (18h) polymicrobial insult appeared to result in the loss of lymphocyte rich white pulp (Figure 2, E, and G). Cerium oxide nanoparticle treatment appeared to attenuate the loss of white pulp in at the 18h time point (Figure 2, G and H).

Cerium Oxide Nanoparticle Treatment Attenuates Sepsis Associated Increases in Splenic HMGB1 Expression

Previous studies have shown sepsis is associated with a massive increase in the splenic levels of HMGB1 (Valdes-Ferrer, Rosas-Ballina et al. 2013). Consistent with this finding, HMGB1 expression was increased with sepsis at 3h and 18h after induction (Figure 3A). Consistent with our histological findings of improved tissue splenic structure, nanoparticle treatment decreased HMGB1 levels at 18h time point (Figure 3A).

Apoptosis is not a Major Pathway for Splenic Cell Death in the Polymicrobial Inoculum Sepsis Model

Although recent data has suggested that lymphocyte apoptosis in the spleen is increased in the cecal ligation and puncture sepsis model (Hotchkiss, Coopersmith et al. 2005) we did not find similar evidence in the CI model. Specifically, sepsis did not appear to increase the levels of apoptotic proteins Bax, cleaved caspase 3 and caspase 3 in the spleen (Figure 4A, B, and C). Similarly, treatment with CeO₂ nanoparticles did not appear to affect splenocyte death.

Cerium Oxide Nanoparticles Attenuate Bacterial Load in The Blood And Peritoneal Fluid of Septic Animals

The number of CFU in the blood and peritoneal fluid were significantly increased at 18h in the septic animals which were decreased with nanoparticle treatment (Figure 5, A-B).

Discussion

Despite considerable improvements in medical technology, millions of people die from sepsis each year. With growing antibiotic resistance and the emergence of new pathogens, the need to develop new safe and effective drugs is of paramount significance. CeO₂ nanoparticles are potent anti-oxidants and that they might be beneficial for the treatment of cancer (Giri, Karakoti et al. 2013), stroke (Estevez, Pritchard et al. 2011) and other diseases characterized by increased systemic ROS levels. Recent studies have shown that cerium oxide nanoparticles are effective able to reduce mortality in Sprague Dawley rats subjected to polymicrobial insult (Manne, Arvapalli et al. 2015). Conversely, other work has suggested that CeO₂ nanoparticles may be toxic to cells and that they might cause tissue damage and remodeling (Aalapati, Ganapathy et al. 2014). Why different studies may arrive at different results is currently unknown, but could be related to differences in nanoparticle size, shape and charge (Das, Dowding et al. 2013). In an effort to better understand the relationship between nanoparticle morphology and biological activity, we first characterized the particles using electron microscopy. Consistent with our previous data (Manne, Arvapalli et al. 2015), nanoparticles were spherical in shape with an average size of 10-30nm in diameter (Figure 1).

Previous studies have shown that there is loss in white pulp (lymphocytes) in spleen with sepsis. To confirm whether CeO₂ nanoparticles can prevent the loss of white pulp, hematoxylin and eosin staining was performed on frozen spleen sections. CeO₂ nanoparticle treatment attenuated the loss of white pulp which appeared to maintain the structural integrity of spleen (Figure 2). Using the mouse CLP sepsis model, Hotchkiss and colleagues, demonstrated that apoptosis is the major mechanism of lymphocyte cell death in spleen with sepsis (Hotchkiss, Tinsley et al. 1999). In particular, they found that sepsis was associated with a significant amount

of CD4⁺ T cell death. To investigate whether a similar phenomenon occurs in our animal model, immunoblotting was performed to evaluate the expression of apoptotic regulators Bax, cleaved caspase 3 and caspase 3. Interestingly, we failed to find any sepsis-associated increase in cell death. Why our results might differ from those found by Hotchkiss and co-workers is not entirely clear, but might be due to difference in the sepsis model used. Unlike the CLP model which exhibits a slow septic progression, the cecal inoculum model is rapid in onset. As such, the cell death seen in the cecal inoculum model may occur via necrosis rather than apoptosis. Similarly, because the T cells make up only a small percentage of the total spleen cell number, it is possible the presence of T cell apoptosis could be obscured or diluted by looking at apoptotic events using whole organ lysates. Repeating these studies after the isolation of specific cell types prior to investigating apoptotic signaling may be beneficial in furthering our understanding of the roll that cellular apoptosis may play in the spleen of animals subjected to the cecal inoculum model of sepsis.

Recent studies have shown that HMGB1, a nuclear protein, is released during late phase of sepsis into circulation and that it is highly correlated with organ failure and sepsis progression (Huang, Tang et al. 2010, Wang, Ward et al. 2014). Several clinical trials have also demonstrated high levels of HMGB1 in blood of septic patients (Angus, Yang et al. 2007) and that administration of anti-HMGB1 antibody has been shown to be organ protective (Qin, Wang et al. 2006). In relation to this, Jared and coworkers, have shown that the removal of the spleen can be protective in some instances of sepsis as it appears to reduce circulating HMGB1 levels (Huston, Wang et al. 2008). Here, for the first time, we show that intervention with CeO₂ nanoparticles can attenuate the levels of HMGB1 in septic spleen (Figure 3). Whether this decrease in HMGB1

alone can explain the increased animal survival seen in CeO₂ treated animals is currently unclear and will require further investigation.

In an effort to extend our findings on the therapeutic efficacy of CeO₂ nanoparticles, we next evaluated the bacterial load in blood and peritoneal fluid of septic animals. Spleen plays an important role in immune mediated bacterial clearance and that loss in splenic function would attenuate the pathogen clearance. Here we found that treatment with the CeO₂ nanoparticles was associated with diminished bacterial load in both the blood and peritoneal fluid (Figure 4).

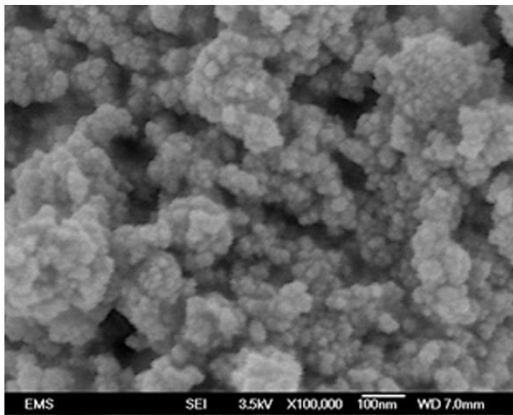
Whether this attenuation is due to a direct effect of CeO₂ nanoparticles on splenic lymphocyte population, the liver Kupffer cells or circulating monocytes is not known and is a potential subject for future investigation.

FIGURES

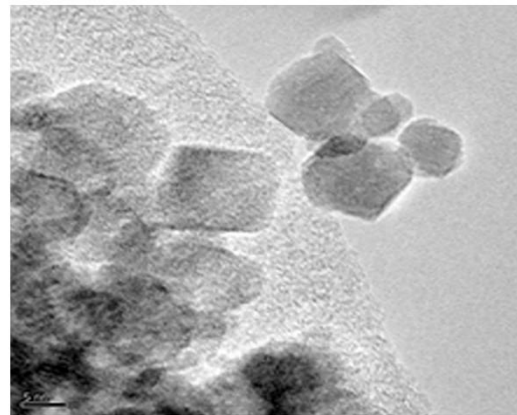
Figure 1 Characterization of CeO₂ nanoparticles.

A) Field emission scanning electron microscopy (FESEM) B) Transmission electron microscopy (TEM) C) Energy dispersive X-ray spectroscopy (EDS).

A)



B)



C)

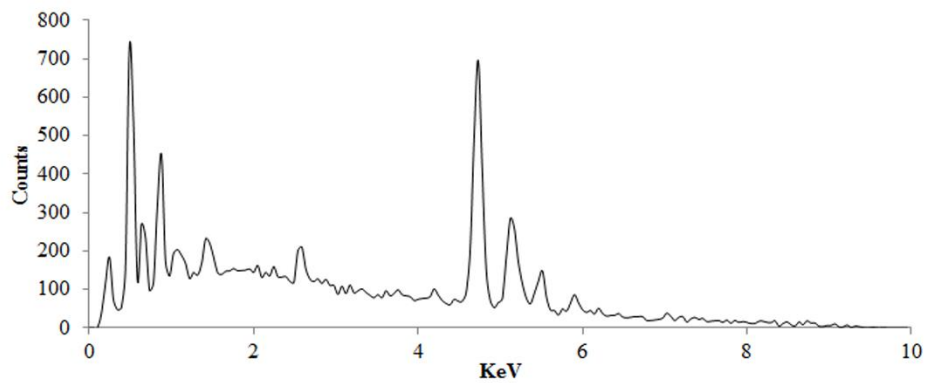


Figure 2 CeO₂ nanoparticles attenuate severe sepsis induced inflammatory damage in spleen.

A-D) Hematoxylin and eosin (H&E) staining of 3h time point spleen sections imaged at 200× magnification - Control, CeO₂, Sepsis, and Sepsis + CeO₂. E-H) Hematoxylin and eosin (H&E) staining of 18h time point spleen sections imaged at 200× magnification - Control, CeO₂, Sepsis, and Sepsis + CeO₂ (n=3/group). Note: loss of white pulp in sepsis 18h (arrow).

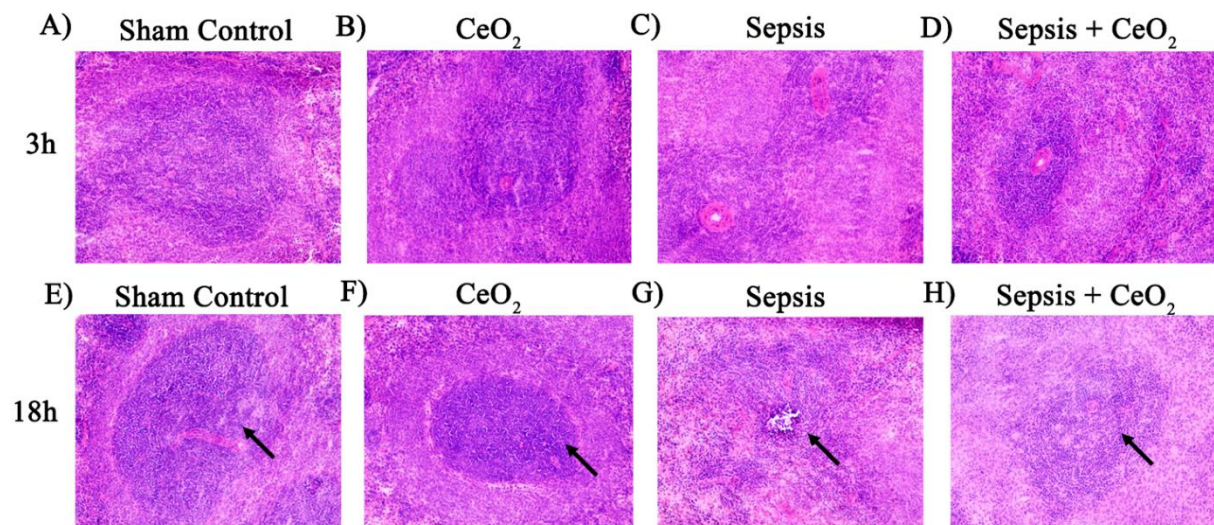


Figure 3 CeO₂ nanoparticles attenuate severe sepsis induced increase in expression of pro-inflammatory cytokine HMGB1.

*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).

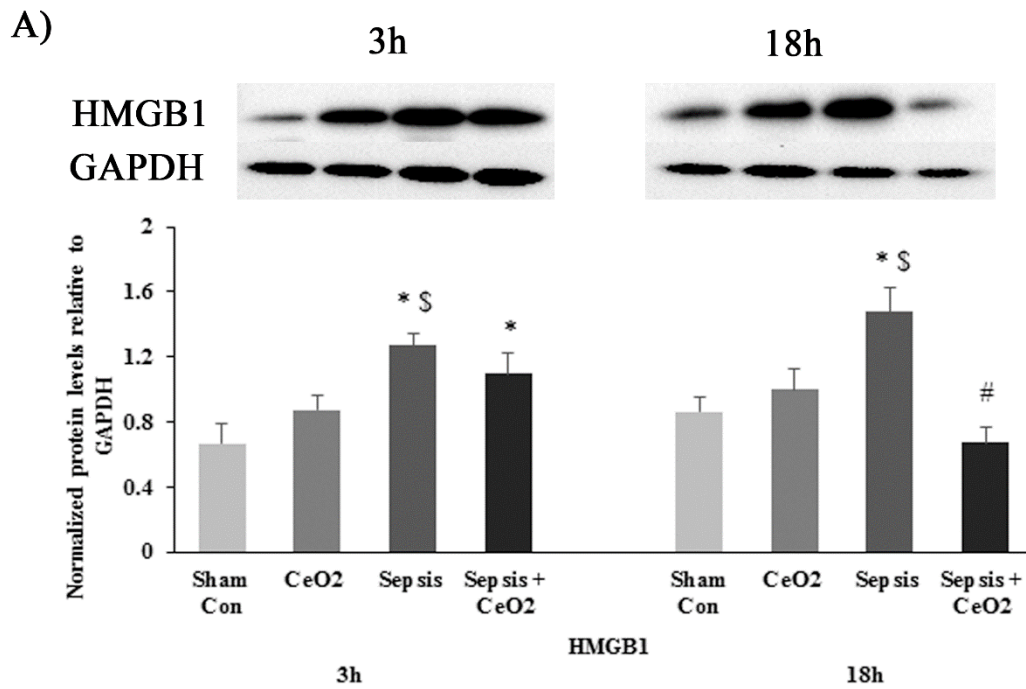


Figure 4 Cecal inoculum method of polymicrobial sepsis does not induce caspase 3 mediated apoptosis in spleen.

A) Total protein levels of Bax at 3h and 18h in Control, CeO₂, Sepsis, and Sepsis + CeO₂ groups. B) Levels of cleaved caspase 3 at 3h and 18h in Control, CeO₂, Sepsis, and Sepsis + CeO₂ groups. C) Total levels of caspase 3 at 3h and 18h in Control, CeO₂, Sepsis, and Sepsis + CeO₂ groups. (n=6/group).

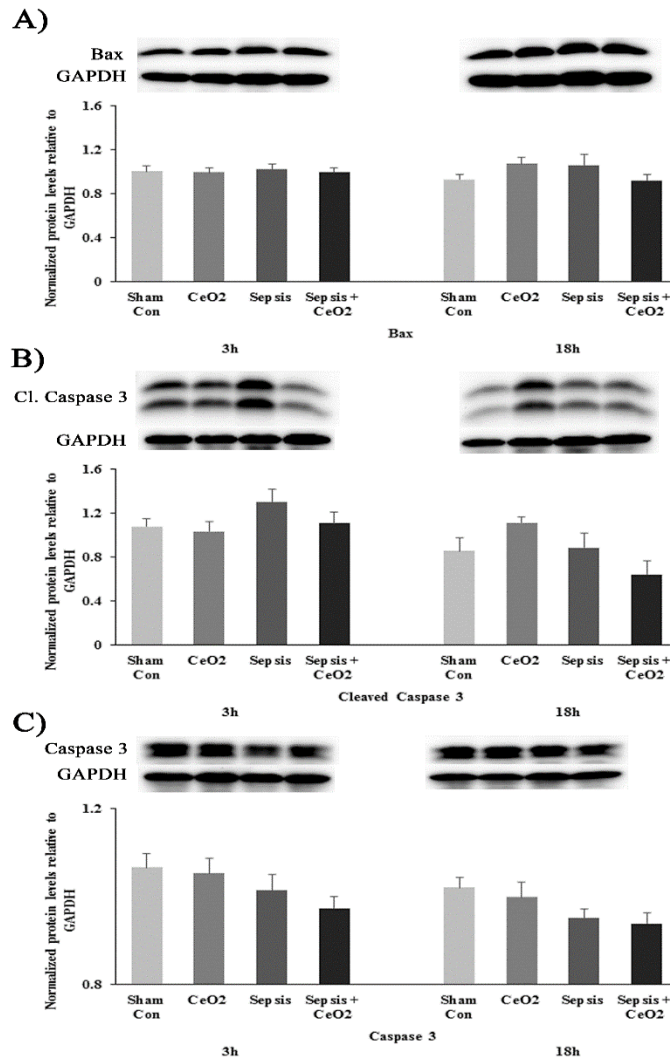
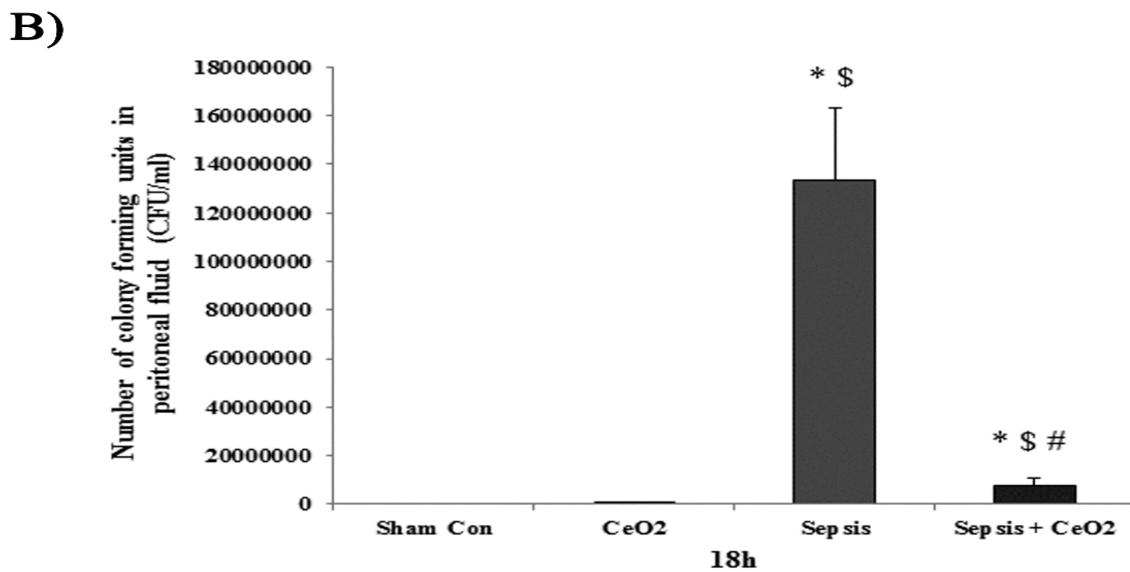
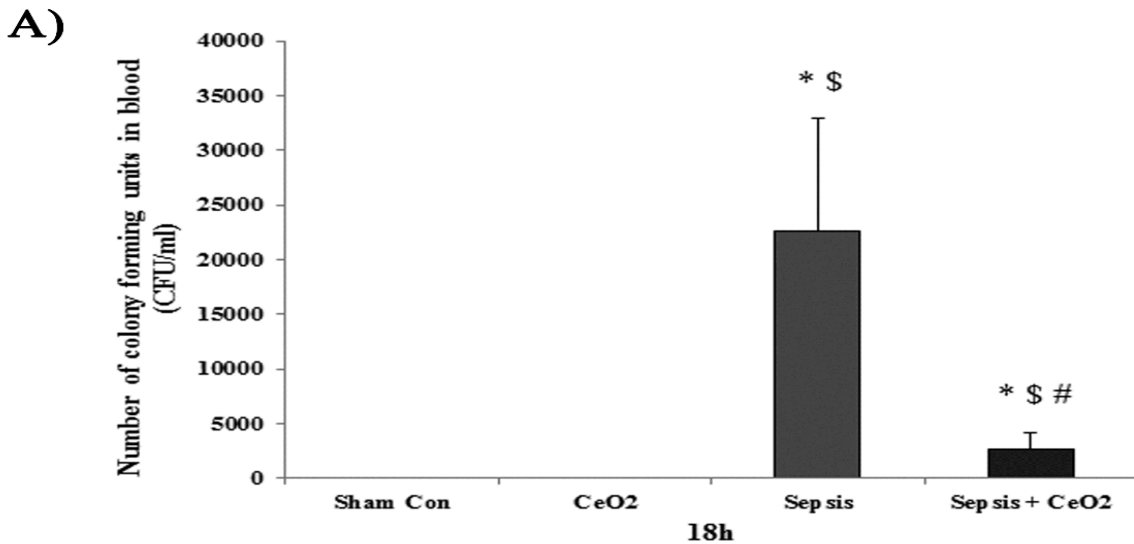


Figure 5 CeO₂ nanoparticles attenuate bacterial load in blood and peritoneal fluid of septic animals.

A) Bacterial load in blood

B) Bacterial load in peritoneal fluid

*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).



CHAPTER 4

CONCLUSIONS

Sepsis is the leading cause of death in non-coronary intensive care unit in United States (Mayr, Yende et al. 2014). Current treatment regimens are largely supportive in nature and fail to address the uncontrolled immune response in severe sepsis. CeO₂ nanoparticles exhibit potent anti-inflammatory activity and that they may have biomedical use for the treatment of stroke (Estevez, Pritchard et al. 2011), cancer (Gao, Chen et al. 2014), hepatic steatosis (Oro, Yudina et al. 2015) and diabetes (Hosseini, Baeeri et al. 2013). These particles can also be used to treat severe sepsis in Sprague Dawley rats that are subjected to either a LPS (Selvaraj, Nepal et al. 2015) or a polymicrobial sepsis insult (Manne, Arvapalli et al. 2015). Here we investigated whether CeO₂ nanoparticles can effect/attenuate splenic damage in Sprague Dawley rats subjected to polymicrobial insult. Our data suggest the following:

1. Histological investigation demonstrated that CeO₂ nanoparticle treatment is associated with an attenuation of sepsis induced splenic damage. Specifically, we found that treatment with CeO₂ nanoparticles decreased the loss of white pulp when compared to septic animals,
2. CeO₂ nanoparticle treatment functioned to decrease sepsis associated increases in splenic HMGB1 expression in the hypo dynamic phase of sepsis.
3. CeO₂ nanoparticle treatment attenuated the bacterial load in the blood and the peritoneal fluid of septic animals.
4. Neither sepsis nor CeO₂ nanoparticle treatment affected the protein expression of apoptotic regulators in the spleen.

Future Directions

The current study investigated whether CeO₂ nanoparticles can attenuate splenic damage in the polymicrobial model of sepsis. While the preliminary results indicate that CeO₂ nanoparticle treatment confers some degree of splenic protection, the exact mechanism of action is currently unclear. Previous studies have shown that CeO₂ nanoparticles accumulate in the spleen (Selvaraj, Nepal et al. 2015) and in the liver Kupffer cells (Tseng, Lu et al. 2012) when administered intravenously. Whether CeO₂ nanoparticles are taken by lymphocyte population or the splenic cords after intravenous injection is not known. Determining the site of CeO₂ localization by the isolation of the different splenic cell types and quantification of ceria levels by induction coupled plasma mass spectroscopy (ICP-MS) is likely to be invaluable in helping to establish where the CeO₂ nanoparticles might exert their beneficial effects. Previous studies by Hotchkiss and colleagues, demonstrated that sepsis is associated with increased apoptosis of splenic CD4⁺, CD8⁺ T cells and CD 19 positive B cells (Hotchkiss, Tinsley et al. 1999). Additional experiments using immunohistochemistry to examine the effects of sepsis and the nanoparticle treatment on the number of these different cell types is needed to further confirm that CeO₂ nanoparticles act by modulation of lymphocyte function.

The sepsis model used in the current study involves administration of a single bolus of known quantity of cecal material into peritoneal cavity. Although polymicrobial in nature, it more closely models the onset of septic symptoms seen in the LPS model rather than that observed in human patients. As such, future experiments using the CLP model which better mimics the progression of sepsis seen in humans are likely need to better establish the potential efficacy of this nanoparticle based treatment. Similarly, in the current study CeO₂ nanoparticles were administered at the same time as sepsis induction. It is not known whether CeO₂

nanoparticles can provide the same degree of therapeutic efficacy when administered during later phases of sepsis that is characterized the presence of severe hypotension and uncontrolled cytokine storm. Further experiments to test this hypothesis would be very beneficial in determining the clinical potential of our approach.

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APPENDIX: LETTER FROM INSTITUTIONAL RESEARCH BOARD



Office of Research Integrity

November 16, 2015

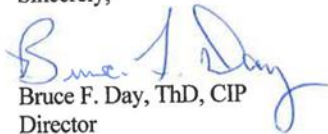
Vendata Bandrupalli
Room #217, Byrd Biotech Building
Marshall University

Dear Mr. Bandrupalli:

This letter is in response to the submitted thesis abstract entitled "*Cerium Oxide Nanoparticles Attenuate Polymicrobial Sepsis Induced Splenic Damage in Male 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 Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction it is not considered human subject research. 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

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