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Toxicological Effects of Cerium Oxide Nanoparticle Aggregates on Caenorhabditis elegans

Steven N. Rogers
stevenrogersemail@gmail.com

Toxicological Effects of Cerium Oxide Nanoparticle Aggregates on *Caenorhabditis elegans*

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Steven N. Rogers

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Dr. Eric Blough, Committee Chairperson

Dr. Philippe Georgel

Dr. Todd Green

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Abstract

Assessing the toxicity and unique reactivity of nanoparticles in biological systems has become an relevant and quickly growing area of environmental toxicology research. The broad use of nanoparticles in industrial and commercial commodities results in exposure of these nano-compounds to the environment, the ecosystems, and humans. While previous data has suggested that cerium oxide (CeO_2) nanoparticles are relatively safe to cultured cells much less is known about the potential toxicity of these materials at the organismal level. In this study we employed transgenic *Caenorhabditis elegans* (*C. elegans)* strains to assess the toxicity of CeO_2 nanoparticles under "real-world" conditions. Our findings indicate that while exposure to aggregated CeO_2 in *C. elegans* has no effect on average life span, it is associated with decreases in nematode body length, progeny count, and increased organismal stress. These findings demonstrate that exposure to aggregated CeO_2 particles (0-17.21 ug/mL) may be associated with diminished organismal fitness in *C. elegans*.

Acknowledgments

This thesis would not have been possible without the tremendous assistance and the support provided by my advisor Dr. Eric Blough, our lab manager Kevin Rice, Dr. Vellaimamy Selvaraj, and my supporting committee Dr. Philippe Georgel and Dr. Todd Green. Each individual was at the ready to provide feedback, instruction, or give suggestions whenever I needed them. Their time, consideration, and willingness to give this project attention regardless of their workloads is greatly appreciated. I would also like to thank the fellow members of the Blough lab, especially Eli Shleser, who taught me all *C. elegans* culturing techniques for this project, as well as Prasanna Manne, who has not only been there to answer my benchwork questions, but those of academic nature as well. Eric Blough has managed to build a lab with a motivational environment filled with workers of diverse backgrounds who show dedication above and beyond what I have come to expect. Their attention to effort and hard work has certainly rubbed off on me. I have gained knowledge in experimental design, courage to pursue a larger scoped project, and understanding of what it takes to be a scientist thanks to my friends and coworkers in my lab.

List of abbreviations and symbols

ENM	Engineered nanomaterial
CeO_2	Cerium oxide
HSP	Heat shock protein
DNA	Deoxyribonucleic acid
TEM	Transmission electron microscopy
DLS	Dynamic Light scattering
ROS	Reactive oxygen species
nm	Nanometer
nM	Nanomolar
HO-1	Heme oxygenase-1
GST	Glutathione s-transferase
TR	Thioredoxin reductase
DLS	dynamic light scattering
TEM	transmission electron microscopy
HRTEM	High resolution transmission electron microscopy
GFP	Green fluorescent protein
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
IL	Interleukin
SOD	superoxide dismutase
FDA	Food and Drug Administration
MTL-2	metallothionein 2
MAPK	mitogen activated protein kinase

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CHAPTER 1

Introduction

Nanotechnology is a quickly evolving field of science and is considered a vital technology of the 21st century [4] [5]. The nanotechnology industry and its various applications are expanding rapidly, with a global market size estimated to be in excess of $1 trillion by the year 2015 [6]. There are currently over 1000 products containing nanomaterials on the market. Increasing nanomaterial use is accompanied by an increased risk of nanomaterial exposure. The long-term goal of nanotoxicology is to understand and categorize nanoparticles by physicochemical and molecular determinants, biodistribution, routes of exposure, and potential genotoxicity [7]. How exposure to nanomaterials may affect biological function is not well understood. Indeed, it was not until 2011 that the FDA began to publish proposed guidelines for evaluating use of nanomaterials. Thus far, interest in the potential toxicity of nanomaterials (nanotoxicity) has been sparse.

Nanoparticles are a type of nanomaterial that are created by the engineered synthesis of a larger bulk compound into smaller particles with a single dimension less than 100 nm [4]. Nanoscale compounds oftentimes have an increased chemical reactivity and higher catalytic ability than that observed in their "bulk" counterparts given their higher surface to volume ratio [5]. How nanoparticles may affect biological function is not well understood. Given the ethical and logistical problems associated with performing toxicity studies in humans the use of animals and cultured cellular constructs is often desirable. The soil nematode *Caenorhabditis elegans* (*C. elegans*) is often employed as a model organism to study environmental toxicology. The value of using nematodes to study nanotoxicology lies in assessing how accumulation of engineered nanomaterials in our environment might affect soil organisms.

Cerium dioxide (CeO_2) is an oxide of the abundant earth metal ceria and is now used in a wide variety of applications in its nanoscale form. These uses include: acting as a catalyst (an

electrolyte material of solid fuel cells) in catalytic converters to convert harmful carbon monoxide to safe carbon dioxide [8], as a ultraviolet blocking material [9], and as an industrial polishing reagent [10]. The biological effects and toxicity of CeO_2 are equivocal. Unlike other metal oxide nanoparticles, recent data has suggested that CeO_2 may be neuroprotective and anti-inflammatory [11-14]. CeO_2 nanoparticles are also thought to function as superoxide dismutase (SOD) and catalase mimetics. Interestingly, additional work has suggested that CeO_2 nanoparticles exhibit the ability to switch valence states between +3 and +4 which may aid in their ability to scavenge reactive oxygen species (ROS) [15, 16]. In contrast, other data has suggested that exposure to CeO_2 nanoparticles leads to increased oxidative stress [17, 18], inflammation, and DNA damage [19-22]. In a similar fashion, it has also been shown that CeO_2 nanoparticles are toxic to organisms in aquatic environments [23, 24].

Purpose

The objective of this research was to examine the toxicological effects of CeO_2 nanoparticles in *C. elegans.* To address this objective, we examined how exposure to different concentrations of CeO_2 nanoparticles affected *C. elegans* longevity, reproduction, and the stress response to exposure to increased temperature. The overall hypothesis of this study is that exposure to CeO_2 nanoparticles will elicit toxicological effects on *C. elegans* in a dose-dependent manner.

Specific aims

Specific Aim #1

To determine if exposure to CeO_2 nanoparticles effects *C. elegans* life span.

Hypothesis

Exposure to CeO_2 nanoparticle will effect *C. elegans* life span in a dose-dependent manner.

Specific Aim #2

To determine if exposure to CeO2 nanoparticles effects the oxidative and heat shock stress response in *C. elegans*, and to determine if exposure to CeO_2 nanoparticles effects the oxidative and heat shock stress response in *C. elegans.*

Hypothesis

Exposure to CeO_2 nanoparticle will effect the oxidative and heat shock stress response in *C. elegans* in a dose-dependent manner.

Specific Aim #3

To determine if exposure to CeO_2 nanoparticles effects *C.elegans* fecundity.

Hypothesis

Exposure to CeO_2 nanoparticles will effect *C. elegans* fecundity in a dose-dependent manner.

Specific Aim #4

To determine if exposure to CeO_2 nanoparticles effects growth inhibition and changes development in *C. elegans*

Hypothesis

Exposure to CeO_2 nanoparticles will effect growth inhibition and change development in *C. elegans.*

Specific Aim #5

To determine if exposure to CeO_2 nanoparticles *C.elegans* is associated with changes in survivability during exposure to elevated environmental temperature.

Hypothesis

Exposure to CeO_2 nanoparticles under increased environmental temperature will alter survivability in *C. elegans.*

CHAPTER 2

REVIEW OF LITERATURE

Introduction

In the following chapter, a review of the literature concerning the present study will be presented. The following areas will be addressed: 1) Nanotechnology and engineered nanomaterials, 2) Structure and function of CeO_2 nanoparticles, 3) The use of *C. elegans* as a model organism and their life cycle, and 4) Use of engineered *C. elegans* strains CL2166 and SJ4005 as models for measuring the toxicological effects of nanoparticle exposure.

Nanotechnology - a major advancement in modern science

The prefix "nano" is derived from the Greek word “nanos” which means “dwarf”. On an absolute scale, a nanometer is equivalent to one billionth of a meter. Nanotechnology is the capability of manipulating matter on a molecular and atomic level, and is considered a major advancement in modern science. With decreasing size, the surface to volume ratio of a compound increases, which is oftentimes associated with increased chemical reactivity and other changes in the physical reactivity of the compound [25]. Applications for nanotechnology include uses in medicine, electronics, molecular self-assembly [26], scanning probe microscopy, and solar cell technology [25] [27].

The origin of nanotechnology is most often attributed to Richard Feynman and his now famous talk, “There's plenty of room at the bottom” [28]. In the 1980s, the completion of the scanning tunneling microscope and atomic force microscope allowed observation and manipulations of molecules on a nano-scale. Since the advancement of these microscopes, many additional techniques for engineering nanomaterials were developed, and rapid progress has been made in nanotechnology [29].

New applications for nanotechnology as well as the increasing ease of manufacturing were so sudden that a flood of engineered nanomaterials in commercial and industrial products began to surface in the 2000s. Incorporating nanomaterials into products appeared to be a way to make these products more efficient. The addition of silver nanoparticles for the prevention of bacterial growth to existing products was amongst the first use of nanoparticles in clothing, bandages, disinfectants and food packaging [30]. Zinc oxide nanoparticles, shown to protect against UV, were put into cosmetics, paint, and other coating materials [31]. To date, materials in both consumer and industrial products are being continually replaced with nanoscale equivalents [32].

As the use of engineered nanomaterials in products has increased, public awareness and controversy over the use and safety of nanoparticles began to develop. In July 2004, the

Royal Society of England published a report titled “Nanoscience and nanotechnologies: opportunities and uncertainties”, and the Federal Drug Administration (FDA) released the 'Nanotechnology Task Force Report” in 2007, which was centered on how to address the manufacturing of nanomaterials, the enforcement of compliance, and additional guidelines regarding the use of nanotechnology in manufactured products [33]. The FDA continues to update guidelines for regulation of nanomaterials, and is now investing in a FDA-wide nanotechnology regulatory science program for enhancing their capabilities at diagnosing nanoparticle safety [33].

Nanotoxicology

Nanotoxicology is the field of science dedicated to studying the toxicity of nanomaterials. The main purpose of nanotoxicology is to uncover threats nanoparticles may pose to the environment and human beings. When dealing with particles on the same scale as our own DNA, biological reactivity of nanomaterials is still uncertain. Indeed, the very same catalytic properties of nanomaterials that may make them therapeutic can also be the same properties that may make them harmful [25]. Nanotoxicology requires further research in order to reach standards for what are considered unnecessary exposure levels and to avoid unintended health risks to humans and the environment [34].

Nanoparticle aggregation: a natural tendency

Both attractive forces such as weak Van der Waals forces or strong, difficult to break, covalent bonds, contribute to nanoparticle aggregation, or agglomeration, depending on the type of attraction. Aggregation is used to describe attraction by weak forces and agglomeration is used to describe attraction by stronger forces. The unique properties of nanoparticles are

related to their size, as well as shape, surface area, charge, solubility, surface chemistry, diffusion rate, and purity [35]. Most of these properties change to some degree when nanoparticles begin to agglomerate/aggregate, and thus may change the reactivity and function of the material when it leaves its nano-scale form. Additionally, it has been shown that in a biological matrix, single particles tend to form agglomerates [36] [37]. It is important to study aggregated versus non-aggregated states of nanoparticles, as aggregation alters size and other physical properties that potentially alter biological reactivity.

The literature shows varying changes in toxicity during nanoparticle aggregation compared to the evenly dispersed particle interactions. Some papers report a decrease in toxicity as aggregated size increases [38] [39], while other show no size dependent changes in toxicity at all [40] [41]. For example, Gosens and coworkers [41], observed no major differences in either pulmonary or system toxicity markers (such as pro-inflammatory cytokines IL-6, MIP-2, and TNF-α) after exposure to agglomerated (250 nm) versus single nanoparticle suspensions (50 nm) of gold in rodents. Both particles were taken up by macrophages, produced the same biological effects and it was determined that smaller particles do not pose a greater hazard than that observed using the single particle suspensions [41]. Similarly, Prasad and colleagues [40] showed that titanium dioxide (TiO_2) exhibits the same chromosomal damage (as seen by micronucleus assay) *in vitro* independent of aggregation state [40]. In contrast, some nanoparticles show size dependent changes in toxicity. Arnold and coworkers [38] reported size-dependent growth inhibition in *C. elegans* after treatment with nano-scale CeO_2 (53.34 ± 3.12 nm) as compared to larger CeO_2 aggregates [38]. Kim and coworkers [39] observed a greater toxic effect of silver nanoparticles on zebrafish as seen by increased embryonic mortality. Additional size-dependant toxicity of silver nanoparticles has also been seen in fruit flies and in human C3A, Caco-2 intestinal epithelial cells, and primary trout hepatocytes [42] [43] . Taken together, these data suggest that more research is essential in

determining which specific nanoparticles are subject to size-dependent toxicity, as each species of nanoparticle has the potential to react differently in aggregated versus non aggregated states.

Cerium oxide: The many applications of ceria

Cerium oxide (CeO_2), is an oxide of the rare earth metal cerium (Figure 1). Ceria has been used traditionally as a polishing reagent [44], and recently in its nanoscale form (CeO_2) in a variety of industrial applications such as gas sensors [45], solar cells [46], chemical catalysts [47], and a fuel additive [48].

Figure 1. Three dimensional structure of CeO_2

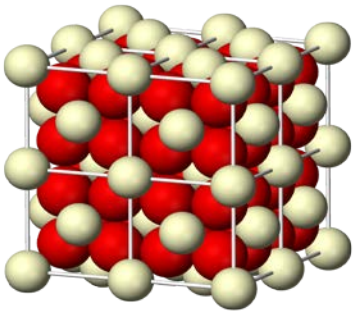

Figure 1. Three dimensional crystal lattice structure of cerium oxide (CeO_2). The light and dark colored spheres represent ceria and oxygen, respectively. As the particle size decreases more of the reactive ceria tend to be positioned on the surface of the lattice which may give rise to increased chemical reactivity [51]

Due to unique ability of ceria to undergo autoregenerative reactions between its, Ce^{3+} and Ce^{4+} oxidation states (Figure 2, [2]), the potential of using this compound for biological applications has been explored. Recent data has suggested that ceria may be neuroprotective [49] and that it may function as an anti-inflammatory [13] and antioxidant [12, 50], however it is currently unknown which pathways CeO2 interacts with to achieve these therapeutic effects.

Cerium oxide nanoparticles exhibit antioxidant activity

CeO_2 changes physiochemical properties upon entering its nano-scale form, also referred to as its nanocrystalline state. As size of a nanoparticle decreases, there is an increase in the ratio of atoms on its surface. This increase of surface atoms changes oxygen stoichiometry, allowing

an increase of trivalent Ce^{3+} ions to appear on the surface of CeO_2. The antioxidant activity of CeO_2 is associated with the presence of Ce^{3+} ions, so as particle size decreases and the ratio of surface ions increases, CeO_2 becomes more capable to undergo antioxidant redox reactions [51].

CeO_2 is unique in that it can undergo cycles of both oxidation and reduction depending on presence or lack of oxygen available and the current valence state of the particle which

Figure 2. Autoregenerative mechanism of CeO_2

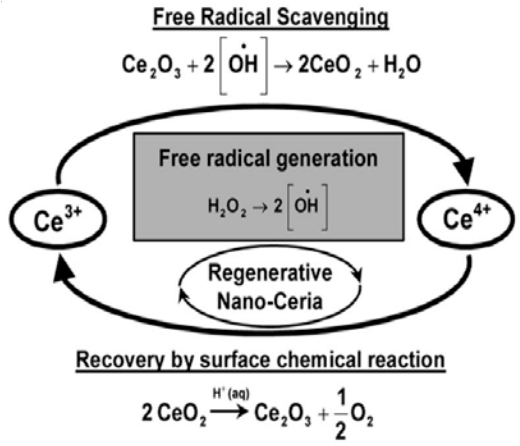

Figure 2. Autoregenerative mechanism of CeO_2.

The autoregenerative mechanism proposed by Das and colleagues (ref), displaying the regenerative properties of CeO_2 when treated with hydrogen peroxide [3]. This model of autoregeneration details how CeO_2 functions as a free radical scavenger [2].

(Ce^{3+} or Ce^{4+}). After oxidation or reduction, cerium returns to an equilibrium state determined by the amount of oxygen vacancies on the crystal lattice structure. The amount of oxygen vacancies are relative to particle size, determines the ability of ceria to give or accept oxygen. These oscillatory transitions between oxidation and reduction states are known as autoregeneration and allows cerium oxide to participate in a variety of chemical reactions [2].

Figure 3. Three step autoregenerative reaction and radical-scavenging mechanism of ceria

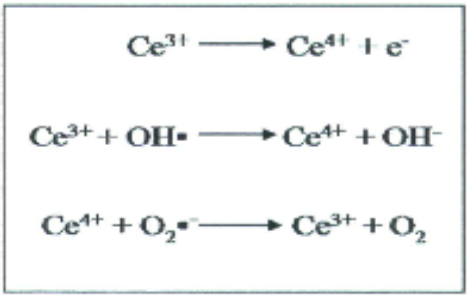

Figure 3. The radical-scavenging properties of CeO_2. is demonstrated in a three step reaction in which ceria can reduce both a hydroxyl radical and a superoxide anion [2].

Reactive oxygen species (ROS) are responsible for a wide range of damaging effects on the body including aging, neurodegenerative disorders, cancer, and inflammation. The unique chemistry of CeO_2 allows for potential function as an antioxidant agent in defense against damage from free radicals and oxidative stress. However, different ionic ratios of Ce^{3+} and Ce^{4+} on the surface of the particle, which is dependent on size of the ceria nanoparticle, plays an important role in the amount of reactivity and the type of catalytic ability. The ionic ratios of Ce^{3+} and Ce^{4+} on the surface determine reactivity, so the size and relative surface area of the CeO_2 particle determines reactivity and potential antioxidant function.

Experiments demonstrating the antioxidant activity for CeO_2

The ability of CeO_2 to react with reactive oxygen species (ROS), especially hydrogen peroxide and superoxide, is associated with catalase mimetic activity and superoxide dismutase (SOD) mimetic activities. Oxidoreductase enzymes such as catalase and super oxide dismutatse (SOD) assist in protecting cells from ROS by neutralizing free radical species. To understand how the ratio of Ce^{3+} and Ce^{4+} ion concentrations in ceria nanoparticles determines

reactivity with superoxide and hydrogen peroxide, experiments were performed to study varying catalytic activity with different ionic ratios of ceria [16]. These experiments revealed that a larger Ce^{3+}/Ce^{4+} ratio decreased the amount of superoxide ion and caused additional competition with ferricytochome-C in reducing superoxides than ceria with a smaller Ce^{3+}/Ce^{4+} ratio suggesting that the amount of Ce^{3+} on the surface of the nanoparticle is what is responsible for SOD properties of CeO_2 [52]. Additional studies were then performed to confirm the redox regeneration of ceria nanoparticles. These experiments revealed that high concentrations of Ce^{4+} promoted superoxide mimetic activity, and that high concentrations of Ce^{3+} lacked this effect [52]. The Ce^{3+} and Ce^{4+} ion concentrations alternate during the autoregenerative process of CeO_2, resulting in catalase and SOD mimetic activities being favored during higher surface ratios of Ce^{3+} and Ce^{4+}, respectively.

Cerium Oxide: Potential therapeutic for inflammation relief

The redox properties of CeO_2 also made it a choice compound to study chronic inflammation therapy, which is a precursor state in many diseases including multiple sclerosis, rheumatoid arthritis, and heart disease [53]. It was shown that CeO_2 attenuates ROS production in J774A murine macrophages and is capable of reducing nitric oxide synthase (iNOS) after challenge with gram-negative bacteria lipopolysaccharide (LPS). When these macrophages were studied under high-resolution transmission electron microscopy (HRTEM), CeO_2 appeared to have crossed the macrophage membrane and deposit in the cytosol as black spots. The same researchers then looked at the effects of CeO_2 distribution and pharmacokinetics after an intravenous injection in mice for thirty days and found no toxic evidence of the compound *in vivo* (assessed by absence of lesions in H&E staining of brain, lungs, liver, kidneys, spleen, and pancreas) [13].

A major player in inflammation response is nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB) [54]. Improper NF-kB regulation is linked to not only inflammation but

cancer, autoimmune diseases, and septic shock [55]. CeO_2 (in bulk or nanoparticle form) has been shown to significantly diminish the expression of several NF-kB inflammatory-related genes including tumor necrosis factor-α (TNF-α), iNOS, interleukin IL-1β and IL-6 in *vitro* (H9c2 cardiomyocytes) [56]. The down regulation of these inflammatory response genes is suggestive of the possibility that CeO_2 may function as an immunomodulator and as a potential mediator of the inflammatory response.

Cerium oxide: neuroprotective capability

Cerium oxide has multiple neuroprotective capabilities which are believed to be involved with its ROS scavenging ability. The potential to relieve free radical production and oxidative stress may be beneficial for combating aging, vision loss, stroke, Alzheimer's and Parkinson's diseases [57], and following conditions of ischemia and reperfusion [49] [57].

Chen and coworkers [58] observed that CeO_2 can lower the ROS production in the retina that can eventually lead to blindness. They concluded that CeO_2 must act as a free radical scavenger and also found that CeO_2 prevents any increase in apoptotic neuronal cells in culture, even after adding hydrogen peroxide. This study was performed *in vitro* and then adapted to an *in vivo* study using albino rats, which are highly sensitive to photon exposure. Histology was performed and no change to the optic nerve was found. This study was then performed with human lens epithelial cells, where it was observed that CeO_2 exposure leads to no DNA damage (as seen by comet assay), no sister chromatin exchanges, or any significant cell damage in general. The authors concluded that CeO_2 may be useful for the potential treatment of cataract disease [59].

Estevez and coworkers reported that CeO_2 can reduce ischemic cell death in mice hippocampal brain slices by 50% and 3-nitrotyrosine, produced by the free radical, peroxynitrite by 70% [49]. Further evidence of cerium oxide as a neuroprotective agent has been shown by its ability to preserve normal neuronal calcium signaling after brain trauma. Calcium signaling

by means of calcium gated voltage-channels is an integral process in neurotransmitter release, and is a major contributing factor to excitotoxic cell death/brain damage as seen in stroke [57]. Taken together, these data suggest that cerium oxide may be a therapeutic option for neuronal damage in multiple neurodegenerative disorders.

CeO_2 toxicity

While the vast majority of research has shown that CeO_2 may be protective, other data has suggested that CeO_2 particles may function as a pro-oxidant depending on environmental conditions and the specific type of cell [12, 60]. Park and coworkers [60] showed that oxidative stress and even cell death is induced by CeO_2 in BEAS-2B epithelial lung cells. A significant decrease (40-50%) in number of viable cells compared to the controls was seen when BEAS-2B cells were exposed to 20 µg/mL CeO_2 (30 nm for 96 hrs). Even at 5 µg/mL and higher concentrations (up to 40 µg/mL), CeO_2 was associated with ROS production as seen by a 75% increase in glutathione levels compared to controls (at 24 hrs). At 40 µg/mL CeO_2, increases in genes related to oxidative stress, such as catalase, glutathione S-transferase (GST), heme-oxygenase 1 (HO-1), and thioredoxin reductase (TR) were observed at 4 and 8 hrs. Despite low concentrations (5 µg/mL) of CeO_2 eliciting an a pro-oxidant effect on BEAS-2B cells, the authors also demonstrated that other cell lines such as T98G, derived from human brain cells, and H9C2 cells, derived from rat cardiomyocytes, had no change in viability. In different cell lines, different oxidative stress genes have been shown to change in levels. For instance, in RAW 264.7 macrophage cells, CeO_2 exposure was not associated with increased HO-1 expression [12], whereas, in BEAS-2B cells, exposure to CeO_2 induced increased HO-1 expression [60].

In addition to *in vitro* work using cultured cells, other studies have examined the effect of CeO_2 nanoparticle exposure using live animals. In these experiments researchers have studied effects of intratracheal administration of CeO_2 given to rats. Their data demonstrated that exposure was associated with pulmonary toxicity, pulmonary fibrosis, hepatic toxicity, and loss

of alveolar macrophage function are all associated with intratracheal installations in a dose-dependent fashion[22, 61-63].

In summary, a review of the current literature demonstrates progress in documenting the unique structure and function of CeO_2 as it relates to toxicity. The toxicity of CeO_2 appears to be selective to cell type or tissue [22] [57] [63], and behaves in a dose-dependent fashion [58-60]. Commensally, other research indicates that CeO_2 has antioxidant activity as both a catalase and superoxide mimetic [3] [51]. The specific reactivity of CeO_2 is altered by its environment [36] [37], size [35], and charge [3], suggesting there is no uniform activity of CeO_2.

Caenorhabditis elegans as a model organism

Caenorhabditis elegans (*C. elegans*) is a 1 mm long transparent free-living roundworm nematode found living in soil of temperate climates around the world. The organism has many uniform biological traits, a conserved genome, and a simple anatomy, making it highly useful to study molecular and developmental biology in addition to developmental toxicology [64].

The *C. elegans* embryo develops and matures through four developmental larval stages during its lifecycle. In the *C. elegans* fully adult stage, it exists as a 959 celled hermaphrodite or a 1031 celled adult male, has a defined reproductive life cycle of 2.5 days, and eats a simple diet of *Escherichia coli* (*E. coli*) bacteria. Additionally, *C. elegans* live roughly two to three weeks [65], making this organism highly useful for longevity and aging studies in particular [66].

Figure 4. Anatomy and reproductive life cycle of *C. elegans*

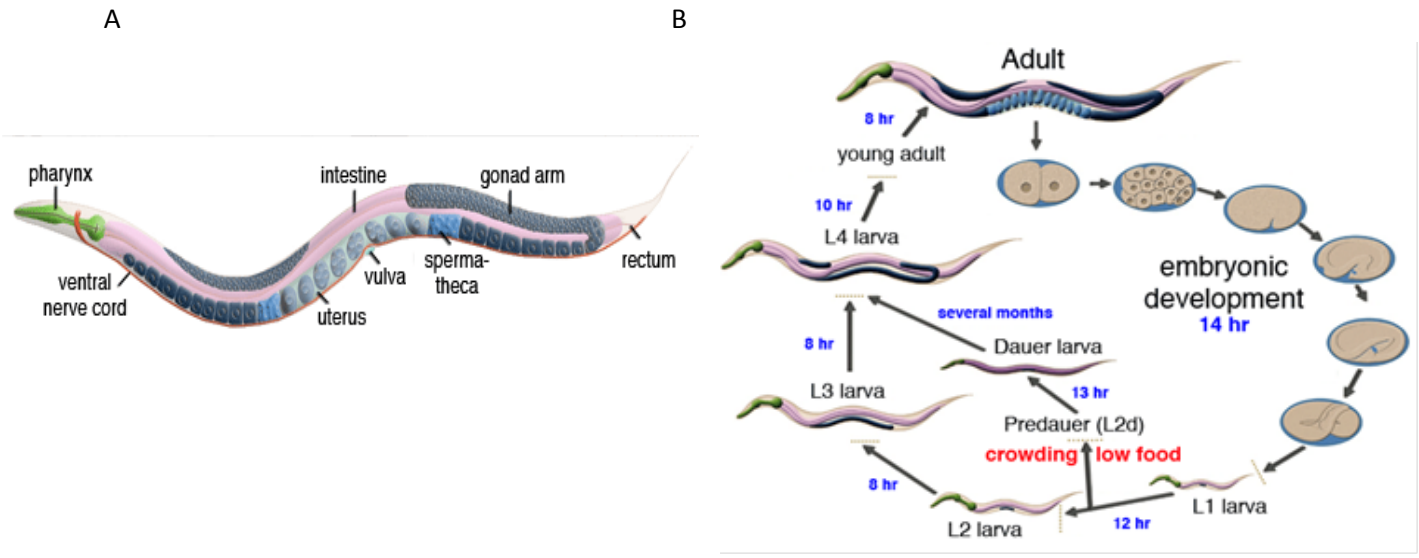

Figure 4. (Panel A) The adult C. elegans is characterized by its simple anatomy, few organs, and visible embryos. (Panel B) After hatching, the nematode develops through four larval stages before becoming an adult. Alternative development pathway for dauer larva results from crowding or low food conditions [1] (Panel B).

A seminal investigation by Sydney Brenner titled "The genetics of *Caenorhabditis elegans*" was published in 1974. This paper summarized about one hundred genes in this nematode and laid the groundwork for studying cellular differentiation as it related to the entire *C. elegans* genome [64, 67]. The complete mapping of the *C. elegans* genome has lead to important research in cancer [68], aging [69, 70], and studies of the developmental nervous system [71]. Importantly, in 2006, Andrew Fire and Craig Mellow won the Nobel prize for physiology and medicine after they discovered interference RNA (RNAi) in a *C. elegans* model [72]. Many of the discoveries in *C. elegans* research surfaced when transgenic toolkits emerged that allowed for the “tagging” of green fluorescent protein (GFP) to a gene of interest [65]. Properly performed, this approach was very powerful, as it allows for the real time analysis of gene expression in the intact living organism. [73]. The development of transgenic *C. elegans* strains, a complete mapping of the *C. elegans* genome, and ease of cultivation has allowed for several discoveries in the areas of developmental biology and toxicology**.**

C. elegans toxicological endpoints

C. elegans have been commonly used as a model organism for studying nanotoxicity as it is sensitive to changes in the soil environment [74] [75]. Traditional assays with *C. elegans* are typically based upon uniform characteristics of their biology, such as an average life expectancy of two weeks, typical reproduction count of 300 offspring, a time-dependent four stage larval development, and measurement of body length (averaging 1mm) [64]. In addition to these well-known assays, other work has recently begun to examine measures of thermotolerance, heat-shock protein expression and oxidative stress as potential indicators of toxicity [76-78].

In 2008, Martin Chalfie won the Nobel prize in chemistry for his work demonstrating GFP expression within transgenic *C. elegans.* Chalfie and coworkers [79] published the seminal paper "Combinatorial marking of cells and organelles with reconstituted fluorescent proteins" in which they successfully created a single component fluorescent system allowing observation of a fluorescent signal expressed in a single cell type. This creation of GFP engineered *C. elegans* allowed researchers to describe real time expression patterns of genes *in vivo,* and became another vital endpoint in studying both nano and ecotoxicity.

Toxicity of nanodiamond exposure (~120 nm) in larval stage L4 worms was studied by Mohan and coworkers [80] using engineered *C. elegans* strains expressing GCS-1::GFP (glucosylceramide) and DAF-16::GFP (the *C. elegans* equivalent gene for human insulin-like growth factor 1, IGF-1). Their finding that nanodiamond exposure failed to induce translocation of GCS-1 and DAF-16 led them to conclude that nanodiamond particles were generally non-toxic under their experimental conditions at a concentration of 0.5 mg/mL.

Qu and coworkers [81] assessed the toxicity of quantum dots (QDs) (5-6 nm) exposure (20nM, 200nM) using engineered *C. elegans* strain CL2120 bearing the *mtl-2::GFP* construct.

The transgenic strain CL2120 allows *in vivo* observation of metallothionein 2 (mtl-2), an intercellular protein regulating metal exposure. By observing fluorescent expression of mtl-2 as a measure of toxicity of metal exposure, the researchers were able to noninvasinvely image the expression of mtl-2 and determine long term toxicity of QD exposure.

The multi-parametric endpoints and particularly, the sensitivity of *C. elegans* to oxidative stress makes the nematode highly useful for studying toxicology as it relates to environmental exposures. This is because nematode population is typically regulated by predators and microbial parasites, their diverse biological interactions place them in many food webs, and they are useful indicators of ecosystem health [82].

Use of C. elegans to study potential toxicity of CeO_2

CeO_2 has already shown to trigger adverse reactions on *C. elegans* [83] [84] [38]. Indeed, a *C. elegans* publication was the first investigation to show that nanoparticles exhibit adverse effects including attenuated lifespan, thermotolerance, and induced oxidative damage (as seen by increased lipofuscin) at such low concentrations (1nM-100 nM) following chronic exposure [52]. Other work observed size-dependent effects of bulk (powder) versus nanoscale (53 ± 3 nm) size particles using equimolar (2.5 - 9.75 mg/L) concentrations of CeO_2 over a 3 day exposure period.

Although informative, this work by itself is still incomplete. Whether different CeO_2 particle sizes or concentrations exhibit similar effects is not yet clear. To address this gap in our understanding, the range of particle concentrations and preparation “state” (agglomerated vs. single particle dispersion) were chosen to represent ecologically “real world” exposure conditions as opposed to those typically employed in the laboratory.

Summary

Nanotechnology is the study of molecules at the nanoscale while nanotoxicology is the study and assessment of the toxic effects elicited by nanomaterials. A long term goal of nanotoxicology is to document and understand all of the toxicological properties of nanomaterials so that undesired exposure and effects to humans and the environment is minimized where possible. CeO_2 appears to convert harmful ROS to neutralized forms and attenuate oxidative stress; however this compound has also been reported to generate oxidative stress and free radicals depending on the type of cell or model it is studied in. The effects of CeO_2 in its aggregated form have not been extensively studied, especially in soil organisms. The incorporation of GFP coupled stress response genes in *C. elegans* may allow the use of fluorescent markers indicative of the heat shock response and ROS production to study the toxic and subtoxic effects of aggregated CeO_2 nanoparticles. Additionally, progress in studying significant *C. elegans* endpoints at micro-concentrations, realistic to environmental accumulation [85], has yet to be achieved, and may prove to be a valuable measurement in assessing environmental exposure and ecotoxicity of CeO_2 nanoparticles.

CHAPTER 3

Research paper to be submitted for publication

Abstract

The continual increase in production and disposal of nanomaterials still raises concerns regarding the safety of nanoparticles on the environmental and human health . Recent data have suggested that cerium oxide (CeO_2) nanoparticles may exhibit both harmful and beneficial effects on biological processes. Herein, we investigated the potential toxicity of CeO_2

nanoparticles in an aggregated state (195 ± 78nm) in *Caenorhabditis elegans* (*C. elegans*) using several different concentrations (0-17.21 µg/mL) on nematode growth medium. Our findings demonstrate that chronic exposure of CeO_2 nanoparticle aggregates are responsible for inducing ROS and heat shock stress response (HSP-4) in *C. elegans*, but don't contribute to change in mortality. However, CeO_2 promoted a significant decrease in fertility, decline in stress resistance as measured by thermotolerance, and shortened worm length ($P<0.05$), in a strain-dependent manner. Overall results obtained from this study reveal the sublethal toxic effects of CeO_2 nanoparticle aggregates on *C. elegans* at "real world" exposure conditions and contribute to the available limited data on CeO_2 environmental toxicity.

Key words: Cerium oxide, *C. elegans,* toxicity, cerium oxide aggregates, cerium oxide agglomerates

Introduction

The use of nanotechnology in industry is rapidly increasing, with a worldwide market size estimated to be in excess of $1 trillion by the year 2015 [86]. Despite the rapid progress and early acceptance of nanotechnology, the potential for adverse health effects in humans and the environment due to prolonged exposure at various concentration levels has not yet been established. Assessing the potential toxicity and the effects of nanoparticles on biological systems has become a relevant and quickly growing area of environmental toxicology research [87].

Due to their smaller size and increased surface to volume ratio, nanomaterials oftentimes exhibit differences in their biological reactivity compared to that observed in "bulk" materials [88]. Indeed, recent data has suggested that material toxicity can vary in a size dependent fashion with smaller features being associated with increased cellular dysfunction [25], [88]. How exposure to nanoparticles may affect the environment and human health are still not fully understood [87].

Ceria is a rare-earth element that in its oxide (CeO_2) form is used as an industrial catalyst, in the automotive industry [8], as a ultraviolet blocking material [9], and an industrial polishing reagent [10]. Data on how CeO_2 may affect biological function when present as a nanoparticle is equivocal. Indeed, recent data has indicated that CeO_2 nanoparticles may be neuroprotective, function as an anti-inflammatory agent, and are non-cytotoxic [11-14]. It is thought that CeO_2 nanoparticles may also function as an antioxidant by acting as superoxide dismutase (SOD) and catalase mimetics [3]. Interestingly, CeO_2 nanoparticles demonstrate an autoregenerative capability to cycle between +3 and +4 valence states, scavenging hydroxyl and superoxide radicals during each cycle [15, 16]. In contrast to these data, other studies have demonstrated that exposure to CeO_2 nanoparticles can lead to increases in oxidative stress [17, 18], cellular inflammation, and DNA damage [19-22], and that CeO_2 nanoparticles are toxic to aquatic organisms [23, 24].

Caenorhabditis *elegans* (*C. elegans*) is a member of the nematode family that exists as free-living roundworm that lives in the soil. Nematodes are the most abundant multicellular animals on earth. The nematode population is typically regulated by predators and microbial parasites, and their diverse biological interactions place them in many food webs [82]. Nematode activity is thought to affect several aspects of plant community composition and succession and their effects on soil processes make indices of nematode assemblage useful indicators of ecosystem health. *C. elegans* is widely used in the laboratory for a variety of different types of investigations given its short life span, transparency, ease of cultivation, and high level of conservation with the vertebrate genome [64]. In the last decade or so, *C. elegans* has begun to be used as a model organism for the investigation of chemical toxicity given its sensitivity to oxidative stress [89]. How exposure to CeO_2 nanoparticles may affect biological function in *C. elegans* is not well understood under environmentally relevant conditions [83]. Recent data has suggested that CeO_2 nanoparticle exposure in *C. elegans* is associated with growth inhibition [3] and decrease in longevity [83]. Although informative, it should be noted that only one size of CeO_2 nanoparticles was investigated. Given that nanoparticle size directly influences chemical and biological reactivity and that toxicological effects are concentration dependent, additional study is warranted. Similarly, while the measurement of growth inhibition and decreased longevity is important to understanding toxicity of CeO_2, how exposure to CeO_2 nanoparticles might affect *C. elegans* larval development and fecundity is not known. This latter fact is particularly important given the potential roles that nematodes play in regulating ecosystem productivity. Therefore, the purpose of this study was to observe multiple endpoints for toxicity of CeO_2 nanoparticles at both different sizes and concentrations. We hypothesized that changes in CeO_2 concentration and size have the potential to alter *C. elegans* development, external stress resistance, reproduction, and even viability. Our data suggest that exposure to CeO_2 nanoparticle aggregates is associated with increased levels of organismal

stress, decreases in fertility, and diminished worm growth. Taken together, these findings suggest that exposure to CeO_2 particles may be toxic to *C. elegans*.

Materials and Methods

Transmission electron microscopy (TEM)

Previously characterized 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 (3.5mg/ml) were prepared in ddH_20 by sonication using a Vibra Cell Sonicator (Sonics & Materials, Inc) at 600 W for 2 min at room temperature. Particles were imaged in their native state to determine the size and shape using an (Hitach-H-7000) electron microscope at 75 keV and a magnification of 50,000x. Particle size was estimated from at least 100 different nanoparticles using ImageJ software.

Dynamic light scattering (DLS)

The hydrodynamic size and size distribution of the CeO_2 nanoparticles was evaluated in ddH_2O water using a Particle Size Analyzer (HORIBA, Model-LB-550) equipped with a He-Ne laser (633nm) using back-scattered light. Experiments were performed in triplicate runs that were performed on three different days with freshly prepared samples.

C. elegans strains and culturing conditions, chemicals and materials

C. elegans strains were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. The CL2166 strain carries a gst-4::GFP reporter allowing fluorescent observation of glutathione S-transferase. The SJ4005 strain exhibits a HSP-4::GFP transgene that exhibits oxidative stress-inducible fluorescence of heat shock protein production HSP-4, (the human equivalent to hsp70) [90]. Age-synchronized populations of *C. elegans* were prepared using standard procedures [91]. Nematode strains were maintained at 20°C using

E*scherichia coli* (*E. coli*) OP50-1 suspensions spread on nematode growth medium (NGM) plates 24 hours prior to nematode transfer to ensure sufficient bacterial lawn growth.

Effect of CeO_2 particle exposure on life span and reproduction

Age-synchronous eggs (d=0) were grown to L4 larval stage and then transferred to OP50-1 coated plates with or without CeO_2 nanoparticles (0 - 17.21 µg/mL). *C. elegans* were transferred to new plates during each day of the reproductive cycle. Just prior to the end of the reproductive phase, nematodes were transferred to new plates every three days. Worms were observed daily and the number of live and dead counted. Nematodes were scored as dead when it no longer responded to being touched with a worm pick made from platinum wire. Nematodes that escaped the bacterial lawn or burrowed into agar were excluded from analysis.

Age-synchronous L4s were transferred to individual NGM plates (0 - 17.21 µg/mL) at the beginning of their reproductive cycle (~2.5 days) and then transferred to new plates every 24 hours. Eggs and L1s from each L4 were counted following each 24 hour plate transfer.

Effect of particle exposure on *C. elegans* growth and development

Age-synchronized *C. elegans* eggs were distributed on OP50-1 *E. coli* lawns with or without exposure to CeO_2 particles. On days 2 and 3, the L4s were counted and removed from each plate. After paralysis using 5 µL of 5% hypochlorite solution, GFP reporter gene expression was observed using a Olympus BX51 fluorescence microscope (Olympus America, Melville, NY). Images were captured under standardized conditions and Image J software was used to quantify mean GFP intensity and animal length.

Effect of particle exposure on thermotolerance

Thermotolerance assays were performed as described by Lithgow [92]. Briefly, three day old nematodes were exposed to 35°C. Surviving worms were counted after 10 hours.

Statistical analysis

Results are presented as mean ± SEM. Comparisons between groups were performed using the *Students t-tests* or one-way analysis of variance (ANOVA) and *post hoc* testing as appropriate. The level of significance accepted *a priori* was $P \leq 0.05$.

Results

Characterization of CeO_2 particles

TEM analysis showed that the CeO_2 particles were spherical/round in shape with a diameter of 195 ± 78 nm in size (Figure 5 A). The mean hydrodynamic diameter of the CeO_2 nanoparticles as measured by dynamic light scattering (DLS) was 184 ± 75.3 nm (Figure 5 B).

Exposure to CeO_2 particles is associated with increased stress but not death

Compared to untreated worms, CeO_2 particle exposure did not affect nematode longevity irrespective of strain in CL2166 or SJ4005 strains at our chosen dosing concentrations. We chose the N2 wild type to verify the survivability results of both GFP transgene strains, and still observed no change in longevity by CeO_2 exposure (Figure 6). In an effort to better understand any potential toxicity of the CeO_2 particles, we next investigated if particle exposure was associated with increased organismal stress using the fluorescent transgenic strains SJ4005 and CL2166. The SJ4005 contains a GFP reporter coupled to HSP-4 production, while the CL2166 strain contains a GFP reporter coupled to GST response genes. Compared to that observed in the unexposed worms, CeO_2 particle exposure appeared to significantly increase HSP-driven fluorescence in a dose-and-time dependent fashion at days 2, 4, and 6 (Figures 7A, 7C; $P<0.05$). Like that seen with the HSP driven GFP reporter strain, CeO_2 particle exposure appeared to exhibit a similar effect in the CL2166 animals (Figures 7B, 7D; $P<0.05$). Taken

together, these data suggest that CeO_2 particle exposure is associated with a significant increase in HSP-4 expression and cellular ROS levels as seen by increased GST-4.

Exposure to CeO_2 particles is associated with diminished egg laying and evidence of delayed maturation.

Age synchronized worms were isolated in individual NGM plates and egg production was counted over the entire reproduction period. Compared to that observed in the unexposed worms, exposure to CeO_2 particles significantly decreased the average daily egg production in the CL2166 but not the SJ4005s stain at days 3 and 5 (Figure 8A, 8C, $P<0.05$) and the total number of eggs produced over a six day period (Figure 8B, 8D, $P<0.05$).

Similar to that seen in egg production, the effects of CeO_2 particle exposure on worm length also appeared to be strain-dependent. Specifically, CeO_2 particle exposure appeared to diminish CL2166 body length early in development (Figure 9B, $P<0.05$) while in the SJ4005 strain, diminished body length was not observed until day 6 (Figure 9A, $P<0.05$). In an effort to understand how CeO_2 particle exposure might cause a decrease in body length, we next examined how exposure affects nematode development. For these experiments, we quantified the number of worms exhibiting L4 development at days 2 and 3 post hatching. Compared to non-exposed control animals, CeO_2 particle exposure at 1.72 µg/mL and above decreased the percentage of L4 worms developed on day 2 and delayed the progression to L4 by day 3 in the SJ4005 strain (Figures 9C, 9D, $P<0.05$).

Exposure to CeO_2 particles is associated with diminished thermotolerance

To determine if CeO_2 increases or diminishes stress load during exposure to elevated temperatures, thermotolerance was chosen to further measure the organisms stress response. Our results show that exposure to CeO_2 particles lowered the ability of the SJ4005 strain but not the CL2155 animals to tolerate elevated temperatures (Figure 10A, 10B, $P<0.05$).

Discussion

The unique chemical and physical properties of nanomaterials have generated considerable interest in industry and more recently, concerns for their potential toxicity. It is thought that engineered nanoparticles may pose a threat to human beings and the environment given their widespread and growing use in everyday products [93]. Importantly, even with the appropriate precautions it remains possible for exposure to occur during each stage of the material lifecycle including production, application, disposal, and recycling [94]. CeO_2 is currently one of fourteen manufactured nanomaterials on the priority list of nanomaterials under investigation by the Organization for Economic Cooperation and Development (OECD) [95]. Here, we examine the effects of CeO_2 particle exposure on *C. elegans* life span, organismal stress levels, organism maturation, and resistance to stress. In contrast to previous reports [83], [84] , we examined the effects of exposure to CeO_2 agglomerates given the fact that nanoparticles frequently undergo agglomeration in the high ionic strength environments oftentimes observed in environmental and biological fluids [96]. The ecologically "real world" exposure conditions used in the present study are in contrast to the vast majority of nanoparticle work where toxicity was examined using sonicated, evenly dispersed particles such as that typically observed only in laboratory settings. The primary findings of this study were that exposure to aggregated CeO_2 nanoparticles was associated with increased organismal stress but not a change in *C. elegans* lifespan, and that the CeO_2 associated increase in stress response resulted in decreased fertility, stunted growth, delays in organismal development, and diminished thermotolerance.

Exposure to CeO_2 particles increases organismal stress

To measure the effect of CeO_2 nanoparticle exposure on *C. elegans* lifespan, age synchronized worms were exposed to a bacterial lawn of OP50-1 *E. Coli* that had been

inoculated with CeO_2 particles. Our results show that exposure to CeO_2 particles had no significant effect on *C. elegans* lifespan even when used at concentrations as high as 17.21 µg/mL. These results, at first glance, were surprising given the previous paper of Zhang and colleagues [83] which demonstrated that exposure to 0.00017 µg/mL was associated with significant increases in the incidence of *C. elegans* mortality. It is possible that differences between the current study and previous work may be related to differences in the size of the nanoparticle used. For example, Zhang and co-workers used particles with a mean particle size of 8.5 ± 1.5 nm whereas in the current study, the mean particle size was measured to be 184 ± 75 nm by DLS and 195 ± 78 nm by TEM. It is thought that as particle size increases, the particle becomes generally less permeable and less catalytic due to larger molecular structure hindering exposure to the CeO_2 active site [25]. Multiple factors, such as pH and the ionic strength of the environment, can cause particle aggregation which can result in the loss of nanoscale properties [97]. This has been shown by Arnold and colleagues who observed that CeO_2 nanoparticles were more toxic than equimolar amounts of "bulk" cerium oxide [38]. Whether the change in particle size is solely responsible for the differences in toxicity observed in the present study and previous is currently unclear and will require further investigation.

Similar to the work of Zhang and colleagues, we found that exposure to CeO_2 particles in *C. elegans* was associated with a toxicological response as demonstrated by increased exposure-induced expression of GFP (Figure 7, Panels A-D). Specifically, we found particle exposure in the SJ4005 strain was associated with an increase in HSP-driven GFP expression (Figure 7, Panels A and C) and that particle treatment in the CL2166 strain induced the ROS-dependent expression of GFP in a concentration dependent manner (Figure 7, Panels B and D). Change in HSP-4 expression is notable as this gene is the mammalian equivalent to immunoglobulin binding protein (BiP, GRP78), an endoplasmic reticulum chaperone in the

hsp70 family of proteins that plays an important role in cellular proliferation, differentiation, and homeostasis [98] [99].

Although beyond the scope of the present study, the reason for the observed increase in stress response by CeO_2 may be related not only to its ability to relieve oxidative stress, but also to cause it. The ability of CeO_2 to cause oxidative stress has been well documented in cell culture [60, 100] and in rats [19]. CeO_2 redox cycling between Ce^{3+} and Ce^{4+} may play a vital role in the generation of damaging oxygen radicals. Using paramagnetic resonance, previous work has demonstrated that CeO_2 nanoparticles in the presence of hydrogen peroxide can cause the formation of hydroxyl radicals and superoxide anions [101]. Just as the beneficial ROS scavenging properties of CeO_2 rely on the number of oxygen vacancies and the Ce^{3+} / Ce^{4+} ratio [2], the oscillatory cycling of giving and taking oxygen appears to work in both directions depending upon the chemical conditions [101]. Whether the creation of hydroxyl and superoxide by CeO_2 explains the increases in organismal stress seen in our GFP analysis as well as diminished *C. elegans* fertility, growth, and development observed in the present study is currently unclear. These findings emphasize the duality of action by CeO_2 seen in the literature, in that it has the capability for both therapeutic and deleterious effects depending on dosage, application, and environment.

Exposure to CeO_2 particles decreases growth and development

It is well known that free radicals can cause deleterious effects on *C. elegans* fertility (fecundity) [102] as well as animal growth and development [103]. Whether exposure to oxygen radicals, by themselves, is the direct cause of these changes or if such alterations are secondary to these elevations in radical levels is currently unclear. For example, Arnold and colleagues observed similar decrease in *C. elegans* growth following CeO_2 exposure which they suggested was due to diminished food intake that was caused by the interactions of CeO_2 and

E. coli [38]. Bearing this in mind, it is possible that changes in development and growth may be related to *C. elegans* food intake, as CeO_2 has a strong affinity to bind to *E. coli* [104] which could, in principle, diminish food intake. Restricted dietary intake has been shown to increase lifespan in *C. elegans* at the expense of prolonging time in dauer stages of the development cycle [105]. Although there may be other factors at play, it is conceivable that the worms exposed to the CeO_2 particles consumed less and that this decrease in food intake may be a contributing factor in the observed decrease in growth and development. Additional experiments, perhaps designed to directly test this assertion, will be useful in proving cause and effect.

It has been previously reported that increased stress plays a role in decreasing growth and development in *C. elegans* [105] [106]. In addition to elevations in organismal stress, another potential reason for the decrease in *C. elegans* growth and development seen in the present study may be related to the ability of CeO_2 to target and down-regulate nitric oxide synthase (NOS) [56]. Nitric oxide is known to be highly conserved between both invertebrate and vertebrate species and it is thought that this molecule plays an important role in neurotransmission, water and salt balance, organismal development, and immune function [107]. Although not measured, it is possible that CeO_2 exposure could diminish NOS and NO levels, which one could predict to cause impairments in nervous system function and *C. elegans* development [108]. Further experiments to directly examine this possibility are needed to establish causation.

Exposure to CeO_2 particles decreases fecundity and ability to endure external stressors.

The measurement of fecundity is one of the most significant toxicological endpoint assays for assessing toxicity in *C. elegans* [109]. Given the nature of our study design it is currently difficult to pinpoint the direct mechanism(s) by which exposure to CeO_2 might decrease fertility although we hypothesize that the increased oxidative stress we observed following CeO_2

exposure is the primary mechanism (Figures 7). Indeed, recent work has demonstrated that nematode stress levels are inversely associated with reproductive capability, along with worm growth and development [110]. This increased stress may also contribute to the diminished thermotolerance we observed following CeO_2 exposure (Figure 10). In summary, our data demonstrate that exposure to CeO_2 particles in *C. elegans* is associated with increased organismal stress, diminished growth, impaired development, and decreased fecundity. The tendency of nanoparticles to favor aggregation such as that observed during "real world" aquatic exposure suggests that CeO_2 may not be as potentially ecotoxic as previously considered when studied in its non-aggregate form. Additional studies on the effect of aggregated versus non-aggregated CeO_2 nanoparticles at varying concentrations and particle sizes, with both soil and aquatic organisms, will be needed to increase our understanding of how CeO_2 particles may affect the environment and those that inhabit it.

Figure 5. CeO_2 nanoparticle characterization

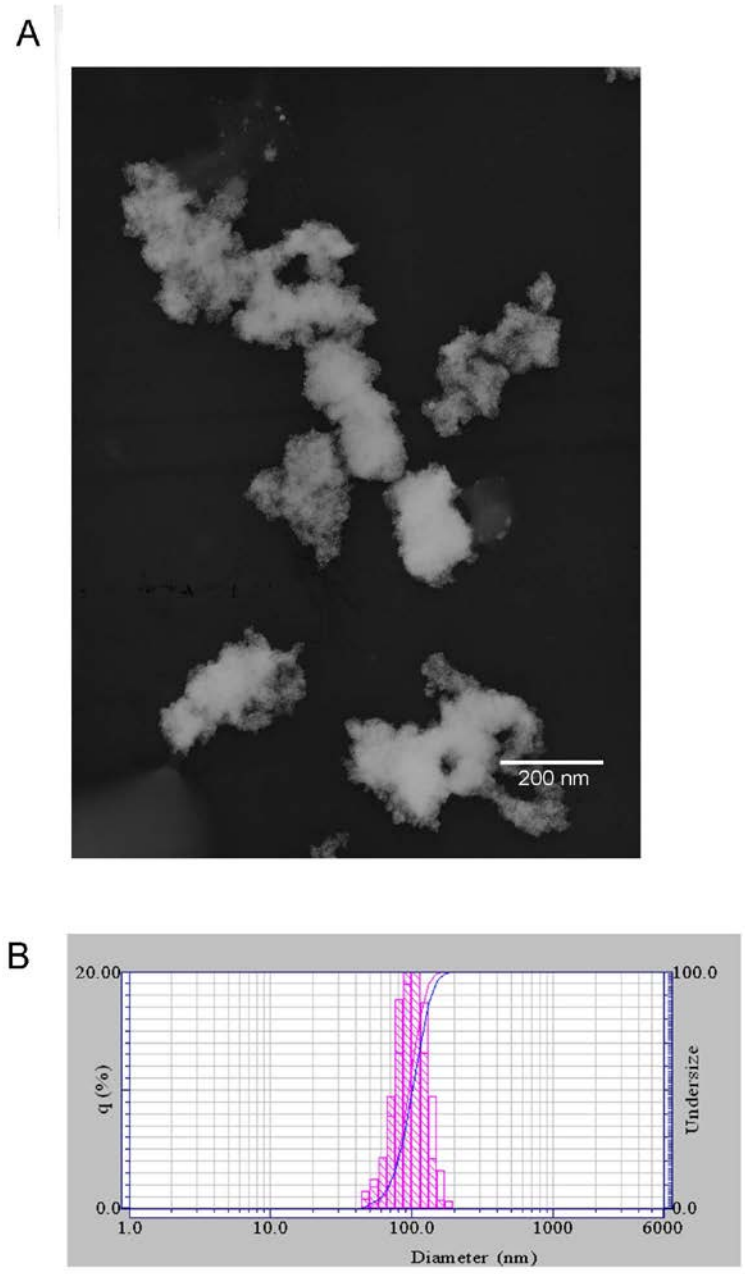

Figure 5. Physical characterization of CeO_2 nanoparticles by TEM and DLS. (A) Transmission electron microscopy (TEM) image of CeO_2 nanoparticles. TEM image was captured using a 0.01 mg/mL CeO_2 suspension. (B) Dynamic light scattering (DLS) measurement of CeO_2 nanoparticles indicates average value of 184 ± 75.3 nm.

Figure 6. Exposure to CeO_2 particles does not affect *C. elegans* longevity.

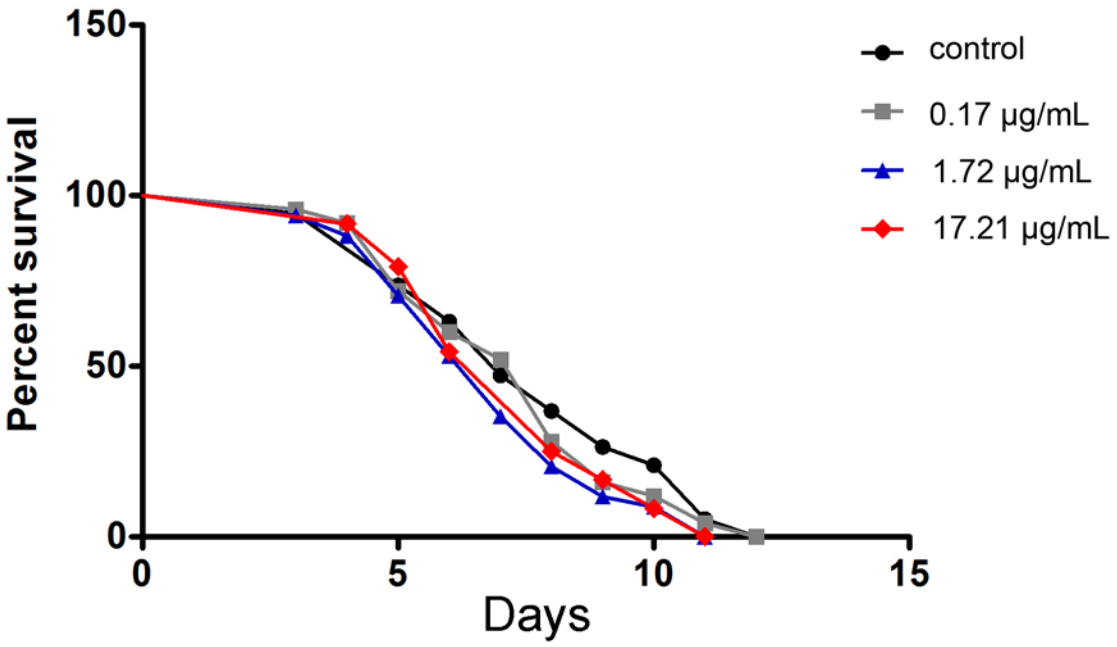

Figure 6. Exposure to CeO_2 particles does not affect *C. elegans* longevity. Strain (A) N2 wildtype showed no significant changes in longevity with CeO_2 particle exposure (0 – 17.21 µg/mL). Experiments were performed in triplicate (n=60-100).

Figure 7. Exposure to CeO_2 particles induces organismal stress

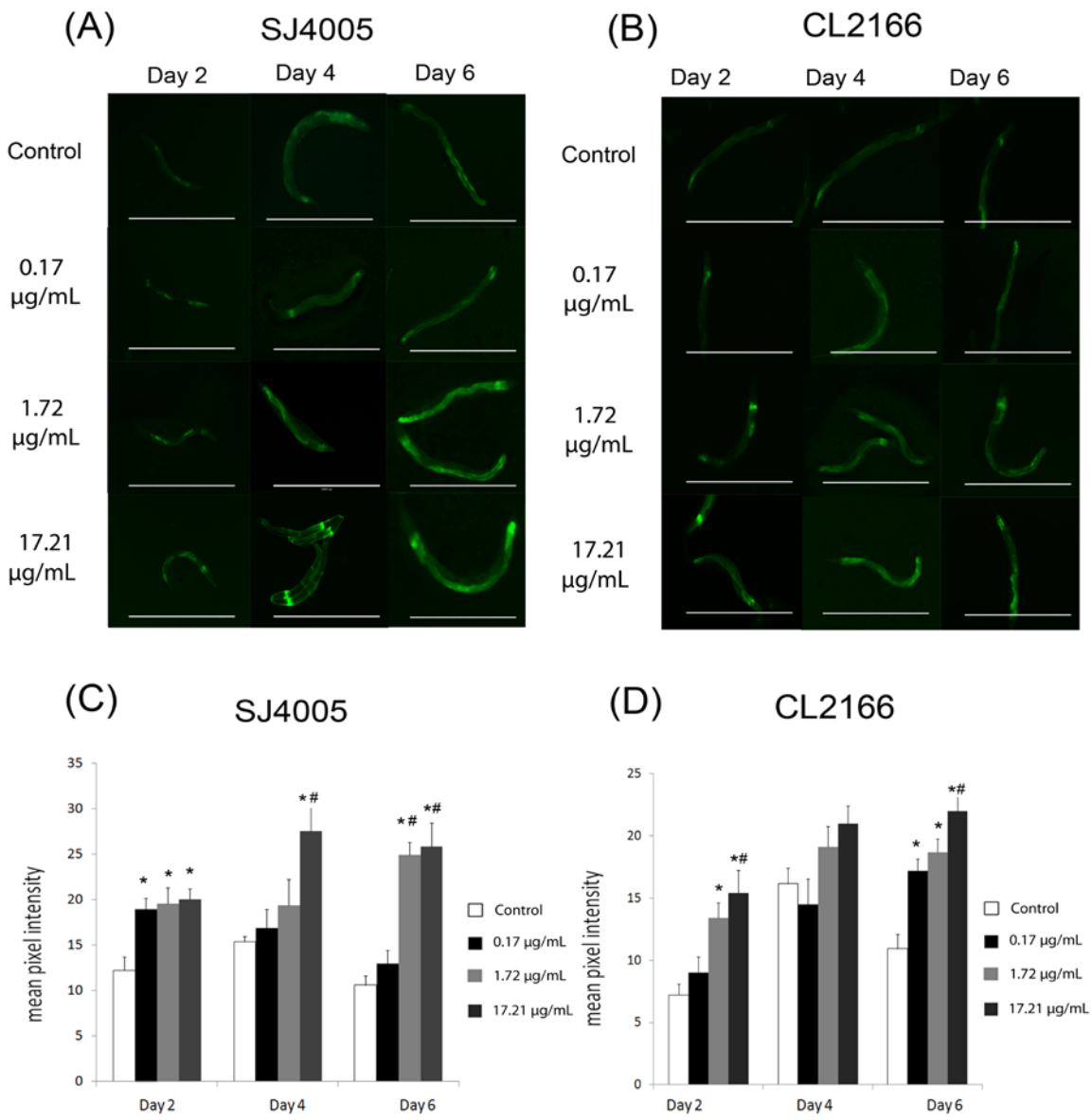

Figure 7. Exposure to CeO_2 particles induces organismal stress. GFP coupled heat shock production (HSP-4) genes from SJ4005 (A) and GFP coupled ROS response (GST-4) from (B) CL2166 were observed (original images at 4x magnification). Scale bar = 1mm. CeO_2 caused an increase of GFP related heat shock protein production (C) in strain SJ4005 and (D) GFP

related ROS expression in (D) strain CL2166. Scale bar equals 1 mm. Average mean pixel intensity as measured in Image j software. Data is expressed as Mean ± SEM relative to the control. n=10-20. * significantly different from control group ($P < 0.05$). # Significantly different from 0.17 µg/mL CeO_2 group ($P < 0.05$).

Figure 8 CeO_2 nanoparticle aggregates induce stress related GFP response

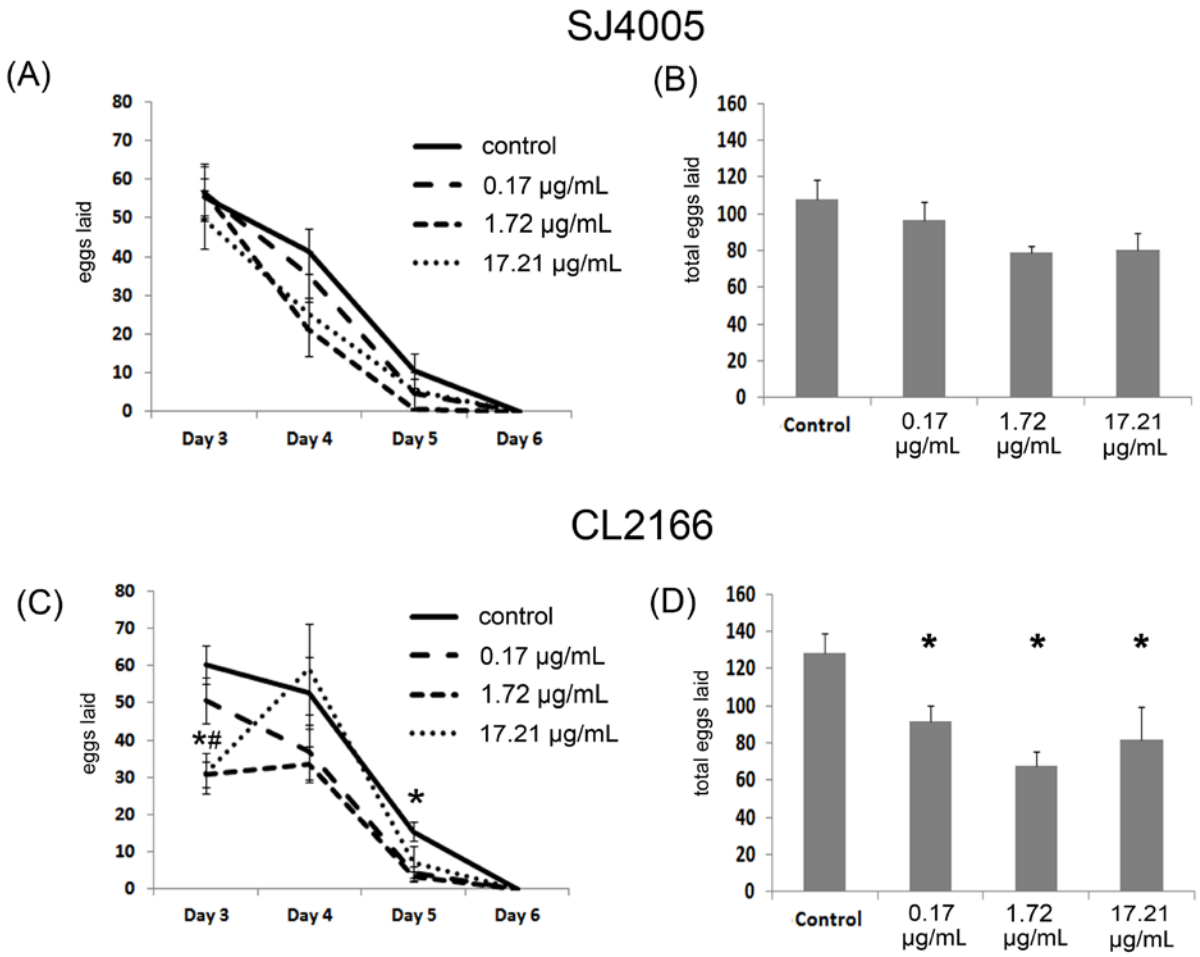

Figure 8. Exposure to CeO_2 particles decreases fecundity. Egg production by individual worms was determined daily and then totaled. CL2166 strain egg production by day (A) and totaled (B). SJ4005 strain egg production by day (C) and totaled (D) n =90 worms. * significantly different from control group ($P < 0.05$).

Figure 9. Exposure to CeO_2 particles affects worm growth and development.

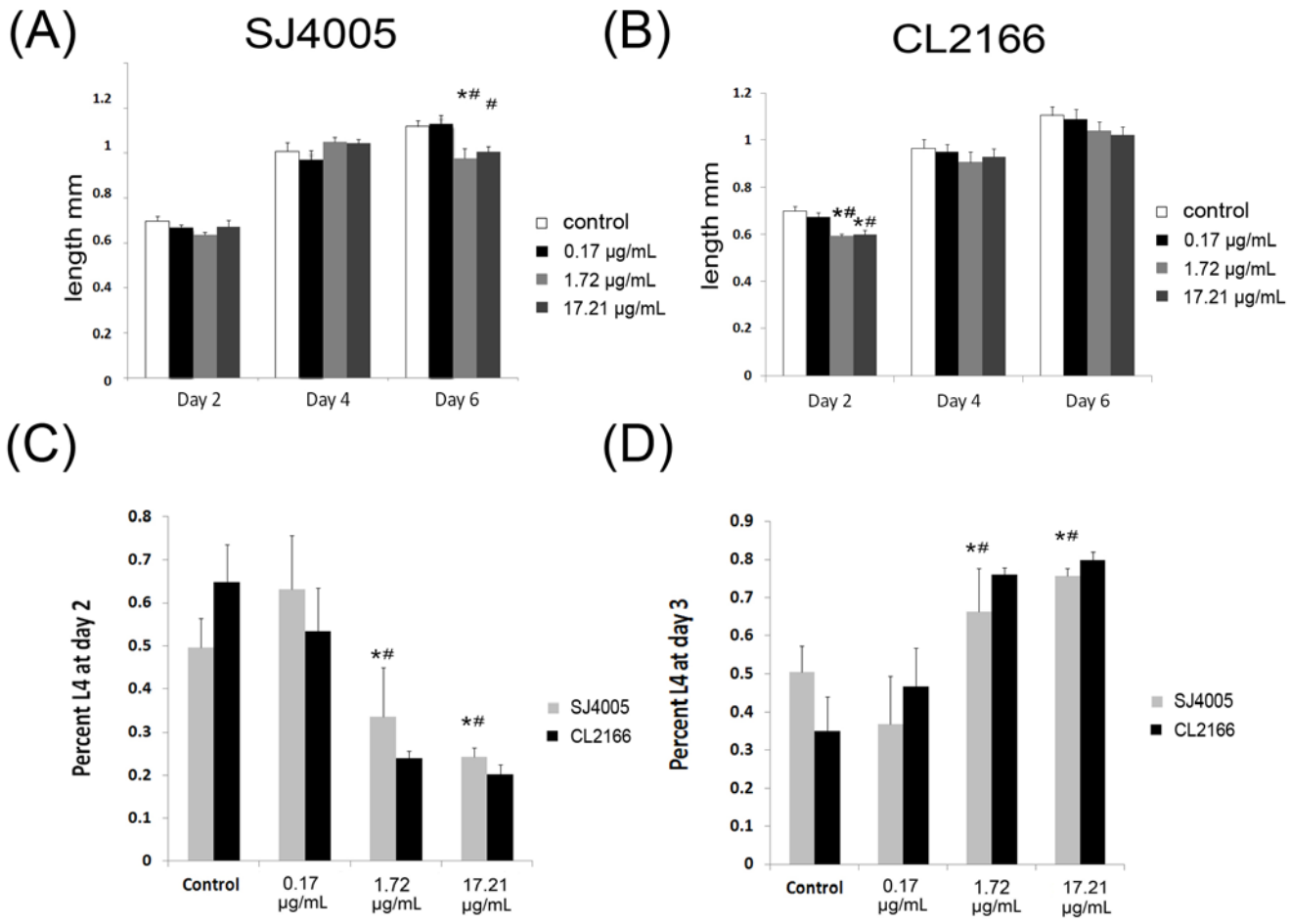

Figure 9. Exposure to CeO_2 particles affects worm growth and development. Cerium oxide particle exposure decreased length of strain (A) CL2166, but not (B)SJ4005. Age synchronized worms were exposed to cerium oxide nanoparticles (0,17-17.21 µg/mL) and monitored until day 2 (C) and day 3 (D) and were marked as being developed L4 or underdeveloped, however CeO_2 nanoparticles had no significant effect on development. n= 150-200. * Significantly different from control group ($P < 0.05$). # Significantly different from 0.17 µg CeO_2 group ($P < 0.05$).

Figure 10. Exposure to CeO_2 particles decreases worm thermotolerance

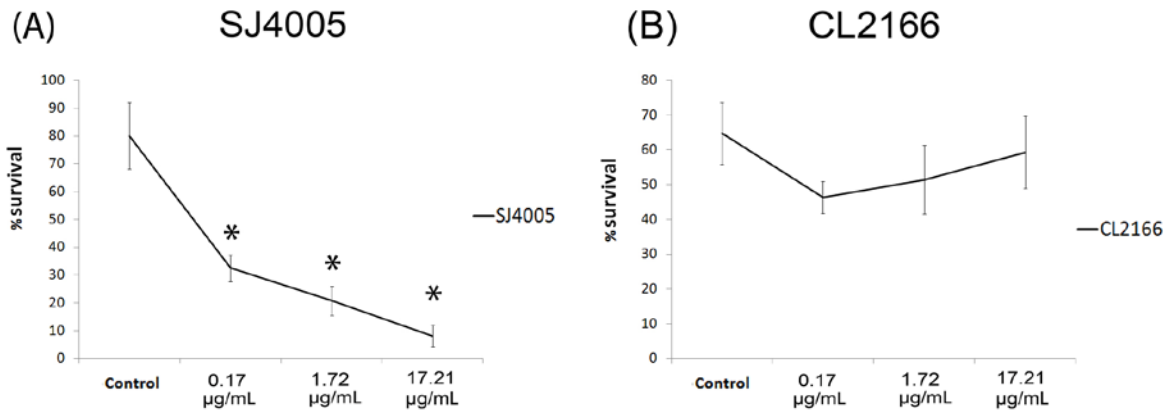

Figure 10. Exposure to CeO_2 particles decreases worm thermotolerance. Age synchronized SJ4005 (Panel A) or CL2166 (Panel B) worms were exposed to CeO_2 particles (0 – 17.21 µm/mL) at 35 °C for 8 hours on day 3 and animal survivability was recorded. SJ4005 strain (A) and CL2166 strain (B). n=60. * Significantly different from control group ($P < 0.05$).

CHAPTER 4

Conclusions

Our data suggest that exposure to CeO_2 particles has no significant influence on organismal lifespan (Figure 6). However, CeO_2 exposure is associated with diminished reproduction (Figure 8), increases in GST-4 and heat shock protein related-signaling (Figure 7), decreases in total nematode length (Figure 9, A,B), and stunted developmental rate (Figure 9 C,D).

Given the nature of our study design it is currently difficult to pinpoint the direct mechanism(s) by which exposure to CeO_2 might decrease *C. elegans* fecundity. We hypothesize that the increased oxidative stress we observe following CeO_2 exposure is the primary mechanism (Figure 7). Indeed, recent work has demonstrated that nematode stress levels are inversely associated with reproductive capability, worm growth and worm development [110]. This increased stress may also contribute to the diminished thermotolerance we observed following CeO_2 exposure (Figure 10). Taken together, these data show that exposure to aggregated CeO_2 is associated with increased organismal stress which most likely contributes, if not directly causes, diminished *C. elegans* growth, impaired development, and decreased fecundity.

To the best of our knowledge, this investigation is the first multi-parametric investigation to examine the ecotoxicity of CeO_2 nanoparticles in their aggregated state in a *C. elegans* model. In contrast to previous reports of evenly dispersed particle suspensions [83], [84], we examined the exposure effects of CeO_2 agglomerates given the fact that nanoparticles frequently undergo agglomeration in the high ionic strength environments oftentimes observed in environmental and biological fluids [96]. Additional studies on the effect of aggregated versus non-aggregated CeO_2 nanoparticles at varying concentrations and particle sizes, with both soil

and aquatic organisms, will be needed to increase our understanding of how CeO_2 particles may affect the environment and those that inhabit it.

Future directions

To expand upon the present research, future research efforts could be centered on the following:

1. Previous data has demonstrated that CeO_2 nanoparticles have a size-dependent antioxidant function [111]. The CeO_2 aggregates studied in this project had a mean size of 184 ± 75.3 nm. To fully describe the potential toxicity of CeO_2 particles, studies using other sizes of nanoparticles in both the aggregated and non-aggregated states should be undertaken.
2. Further examination of the genes involved in CeO_2 toxicity response in *C. elegans* could give insight to its mechanism of action. Experiments with additional GFP-containing transgenic *C. elegans* could potentially reveal additional biological side effects of CeO_2 exposure.
3. The effects of CeO_2 aggregates on other aquatic organisms such as zebrafish or Drosophila would provide additional insight to the ecotoxicity of the particle.
4. Agar nematode growth medium was used in this study. Alternatively, many *C. elegans* papers study exposure effects of compounds in liquid culture. All assays performed in this study could be adapted for liquid culture of *C. elegans*.
5. CeO_2 related activation of mitogen activated protein kinase (MAPK) signaling or markers of inflammation such as NF-kB could be more closely examined by analysis of protein and mRNA levels in the nematode.

References

1. *What is C. elegans?* 2008 2008; 2008:[Available from: http://www.sfu.ca/biology/faculty/hutter/hutterlab/research/Celegans.html.
2. Hardas, S.S., et al., *Brain distribution and toxicological evaluation of a systemically delivered engineered nanoscale ceria.* Toxicol Sci, 2010. **116**(2): p. 562-76.
3. Das, M., et al., *Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons.* Biomaterials, 2007. **28**(10): p. 1918-25.
4. Schulte, P.A., et al., *Issues in the development of epidemiologic studies of workers exposed to engineered nanoparticles.* J Occup Environ Med, 2009. **51**(3): p. 323-35.
5. Yildirimer, L., et al., *Toxicology and clinical potential of nanoparticles.* Nano Today, 2011. **6**(6): p. 585-607.
6. Hood, E., *Nanotechnology: looking as we leap.* Environ Health Perspect, 2004. **112**(13): p. A740-9.
7. Arora, S., J.M. Rajwade, and K.M. Paknikar, *Nanotoxicology and in vitro studies: the need of the hour.* Toxicol Appl Pharmacol, 2012. **258**(2): p. 151-65.
8. Li, J., et al., *Facile synthesis of 3D flowerlike CeO2 microspheres under mild condition with high catalytic performance for CO oxidation.* J Colloid Interface Sci, 2011. **360**(1): p. 93-9.
9. 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.
10. 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.
11. Brunner, T.J., et al., *In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility.* Environ Sci Technol, 2006. **40**(14): p. 4374-81.

12. Xia, T., et al., *Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties.* ACS Nano, 2008. **2**(10): p. 2121-34.

13. Hirst, S.M., et al., *Anti-inflammatory properties of cerium oxide nanoparticles.* Small, 2009. **5**(24): p. 2848-56.

14. Lee, T.L., et al., *Accessing the genomic effects of naked nanoceria in murine neuronal cells.* Nanomedicine, 2012. **8**(5): p. 599-608.

15. Celardo, I., E. Traversa, and L. Ghibelli, *Cerium oxide nanoparticles: a promise for applications in therapy.* J Exp Ther Oncol, 2011. **9**(1): p. 47-51.

16. Korsvik, C., et al., *Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles.* Chem Commun (Camb), 2007(10): p. 1056-8.

17. Eom, H.J. and J. Choi, *Oxidative stress of CeO2 nanoparticles via p38-Nrf-2 signaling pathway in human bronchial epithelial cell, Beas-2B.* Toxicol Lett, 2009. **187**(2): p. 77-83.

18. Horie, M., et al., *Cellular responses induced by cerium oxide nanoparticles: induction of intracellular calcium level and oxidative stress on culture cells.* J Biochem, 2011. **150**(4): p. 461-71.

19. Srinivas, A., et al., *Acute inhalation toxicity of cerium oxide nanoparticles in rats.* Toxicol Lett, 2011. **205**(2): p. 105-15.

20. Wingard, C.J., et al., *Mast cells contribute to altered vascular reactivity and ischemia-reperfusion injury following cerium oxide nanoparticle instillation.* Nanotoxicology, 2011. **5**(4): p. 531-45.

21. Lanone, S., et al., *Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines.* Part Fibre Toxicol, 2009. **6**: p. 14.

22. Ma, J.Y., et al., *Cerium oxide nanoparticle-induced pulmonary inflammation and alveolar macrophage functional change in rats.* Nanotoxicology, 2011. **5**(3): p. 312-25.

23. Van Hoecke, K., et al., *Fate and effects of CeO2 nanoparticles in aquatic ecotoxicity tests.* Environ Sci Technol, 2009. **43**(12): p. 4537-46.

24. Rodea-Palomares, I., et al., *Physicochemical characterization and ecotoxicological assessment of CeO2 nanoparticles using two aquatic microorganisms.* Toxicol Sci, 2011. **119**(1): p. 135-45.

25. Buzea, C., Pacheco, II, and K. Robbie, *Nanomaterials and nanoparticles: sources and toxicity.* Biointerphases, 2007. **2**(4): p. MR17-71.

26. Vijayamohanan, K. and M. Aslam, *Applications of self-assembled monolayers for biomolecular electronics.* Appl Biochem Biotechnol, 2001. **96**(1-3): p. 25-39.

27. Davis, S.S., *Biomedical applications of nanotechnology--implications for drug targeting and gene therapy.* Trends Biotechnol, 1997. **15**(6): p. 217-24.

28. Feynman, R., *Theres Plenty of Room at the Bottom.* Caltech Engineering and Science, 1960. **23:5**: p. 22-36.

29. *PEN* [Webpage] 2005 [cited 2013 Septermber 10,2013]; Webpage]. Available from: http://www.nanotechproject.org/about/mission/.

30. Seltenrich, N., *Nanosilver: weighing the risks and benefits.* Environ Health Perspect, 2013. **121**(7): p. A220-5.

31. Yu, J.X. and T.H. Li, *Distinct biological effects of different nanoparticles commonly used in cosmetics and medicine coatings.* Cell Biosci, 2011. **1**(1): p. 19.

32. Kessler, R., *Engineered nanoparticles in consumer products: understanding a new ingredient.* Environ Health Perspect, 2011. **119**(3): p. a120-5.

33. Ventola, C.L., *The nanomedicine revolution: part 3: regulatory and safety challenges.* P T, 2012. **37**(11): p. 631-9.

34. Mahmoudi, M., et al., *Assessing the in vitro and in vivo toxicity of superparamagnetic iron oxide nanoparticles.* Chem Rev, 2012. **112**(4): p. 2323-38.

35. Drobne, D., *Nanotoxicology for safe and sustainable nanotechnology.* Arh Hig Rada Toksikol, 2007. **58**(4): p. 471-8.

36. Park, M.V., et al., *In vitro developmental toxicity test detects inhibition of stem cell differentiation by silica nanoparticles.* Toxicol Appl Pharmacol, 2009. **240**(1): p. 108-16.

37. Murdock, R.C., et al., *Characterization of nanomaterial dispersion in solution prior to in vitro exposure using dynamic light scattering technique.* Toxicol Sci, 2008. **101**(2): p. 239-53.

38. Arnold, M.C., et al., *Cerium oxide nanoparticles are more toxic than equimolar bulk cerium oxide in Caenorhabditis elegans.* Arch Environ Contam Toxicol, 2013. **65**(2): p. 224-33.

39. Kim, K.T., et al., *Silver nanoparticle toxicity in the embryonic zebrafish is governed by particle dispersion and ionic environment.* Nanotechnology, 2013. **24**(11): p. 115101.

40. Prasad, R.Y., et al., *Effect of Treatment Media on the Agglomeration of Titanium Dioxide Nanoparticles: Impact on Genotoxicity, Cellular Interaction, and Cell Cycle.* ACS Nano, 2013.

41. Gosens, I., et al., *Impact of agglomeration state of nano- and submicron sized gold particles on pulmonary inflammation.* Part Fibre Toxicol, 2010. **7**(1): p. 37.

42. Gorth, D.J., D.M. Rand, and T.J. Webster, *Silver nanoparticle toxicity in Drosophila: size does matter.* Int J Nanomedicine, 2011. **6**: p. 343-50.

43. Gaiser, B.K., et al., *Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments.* Environ Health, 2009. **8 Suppl 1**: p. S2.

44. Artells, E., et al., *Exposure to cerium dioxide nanoparticles differently affect swimming performance and survival in two daphnid species.* PLoS One, 2013. **8**(8): p. e71260.

45. Moos, R., et al., *Resistive oxygen gas sensors for harsh environments.* Sensors (Basel), 2011. **11**(4): p. 3439-65.

46. Corma, A., et al., *Hierarchically mesostructured doped CeO2 with potential for solar-cell use.* Nat Mater, 2004. **3**(6): p. 394-7.

47. Fotopoulos, A., et al., *One pot synthesis and characterization of ultra fine CeO2 and Cu/CeO2 nanoparticles. Application for low temperature CO oxidation.* J Nanosci Nanotechnol, 2011. **11**(10): p. 8593-8.

48. Ou, D.R., et al., *Microstructural and metal-support interactions of the Pt-CeO2/C catalysts for direct methanol fuel cell application.* Langmuir, 2011. **27**(7): p. 3859-66.

49. 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.

50. Tarnuzzer, R.W., et al., *Vacancy engineered ceria nanostructures for protection from radiation-induced cellular damage.* Nano Lett, 2005. **5**(12): p. 2573-7.

51. Yuan, Q., et al., *Controlled synthesis and assembly of ceria-based nanomaterials.* J Colloid Interface Sci, 2009. **335**(2): p. 151-67.

52. Pirmohamed, T., et al., *Nanoceria exhibit redox state-dependent catalase mimetic activity.* Chem Commun (Camb), 2010. **46**(16): p. 2736-8.

53. Singh, V.K., et al., *Modulation of autoimmune diseases by nitric oxide.* Immunol Res, 2000. **22**(1): p. 1-19.

54. Czyz, M., *[Specificity and selectivity of the NFkappaB response].* Postepy Biochem, 2005. **51**(1): p. 60-8.

55. Haddad, J.J. and N.E. Abdel-Karim, *NF-kappaB cellular and molecular regulatory mechanisms and pathways: therapeutic pattern or pseudoregulation?* Cell Immunol, 2011. **271**(1): p. 5-14.

56. 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.

57. Singh, N., C.A. Cohen, and B.A. Rzigalinski, *Treatment of neurodegenerative disorders with radical nanomedicine.* Ann N Y Acad Sci, 2007. **1122**: p. 219-30.

58. Chen, J., et al., *Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides.* Nat Nanotechnol, 2006. **1**(2): p. 142-50.

59. Pierscionek, B.K., et al., *Nanoceria have no genotoxic effect on human lens epithelial cells.* Nanotechnology, 2010. **21**(3): p. 035102.

60. Park, E.J., et al., *Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells.* Toxicology, 2008. **245**(1-2): p. 90-100.

61. Ma, J.Y., et al., *Induction of pulmonary fibrosis by cerium oxide nanoparticles.* Toxicol Appl Pharmacol, 2012. **262**(3): p. 255-64.

62. 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.

63. Toya, T., et al., *Pulmonary toxicity induced by intratracheal instillation of coarse and fine particles of cerium dioxide in male rats.* Ind Health, 2010. **48**(1): p. 3-11.

64. Brenner, S., *The genetics of Caenorhabditis elegans.* Genetics, 1974. **77**(1): p. 71-94.

65. Praitis, V. and M.F. Maduro, *Transgenesis in C. elegans.* Methods Cell Biol, 2011. **106**: p. 161-85.

66. Lapierre, L.R. and M. Hansen, *Lessons from C. elegans: signaling pathways for longevity.* Trends Endocrinol Metab, 2012. **23**(12): p. 637-44.

67. Sulston, J.E. and H.R. Horvitz, *Post-embryonic cell lineages of the nematode, Caenorhabditis elegans.* Dev Biol, 1977. **56**(1): p. 110-56.

68. Miwa, J., et al., *Tumor promoters specifically and reversibly disturb development and behavior of Caenorhabditis elegans.* J Cancer Res Clin Oncol, 1982. **104**(1-2): p. 81-7.

69. Mishur, R.J., J.A. Butler, and S.L. Rea, *Exometabolomic Mapping of Caenorhabditis elegans: A Tool to Noninvasively Investigate Aging.* Methods Mol Biol, 2013. **1048**: p. 195-213.

70. Johnson, T.E., *Aging can be genetically dissected into component processes using long-lived lines of Caenorhabditis elegans.* Proc Natl Acad Sci U S A, 1987. **84**(11): p. 3777-81.

71. Sasakura, H. and I. Mori, *Behavioral plasticity, learning, and memory in C. elegans.* Curr Opin Neurobiol, 2013. **23**(1): p. 92-9.

72. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(6669): p. 806-11.

73. Tsien, R.Y., *The green fluorescent protein.* Annu Rev Biochem, 1998. **67**: p. 509-44.

74. Zhang, Y., et al., *Selection of reliable reference genes in Caenorhabditis elegans for analysis of nanotoxicity.* PLoS One, 2012. **7**(3): p. e31849.

75. Coolon, J.D., et al., *Caenorhabditis elegans genomic response to soil bacteria predicts environment-specific genetic effects on life history traits.* PLoS Genet, 2009. **5**(6): p. e1000503.

76. Spiro, Z., et al., *RNA interference links oxidative stress to the inhibition of heat stress adaptation.* Antioxid Redox Signal, 2012. **17**(6): p. 890-901.

77. Walker, G.A., et al., *Heat shock protein accumulation is upregulated in a long-lived mutant of Caenorhabditis elegans.* J Gerontol A Biol Sci Med Sci, 2001. **56**(7): p. B281-7.

78. Walker, G.A., D.W. Walker, and G.J. Lithgow, *Genes that determine both thermotolerance and rate of aging in Caenorhabditis elegans.* Ann N Y Acad Sci, 1998. **851**: p. 444-9.

79. Zhang, S., C. Ma, and M. Chalfie, *Combinatorial marking of cells and organelles with reconstituted fluorescent proteins.* Cell, 2004. **119**(1): p. 137-44.

80. Mohan, N., et al., *In vivo imaging and toxicity assessments of fluorescent nanodiamonds in Caenorhabditis elegans.* Nano Lett, 2010. **10**(9): p. 3692-9.

81. Qu, Y., et al., *Full assessment of fate and physiological behavior of quantum dots utilizing Caenorhabditis elegans as a model organism.* Nano Lett, 2011. **11**(8): p. 3174-83.

82. Yeates, G.W., *Nematodes in Ecological Webs.* eLS, 2010.

83. Zhang, H., et al., *Nano-CeO2 exhibits adverse effects at environmental relevant concentrations.* Environ Sci Technol, 2011. **45**(8): p. 3725-30.

84. Roh, J.Y., et al., *Ecotoxicological investigation of CeO(2) and TiO(2) nanoparticles on the soil nematode Caenorhabditis elegans using gene expression, growth, fertility, and survival as endpoints.* Environ Toxicol Pharmacol, 2010. **29**(2): p. 167-72.

85. Walser, T., et al., *Persistence of engineered nanoparticles in a municipal solid-waste incineration plant.* Nat Nanotechnol, 2012. **7**(8): p. 520-4.

86. Mina, K., L. Fritschi, and M. Knuiman, *A valid semiquantitative food frequency questionnaire to measure fish consumption.* Eur J Clin Nutr, 2007. **61**(8): p. 1023-31.

87. Aschberger, K., et al., *Analysis of currently available data for characterising the risk of engineered nanomaterials to the environment and human health--lessons learned from four case studies.* Environ Int, 2011. **37**(6): p. 1143-56.

88. Limbach, L.K., et al., *Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations.* Environ Sci Technol, 2005. **39**(23): p. 9370-6.

89. Boyd, W.A. and P.L. Williams, *Comparison of the sensitivity of three nematode species to copper and their utility in aquatic and soil toxicity tests.* Environ Toxicol Chem, 2003. **22**(11): p. 2768-74.

90. Arsenovic, P.T., et al., *Depletion of the C. elegans NAC engages the unfolded protein response, resulting in increased chaperone expression and apoptosis.* PLoS One, 2012. **7**(9): p. e44038.

91. Stiernagle, T., *Maintenance of C. elegans.* WormBook, 2006: p. 1-11.

92. Lithgow, G.J., et al., *Thermotolerance of a long-lived mutant of Caenorhabditis elegans.* J Gerontol, 1994. **49**(6): p. B270-6.

93. Aitken, R.J., et al., *Manufacture and use of nanomaterials: current status in the UK and global trends.* Occup Med (Lond), 2006. **56**(5): p. 300-6.

94. Macphail, R.C., E.A. Grulke, and R.A. Yokel, *Assessing nanoparticle risk poses prodigious challenges.* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2013. **5**(4): p. 374-87.

95. Simonelli, F., et al., *Cyclotron production of radioactive CeO(2) nanoparticles and their application for in vitro uptake studies.* IEEE Trans Nanobioscience, 2011. **10**(1): p. 44-50.

96. Chatterjee, S., A. Bandyopadhyay, and K. Sarkar, *Effect of iron oxide and gold nanoparticles on bacterial growth leading towards biological application.* J Nanobiotechnology, 2011. **9**: p. 34.

97. Van Hoecke, K., et al., *Aggregation and ecotoxicity of CeO(2) nanoparticles in synthetic and natural waters with variable pH, organic matter concentration and ionic strength.* Environ Pollut, 2011. **159**(4): p. 970-6.

98. Mayer, M.P. and B. Bukau, *Hsp70 chaperones: cellular functions and molecular mechanism.* Cell Mol Life Sci, 2005. **62**(6): p. 670-84.

99. Haas, I.G., *BiP (GRP78), an essential hsp70 resident protein in the endoplasmic reticulum.* Experientia, 1994. **50**(11-12): p. 1012-20.

100. Kim, I.S., M. Baek, and S.J. Choi, *Comparative cytotoxicity of Al2O3, CeO2, TiO2 and ZnO nanoparticles to human lung cells.* J Nanosci Nanotechnol, 2010. **10**(5): p. 3453-8.

101. Heckert, E.G., S. Seal, and W.T. Self, *Fenton-like reaction catalyzed by the rare earth inner transition metal cerium.* Environ Sci Technol, 2008. **42**(13): p. 5014-9.

102. Goldstein, P. and T. Modric, *Transgenerational, ultrastructural analysis on the antioxidative effects of tocopherol on early gametogenesis in Caenorhabditis elegans grown in 100% oxygen.* Toxicol Appl Pharmacol, 1994. **124**(2): p. 212-20.

103. Yasuda, K., et al., *Interrelationships between mitochondrial fusion, energy metabolism and oxidative stress during development in Caenorhabditis elegans.* Biochem Biophys Res Commun, 2011. **404**(3): p. 751-5.

104. 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.

105. Fielenbach, N. and A. Antebi, *C. elegans dauer formation and the molecular basis of plasticity.* Genes Dev, 2008. **22**(16): p. 2149-65.

106. Fukuyama, M., et al., *C. elegans AMPKs promote survival and arrest germline development during nutrient stress.* Biol Open, 2012. **1**(10): p. 929-36.

107. Jacklet, J.W., *Nitric oxide signaling in invertebrates.* Invert Neurosci, 1997. **3**(1): p. 1-14.

108. Hobert, O., *WormBook*, in *Specification of the nervous system*, T.C.e.R. Community, Editor 2005.

109. Handy, R.D., et al., *Ecotoxicity test methods for engineered nanomaterials: practical experiences and recommendations from the bench.* Environ Toxicol Chem, 2012. **31**(1): p. 15-31.

110. Richards, J.L., et al., *A quantitative model of normal Caenorhabditis elegans embryogenesis and its disruption after stress.* Dev Biol, 2013. **374**(1): p. 12-23.

111. Shcherbakov, A.B., et al., *[Nanocrystaline ceria based materials--perspectives for biomedical application].* Biofizika, 2011. **56**(6): p. 995-1015.

Appendix

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

March 11, 2013

Steven N. Rogers
1227 7th St.
Huntington, WV 25701

Dear Mr. Rogers:

This letter is in response to the submitted thesis abstract titled "Toxicological Effects of Cerium Oxide Nanoparticles on Caenorhabditis Elgans." 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
Office of Research Integrity

Curriculum vita

Steven Rogers

stevenrogersemail@gmail.com

1227 7th st.

Huntington, WV 25701

Education

Bachelor of Arts, Marshall University, Huntington, WV, May 2010 Major: Biotechnology.

GPA 3.42; Honors

Completed courses in the Biomedical Sciences Graduate Program:

BSC 517 (Biostatistics graduate level)

BMS 600 (Principals of biochemistry)

BMS 651 (Cancer biology)

BMS 660 (Communications)

BMS 661 (Communications II)

BMS 628 (Neuroscience)

BMS 629 (Neuroscience II) Research Rotations

Cumulative GPA: 3.0

Research Experience

I. Researcher, **Center for Bioengineering and Biomanufacturing Commercialization,**

Marshall University, May 2008-Dec 2009

- Developed an Alpha-actinin 3 screening test to detect a single nucleotide

polymorphism to be used as a teaching exercise at Marshall University in IST 341 Human Genetics.

- Created educational instructional videos for many laboratory instruments for CBBC training purposes.
- Attended BIO, the international biotech convention in Atlanta, Georgia 2009.

II. Researcher, **Center for Diagnostic Nanosystems,** Marshall University, Jan 2010-present.

Awards/ Scholarships

- Undergraduate studies were fully paid by WV Promise scholarship award and MU scholarship programs.
- Received a $500 undergraduate research scholarship award from Marshall University College of science for work on a luciferase translation inhibition assay for characterizing activity of Pokeweed antiviral protein (PAP).
- Received the best academic performance of the year award for the 2010-2011 school year from Joan C. Edwards School of Medicine/ Marshall University's Biomedical Sciences Program.
- Was the recipient for Marshall Universities Biomedical Science graduate student organization $500 scholarship award for leadership and performance for the 2011-2012 year.

Teaching experience

- Taught multiple classes of IST 340 (DNA technology) a laboratory research based course at Marshall University.

Community Services

Volunteer at Central City Market through Huntington CCSO in April 2010.

- Have helped organize, promote, and/or perform fund raisers for the Huntington library, jewel City Roller Girls, Huntington Music and Arts Fest, and “Music for Monica.”