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Toxicological Assessment of Intra-tracheal Instilled Nanoceria on Kidneys of Sprague-Dawley Rats

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Toxicological assessment of intra-tracheal instilled nanoceria on kidneys of Sprague-Dawley

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ABBREVIATIONS

ANOVA   Analysis of variance on ranks

Bax   Bcl-2 associated X protein

Bcl-2   B-cell lymphoma 2

BSA   Bovine serum albumin

CeO_2 Cerium Oxide

ECL   Enhanced chemiluminiscence

ERK   Extracellular signal-regulated kinases

JNK   c-Jun N- terminal kinase

MAPK   Mitogen - activated protein kinase

ROS   Reactive oxygen species

SEM   Standard Error Mean

SDS-PAGE   Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

TBS   Tris buffered saline

TBST   Tris buffered saline with 0.5% tween

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ABSTRACT

Recent studies suggest that use of nanoceria in industry is associated with increased risk of human and environmental exposure. How inhaled nanoceria might affect the kidneys is not yet known. To investigate this possibility, Sprague Dawley rats were exposed to a single dose of 7mg/kg body weight cerium oxide nanoparticles by intratracheal instillation. Animals were sacrificed at day 1, 3, 14, or 28 days after exposure and the kidneys collected for histological and biochemical analysis. No significant difference was observed in kidney to body weight ratio between the different groups. Hematoxylin and eosin staining did not reveal any significant changes in kidney morphology. Compared to control animals, immunoblot analysis revealed a significant decrease in the Bax/Bcl2 ratio, a decreased ratio of phosphorylated to total JNK, and a diminished phosphorylated to total p44/42 ratio. There was a decrease in caspase-3 activity in the 28 day exposure group. The data obtained from this study indicate that the inhalation of cerium oxide nanoparticles is not associated with significant nephrotoxicity. Further studies of longer duration or using higher dosages of cerium oxide may be needed to confirm this conclusion.

Key words: Nanoceria, Kidneys, Oxidative stress, Intratracheal instillation

CHAPTER 1

Introduction

Nanotechnology is the study of manipulating material at molecular scale to develop novel engineering products with enhanced physical and chemical properties. Of particular biomedical interest is the potential use of nanoparticles. Nanoparticles can be defined as ultrafine particles ranging in between 1 - 100 nm in size with varying physical and chemical properties (73). Currently, nanoparticles are being used for a wide variety of purposes and have found homes in the automobile, diesel and medical industries (49). It is well known that nanoparticles exhibit different chemical and physical properties than their macroscopic counterparts (24). Although not fully understood, it is thought that these differences are due, at least in part, to the greater surface area to volume ratio seen in nanoparticles (15). Although potentially beneficial, like many materials, it is important to note that nanoparticles may also have non-beneficial effects. Although the manufacture of nanoparticles is tightly controlled, there is still the possibility for environmental and human exposure. For example, a recent study by Song et al. (2009) gave insight into the potential hazards of nanoparticles to people who work in factory that uses polyacrylate (63). This study showed that the workers of this factory suffered from nonspecific pulmonary inflammation, pulmonary fibrosis and foreign-body granulomas of lung pleura which lead to their death (63).

Cerium oxide (CeO_2) nanoparticles are used as a fuel additive, as an insulating layer on silicon substrates, oxygen pumps, clothes, and other ceramic products (60). Cerium oxide is a rare earth oxide with a fluorite lattice structure (12). Baalousha et al. (2012) demonstrated that cerium oxide nanoparticles contain a mixture of Ce^{3+} and Ce^{4+} cations which are responsible for

their different physico-chemical properties (8). Exposure to CeO_2 nanoparticles can be toxic to cells (37). For example, Horie and coworkers demonstrated that CeO_2 nanoparticles can induce oxidative stress in human keratinocyte cells. Supporting this contention, Kennedy et al. (2009) showed that CeO_2 nanoparticle exposure increases the concentration of reactive oxygen species in human vascular endothelial cells (40). Cerium oxide nanoparticles are present in diesel exhaust (52). Recent *in vivo* studies have demonstrated that CeO_2 nanoparticles can cause lung inflammation and fibrosis in a concentration dependent manner (48). A similar study involving intratracheal exposure of nanoceria exposure to Sprague-Dawley rats resulted in elevation of serum alanine transaminase levels, reduced albumin levels and a diminished sodium-potassium ratio. Moreover, intratracheal exposure also resulted in decreased serum triglyceride levels and dose-dependent hydropic degeneration and sinusoidal dilatation of hepatocytes (48). Whether inhaled CeO_2 nanoparticles are capable of causing damage to other vital organs besides the lung and liver is not known.

Significance

With the increasing use of nanomaterials in industry and everyday life there is an increasing risk of exposure. As such, it is crucial to determine the toxicity of nanomaterials in order to protect life and the environment. CeO_2 nanoparticles are widely used in diesel fuel additives and in the manufacturing industry. Recent studies have suggested that the inhalation of CeO_2 nanoparticles is associated with lung fibrosis and liver toxicity. Whether CeO_2 nanoparticles can also cause damage to the kidneys has, to our knowledge, not been investigated.

Hypothesis

The main objective of this study is to investigate the potential toxicological effects of inhaled cerium oxide nanoparticles on kidney structure and function. We hypothesize that inhaled CeO_2 nanoparticles can cross the lung barrier and enter into the general circulation. Once in the blood, we hypothesize that CeO_2 nanoparticles will travel to the kidneys and cause changes in kidney structure and function. To investigate this hypothesis the following specific aims will be addressed:

Specific aim 1:

To determine if inhaled cerium oxide nanoparticles can alter kidney structure.

Hypothesis 1:

The inhalation of cerium oxide nanoparticles will be associated with change in kidney structure.

Specific aim 2:

To determine if inhaled cerium oxide nanoparticles can induce oxidative damage and kidney cell death.

Hypothesis 2:

The inhalation of cerium oxide nanoparticles will be associated with elevations in renal oxidative stress and cell death.

CHAPTER 2

This chapter will focus on the current literature available that is relevant to the current study. Emphasis will be placed on the following topics: (i.) Nanotechnology and its potential role in medicine, (ii.) Commercial applications of nanoceria, (iii.) Molecular mechanism involved in oxidative stress and apoptosis, and (iv.) Nanoceria and oxidative stress.

Nanotechnology and its potential role in medicine

The term nanotechnology was coined by Norio in 1974 in his work related to sputter machining (74). It is thought that the first commercial application of nanotechnology was the development of the atomic force microscope in 1986 (74). Although there are no predetermined limits, it is generally accepted that nanomaterials are between 1 - 100 nm in size. Nanotechnology is a rapidly growing field. It has been posited that nanotechnology will have applications in a number of different fields including engineering, medicine, and energy production (9).

One potential application of nanotechnology in medicine is the use of nanomaterials for theranostic purposes. Gold nanoparticles, silica nanospheres, superparamagnetic iron oxide nanoparticles, and fluorophore-doped silica nanoparticles are currently being used for diagnostic purposes (4, 29). Recent work by Taylor et al. (2008) demonstrated that modified silica nanospheres can be used as T1 and T2 contrast agents for intravascular and soft tissue magnetic resonance imaging (66). Unlike conventional contrast agents, gold nanoprobes that target specific antigens present on cancer cells can be used for the detection of cancer with computed tomography (55). Other work is studying the applications of nanoparticles for the

treatment of brain tumors (72), the management of atherosclerosis (47), in orthopedics (75), and to combat infectious agents (10). Nanoparticles are also being used as vehicles for sustained release of drugs in prostate cancer (68).

Although possibly beneficial, the potential widespread use of nanomaterials has begun to give rise to concern about their toxicity. For instance, Chu et al. (2010) demonstrated that fluorescent quantum dots can be transferred from female mice to their fetuses across the placental barrier and that this transfer can cause the death of the fetus (17). Similarly, Zhang et al. (2011) showed that zinc oxide and titanium dioxide nanoparticles are toxic to human fetal lung fibroblasts due to their ability to induce mitochondrial dysfunction (77). Amongst the several different types of nanoparticles currently in use the potential toxicity of nanoceria is becoming increasingly questioned due to its widespread commercial use. How, or if, cerium nanoparticles may affect cellular function is not well understood.

Commercial applications of nanoceria

Nanoceria are sub-microscopic cerium oxide particles ranging in between 1 - 100 nm in size. They are used in a range of industrial products including sun screens where they act as UV shields (78), in fuel cells, as diesel additives (13), and as polishing agents (43). Cerium oxide nanoparticles exist in a fluorite lattice structure which cycle between Ce^{+3} and Ce^{+4} states. Whereas the Ce^{+4} state is stable in conformation, the Ce^{+3} state is more reactive (14).

The reactive state of cerium oxide is being exploited for catalytic activity. For the addition of cerium oxide nanoparticles (sold as Envirox, Cerica Corporation, Ontario, Canada), to diesel fuel can be used to improve mileage and reduce particulate emissions (52). It is

thought that cerium oxide nanoparticles can function as a catalyst to aid in the combustion of hydrocarbons and soot, thereby reducing the amount of pollutants emitted from vehicles (53). However, other work has demonstrated that cerium oxide nanoparticles are being released from in the diesel exhaust (52).

Molecular mechanism involved in oxidative stress and apoptosis

Oxidative stress results from an imbalance between the amount of reactive oxygen species and the amount of antioxidants in a living organism (32). If excessive, oxidative stress can result in damage to DNA, protein misfolding, mitochondria dysfunction, and damage to the cell membrane which can lead to cell death (42). Reactive oxygen species can be generated from both exogenous and endogenous sources. Although production of reactive oxygen species in minimal quantities is oftentimes beneficial, such as that produced by white blood cells to kill invading pathogens (1), too much oxidative stress can threaten cellular function.

The mechanisms underlying reactive oxygen species-mediated signal transduction involve alterations in the expression of transcription factors and the activation of protein kinases and phosphatases (44) (27). One family of molecules thought to be activated in the presence of reactive oxygen species is the mitogen activated protein kinases (MAPK) (59). Among the different MAPK family members, the extracellular signal-regulated kinases (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) undergo phosphorylation in response to increases in cellular oxidative stress (11). The MAPK proteins are involved in several different processes including the cellular differentiation, and the regulation of cell survival and death (76). If

excessive and sustained, the increased activation of MAPK proteins can lead to uncontrolled cell proliferation or death (41).

Oxidative stress can activate ERK1/2 MAPK proteins through tyrosine kinase signaling (30). Similarly, hydrogen peroxide can cause the activation of ERK1/2 MAPK proteins through the ligand-independent phosphorylation of growth factor receptors (67). Silver nanoparticles can cause the activation of JNK MAPK signaling and that this response appears to be mediated through elevations in cellular reactive oxygen species (38).

In addition to increases in the amount of reactive oxygen species, MAPK proteins can undergo activation through the inhibition of MAPK phosphatases. Kamata et al. (2005) observed that reactive oxygen species can activate JNK MAPK signaling through the inhibition of MAPK phosphatases (39). Similarly, Liu et al. (2010) observed that reactive oxygen species can activate JNK MAPK signaling in cultured fibroblasts by decreasing the activity of the MAPK phosphatase MPK-1 (46). How cellular exposure to cerium oxide nanoparticles can result in the activation of MAPK signaling has, to our knowledge, not been studied extensively.

Apoptosis is a mechanism whereby extracellular and intracellular signals such as hormones, cytokines, and inflammatory mediators coordinately act to cause cell death in a defined order (25). Apoptosis can be induced by increases in the amount of oxidative stress (19). It is thought that high levels of reactive oxygen species can damage the mitochondrial membrane and cause the release of cytochrome C (7). Once released from the mitochondria, cytochrome C binds with and activates apoptotic protease activating factor-1 which can lead to the activation of caspase - 3 and cell death (70).

Oxidative stress-induced cell death may be mediated by MAPK signaling (71). The ERK1/2, p38, and JNK MAPK are thought to undergo activation (phosphorylation) under conditions of increased cellular stress. Tsuruta et al. (2004) howed that JNK activation is required for Bax to induce the release of cytochrome C from the mitochondria and that this process requires the phosphorylation of 14-3-3 proteins (69). Similarly, Deschesnes and coworkers (2001) showed that p38 MAPK is required for membrane blebbing and nuclear condensation (23). Ravindran et al. (2011) demonstrated that elevations in reactive oxygen species is associated with the activation of p38 MAPK and JNK-MAPK which leads to the activation of caspase - 3 and cell death (58). Exposure to nickel oxide nanoparticles can induce cellular death through increased levels of reactive oxygen species and that this process is mediated by the activation of caspase 3 (62). Chromic oxide nanoparticles caused cell death in a caspase - 3 dependent manner (36). Whether exposure of renal cells to cerium oxide nanoparticles can cause cell death has, to our knowledge, not been investigated.

Nanoceria and oxidative stress

It is currently unclear whether exposure to cerium oxide nanoparticles is associated with increases or decreases in reactive oxygen species. Nanoceria can be used to protect neuronal cells from oxidative cell death (18), treat diabetes (56), and to protect cardiomyoctes from cigarette smoke induced increases in oxidative stress (51). Nonetheless, Park et al. (2008) showed that nanoceria increases the level of oxidative stress in BEAS-2B cells (54). Similarly, other studies indicate nanoceria exposure is associated with increased oxidative stress in human bronchial epithelial cells and that this process occurs through the activation of p38-Nrf-

2 signaling (26). Similarly, cerium oxide nanoparticles have also been shown to cause the production of malondialdehyde, increase the amount of lactate dehydrogenase and cause lipid peroxidation in cultured cells (45). The reasons for disparate findings between studies is currently unclear but may be related to differences in the size or type of cerium oxide nanoparticle used. Whether the exposure of renal cells to cerium oxide nanoparticles is associated with increases in oxidative stress, to our knowledge, not been investigated.

Summary

The gaining popularity of nanoceria for its catalytic properties warrants further investigation before they can be used for biomedical purposes. Thus far, evidence exists suggesting that cerium nanoparticles can both cause and ameliorate increases in oxidative stress. Studies have also shown that increased oxidative stress is associated with the activation of MAPK signaling which can cause cell death. Whether exposure of renal cells to cerium oxide nanoparticles causes increases in oxidative stress, MAPK activation or cell death is currently unclear.

CHAPTER 3

ABSTRACT

The growing use of nanoceria in industry is associated with increased risk of human and environmental exposure. How inhaled nanoceria might affect the kidneys is not yet known. To investigate this possibility, Sprague Dawley rats were exposed to a single dose of 7 mg/kg body weight cerium oxide nanoparticles by intratracheal instillation. Animals were sacrificed at day 1, 3, 14, or 28 days after exposure and the kidneys collected for histological and biochemical analysis. No significant difference was observed in kidney to body weight ratio between the different groups. Hematoxylin and eosin staining did not reveal any significant changes in kidney morphology. Compared to control animals, immunoblot analysis revealed a significant decrease in the Bax/Bcl2 ratio, a decreased ratio of phosphorylated to total JNK, and a diminished phosphorylated to total p44/42 ratio. There was a decrease in caspase-3 activity in the 28 day exposure group. The data obtained from this study indicate that the inhalation of cerium oxide nanoparticles is not associated with significant nephrotoxicity. Further studies of longer duration or using higher dosages of cerium oxide may be needed to confirm this conclusion.

Key words: Nanoceria, Kidneys, Oxidative stress, Intratracheal instillation

INTRODUCTION

Nanotechnology is a fast growing multidisciplinary science that has the potential to transform the fields of medicine. Nanoparticles are refined bulk materials that are between 1 and 100 nm in size. Cerium oxide nanoparticles have been used in a variety of different industries including the manufacturing of fuel cells (5), in sun screens (45), and as diesel additives (52). Recent data have also suggested that cerium oxide nanoparticles may exhibit the ability to scavenge reactive oxygen species (34).

Cerium oxide nanoparticles exist in a fluorite lattice structure and transit between Ce^{+3} and Ce^{+4} oxidation states. Ce^{+3} state is responsible for anti-inflammatory and ROS scavenging mechanisms (14). *In vivo* and *in vitro* studies have demonstrated that cerium oxide nanoparticles can protect neuronal cells from apoptotic cell death (22), reduce nitric oxide levels in macrophages (35), protect the myocardium from oxidative stress- induced remodeling (50) and reduce glucose levels in diabetic rats (56). Nanoceria can also induce lung inflammation (64), hepatic damage (48) and increase oxidative stress in cultured BEAS-2B cells (54). Whether nanoceria can cause damage to the kidneys has, to our knowledge, not been investigated.

It is thought that the primary route of unintended nanoparticle exposure is through inhalation. Previous work has demonstrated that intratracheal instillation of nanoceria is associated with lung and liver damage (48). Herein, we attempt to extend these findings and examine if the inhalation of cerium oxide nanoparticles can cause renal damage. Using the same animals and tissues used in our previous work (48) we now examine if the inhalation of ceria is associated with changes in kidney structure, evidence of oxidative stress, and kidney

apoptosis. Unlike that seen with the liver and lung, our data suggest that the inhalation of cerium oxide nanoparticles is not associated with changes in kidney structure or function in male Sprague Dawley rats.

MATERIALS AND METHODS

Animals

Five week aged male Sprague-Dawley rats (n=6 per group) were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA, USA). All animals were in the range of 150 - 180 grams approximately. Animals were housed two per cage under a 12H:12H dark light cycle at 22 ± 2 °C. Rats were fed standard rodent chow and water *ad libitum*. Animals were acclimatization for two weeks prior to study. During the acclimatization period, the animals were observed for weight loss, anorexia and other signs of ill-health. All procedures were performed according to the Marshall University Institutional Animal Care and Use Committee guidelines.

Materials

Bax (#2772), Bcl2 (#2870s), phosphor p38 MAPK $^{Thr180/Tyr182}$ (#9216), p38 MAPK (#9212), phospho p44/42 MAPK $^{Thr202/Tyr204}$ (#9102), p44/p42 MAPK (#9102), phospho SAPK/JNK $^{Thr183/Tyr185}$ (#9251s), SAPK/JNK (#9252), Caspase3 (#9662) were purchased from Cell Signaling Technology(Beverly, MA). HeLa whole cell lysate (sc-2200) and L6+ IGF lysate (sc-24727) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhance chemiluminescence (ECL) western blotting detection reagent (#RPN2108) was purchased from Amersham Biosciences (Piscataway, NJ) and restore western blot stripping buffer (#21063) was obtained from Pierce

(Rockford, IL). Protease (#P834) and phosphatase inhibitors (#P5726) were purchased from Sigma-Aldrich, Inc., St. Louis, MO. All other chemicals that were used for this study were purchased from Fisher Scientific (Hanover, IL).

Characterization of nanoceria

Cerium oxide nanoparticles used for this study were purchased from Sigma-Aldrich (St Louis, MO). The particles used in this study were approximately 20nm in size and were characterized using a Hitachi Model S-4800 Field Emission scanning electron microscope (Schaumburg, IL, USA) at 5 and 20 kV.

Nanoceria instillation and tissue collection

Nanoceria instillation was performed as detailed previously (48). Animals were observed for recovery from anesthesia before being placed into the cages. Animals were humanely sacrificed at either 1, 3, 14, or 28 days following exposure. Kidneys were removed of their capsules, trimmed of connective tissue and rapidly weighed. A small portion of each kidney was preserved in 10% formalin for histological purpose and the remaining tissue was frozen in liquid nitrogen and stored at -80 °C for subsequent use.

Induction Coupled Plasma- Mass Spectrophotometry

Nanoceria content in Kidney was estimated by induction coupled plasma-mass spectrometry (ICP-MS) at Elemental Analysis Inc (Lexington, KY) according to the standard protocol (21). Kidney samples (n = 4 per group) were prepared using EPA method 3050B for the

analysis of total cerium by ICP-MS. A 2.5 g sample was weighed and digested with concentrated nitric acid, 30% hydrogen peroxide, and concentrated hydrochloric acid. Blank, control sample, a laboratory duplicate, and a predigestion matrix spike were prepared for each sample. After digestion, the extracts and the quality control samples were diluted to a final volume of 50 mL before analysis using an Agilent 7500cx ICP-MS. The instrument was calibrated for Ce-140 with 0, 0.1, 1.0, 10.0, and 100 µg/L standards prepared from a certified reference standard traceable to National Institute of Standards and Technology reference materials. A second source calibration verification standard traceable to National Institute of Standards and Technology reference materials was analyzed to verify the calibration standards. A continuing calibration verification standard and a continuing calibration blank were analyzed at the beginning of the run, after every ten samples, and at the conclusion of the run.

Estimation of blood urea nitrogen and creatinine

Blood was collected by cardiac puncture into a serum collection tube (BD Vacutainer®) and centrifuged at 800× g for 15 minutes. Serum was collected and used for biochemical assays using an Abaxis VetScan® analyzer (Abaxis, UnionCity, CA). Serum biochemical parameters, ie, blood urea nitrogen and creatinine were evaluated with a Comprehensive Diagnostic Profile Disk.

Hematoxylin and Eosin Staining

Paraffin blocks from each kidney were sectioned (4-6 microns), collected on glass slides and allowed to dry before placing in an oven at 60$^{\text{o}}$C for 45 minutes. Sections were

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deparaffinized with xylene for 10 minutes followed by dehydration with ascending grades of alcohol. Sections were then washed with distilled water three times and rinsed with phosphate buffer saline twice. Sections were stained with hematoxylin for 1 minute followed by rinsing in running tap water for 15 minutes and decolorization with acid alcohol. The sections were then stained with eosin for 10 minutes and washed with distilled water. Images were taken using bright field microscope (Olympus) at 40X to evaluate kidney morphology.

Immunoblotting

Kidney slices (100 - 150 mg) were homogenized in T-PER lysis buffer for 1 minute followed by sonication at 10w (4 x 30 sec cycles) as outlined previously (48). Protease and phosphatase inhibitors were added to T-PER to prevent protein degradation. Protein extracted from each sample was quantitatively estimated by 660 nm assay (Thermo Scientific, Rockford, IL) in triplicate. Samples were diluted to a final concentration of 2.5 µg/ml using 2X Lamelli buffer and then boiled for 5 minutes. Forty micrograms of protein were loaded onto 10% PAGEr Gold Precast gels (Lonza, Rockland, ME). Separated proteins were transferred onto nitrocellulose membrane and stained with a RAPID Stain protein stain reagent (G-Biosciences, St. Louis, MO, USA) to ensure equal loading of protein in all lanes. Membranes were blocked with 5% milk in Tris Buffered Saline (TBS) containing 0.05% Tween-20 (TBST) and determination of equal loading between lanes and membranes were determined by using beta actin as loading control. Protein detection was performed as outlined by the antibody manufacturer while immunoreactive bands were visualized with ECL (Amersham Biosciences). Exposure time was adjusted at all times to keep the integrated optical densities within a linear and non-saturated

range, and band signal intensity was quantified by using imaging software (AlphaEaseFC). Biotinylated ladder (Cell Signaling) was used as molecular mass standards and Hela Whole cell lysates along with L6 + IGF were included as positive controls. Membranes were stripped and re-probed for beta actin for direct comparisons between the concentration levels of different signaling molecules, with Restore western blot stripping buffer as detailed by the manufacturer (Pierce, Rockford, IL).

Statistical analysis

Results are presented as mean ± SEM. Data were analyzed by using the Sigma Stat 11.0 Statistical program. Data were analyzed using a one-way ANOVA followed by the Student Newman-Keuls post-hoc testing when appropriate. $P < 0.05$ was considered to be statistically significant.

RESULTS:

Morphological changes in kidney structure with nanoceria treatment

Exposure to cerium oxide nanoparticles was not associated with any significant change in the kidney to body weight ratio ($P<0.005$.,Table 1). Similarly, hematoxylin and eosin staining did not reveal any significant change in kidney histology following cerium oxide nanoparticle treatment (Figures 1 and 2).

Nanoceria content in Kidney

Amount of nanoceria at 90 days after instillation of nanoceria was determined through ICP-MS. The control group had no detectable levels of cerium oxide while the treated day 90 group had 0.14 ± 0.02 ppm of cerium oxide nanoparticles. Estimation of nanoceria in earlier treated groups were not performed based on the absence of changes in kidney structure and function (Table 2).

Serum biochemical markers for kidney damage

Levels of blood urea nitrogen and creatinine were estimated from serum which indicates the functional activity of kidney. Compared with control animals, nanoceria instillation did not alter the levels of BUN nor creatinine in animals exposed to nanoceria after 1, 3, 14, 28 days ($P<0.005$.,Table 3).

Alterations in molecular markers of oxidative stress

Mitogen-activated protein kinases are stress activated serine/threonine-specific protein kinases that are involved in the regulation of gene expression, cell survival, and apoptosis (27). Increased levels of activated MAPK members are suggestive of oxidative stress. Compared to control animals, cerium oxide exposure did not alter the ratio of phosphorylated p38 MAPK to total p38 MAPK (Figure 3). Conversely, exposure to cerium oxide nanoparticles for 1, 3, 14 and 28 days decreased the ratio of phosphorylated to total p44/42 MAPK by 19%, 19%, 10%, and 25%, respectively ($P<0.05$, Figure 4). Similarly, cerium oxide nanoparticle exposure decreased

the ratio of phosphorylated to total JNK by 27%, 44%, 42% and 45% at days 1, 3, 14, and 28 (P<0.05; Figure 5).

Expression of apoptotic markers

The ratio of Bax to Bcl2 is an important determinant of intrinsic pathway of apoptosis which results from unregulated increase of mitochondrial membrane permeability. An increase in ratio of Bax/Bcl2 signifies mitochondrial damage which leads to death of cell. Compared to control animals, exposure to cerium oxide nanoparticles decreased the ratio of Bax/Bcl2 ratio by 44%, 59%, 31%, and 20% in day 1, 3, 14, and 28 exposure groups (Figure 6). These changes in the Bax/Bcl2 ratio were not associated with evidence of increased caspase 9 (Figure 7) however caspase 3 activity was significantly decreased in the 28 day exposure group (Figure 8).

DISCUSSION

It is essential to know if the inhalation of cerium oxide nanoparticles was associated with changes in kidney structure and function. Previous work has suggested that cerium oxide nanoparticles can cause lung fibrosis and liver damage (2, 48). The data obtained so far indicate that the inhalation of cerium oxide nanoparticles (7 mg/kg body weight) does not cause significant damage to structure of Sprague Dawley rat kidney up to 28 days after exposure. In addition, we also observed that exposure to cerium oxide nanoparticles did not result in MAPK activation and the induction of cellular apoptosis. Taken together these data suggest that cerium oxide nanoparticles do not exhibit toxic effects to the rat kidney.

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Toxicological effects of nanoceria on human and animal health are a major concern to be addressed due to their widespread usage in commercial products. We did not find any effect of exposure to cerium oxide nanoparticles on kidney weight (Table 1) or kidney function and structure as determined through absence of changes in levels of blood urea nitrogen, creatinine in serum (Table 3) and inflammatory foci by hemtoxylin and eosin staining (Figures 1, 2). These data are consistent with the possibility that the inhalation of cerium nanoparticles is not associated with bioaccumulation in the kidneys. In contrast to the present study, work conducted by Asati et al. (2010) demonstrated that nanoceria are internalized into lysosomes of cultured kidney (HEK293) cells where they can cause cytotoxic (6). Why we did not see toxicity *in vivo* is currently unclear but may be due to the possibility that nanoceria are not able to leave the blood and accumulate in kidneys because of their size or charge (16). Further investigation to determine if nanoceria can accumulate in the kidney may be warranted to better understand potential cause and effect relationships.

It is currently unclear whether nanoceria exposure is associated with diminished (18, 20, 28, 37, 57) or increased oxidative stress (48, 64). Herein, we examined the phosphorylation of MAPK proteins as a surrogate marker of oxidative stress (59). Our analysis of p38 MAPK phosphorylation did not reveal any significant differences between the nanoceria exposed and control animals (Figure 3). Conversely, the ratio of phosphorylated to total p44/42 MAPK was considerably decreased with cerium oxide nanoparticle exposure compared to that observed in the control animals (Figure 4). Similar findings were also found with respect to the ratio of phosphorylated to total JNK levels (Figure 5). Our findings are in contrast to the work of Eom and Choi who demonstrated increased MAPK activation in BEAS-2B cells following exposure to

cerium oxide nanoparticles (26). Why differences may exist between studies is currently unclear but may be related to differences in cell type or type of cerium oxide nanoparticle used.

Reactive oxygen species, if excessive, can overwhelm anti-oxidant defenses mechanisms which can cause apoptotic cell death and organ failure (3, 61). It is well known that intrinsic pathway of apoptosis is the general mechanism of cell death through unregulated increase of mitochondrial membrane permeability characterized by higher Bax to Bcl2 ratio (33). Herein, we found decreased levels in the ratio of Bax / Bcl-2 in different time points of nanoceria exposed groups compared to controls which are in concomitant to changes in MAPK members that we observed (Figure 6). This observation is in contrast to the work of Sarkar et al., (2011) who demonstrated an increase in Bax / Bcl-2 ratio following the exposure of kidney cells to copper nanoparticles and to bulk copper (31).

To extend upon our finding showing a diminished Bax/Bcl-2 ratio following cerium oxide nanoparticle exposure we next examined cellular isolates for evidence of caspase 9 and caspase 3 activity. These biomarkers were chosen for analysis on the basis of previous work showing that caspase-3 is an end stage markers for apoptotic cell death (25). Our analysis of caspase 9 by immunoblotting did not reveal any statistical differences between the control and nanoceria exposed groups (Figure 7). Nonetheless, the activity of caspase 3 was slightly lower in the protein isolates from animals that had been exposed to cerium oxide nanoparticles for 28 days (Figure 8). Taken together, these data, like our findings for Bax/Bcl-2 suggest that the inhalation of cerium oxide nanoparticles is not associated with increased renal cell death.

The data of the current study demonstrate that the inhalation of cerium nanoparticles in male Sprague Dawley rats is not associated with renal damage for at least the first 28 days of

exposure. Why our findings may differ from previous work is currently unclear but may be related to differences in model used (*in vivo* vs. *in vitro*), dosage or degree of exposure, type of cerium oxide nanoparticle examined or the time point of observation. Given these uncertainties, further evaluation may be warranted before reaching a final recommendation regarding the potential renal toxicity of cerium oxide nanoparticle exposure.

Table 1: Effects of nanoceria exposure on kidney and body weights

Animals	Kidney weight (gms)	Body weight (gms)	Kidney to body weight ratio
Controls	2.91 ±0.13	319.67 ± 6.49	0.91 ± 0.033
1 day exposure	2.73 ± 0.13	319.67 ± 6.2	0.871 ± 0.031
3 day exposure	2.95 ± 0.12	331.67 ± 9.83	0.892 ± 0.035
14 day exposure	3.10 ± 0.14	332.33 ± 8.60	0.934 ± 0.031
28 day exposure	3.22 ± 0.12	403.66 ± 11.81	0.797 ± 0.016

Values are expressed as mean ± SEM

Table 2: Nanoceria concentration in kidneys

Control Group	0ppm
90 day cerium oxide instilled group	0.14±0.02ppm

Nanoceria content as determined through ICP-MS

Table 3: Effects of nanoceria exposure on levels of BUN and creatinine in serum

Animals	BUN (mg/dl)	Creatinine (mmg/dl)
Controls	17.2±0.3	0.3±0.0
1 day exposure	17.4±0.7	0.3±0.0
3 day exposure	16.5+0.9	0.2±0.0
14 day exposure	16.8±1.0	0.3±0.0
28 day exposure	18.8±0.4	0.3±0.0

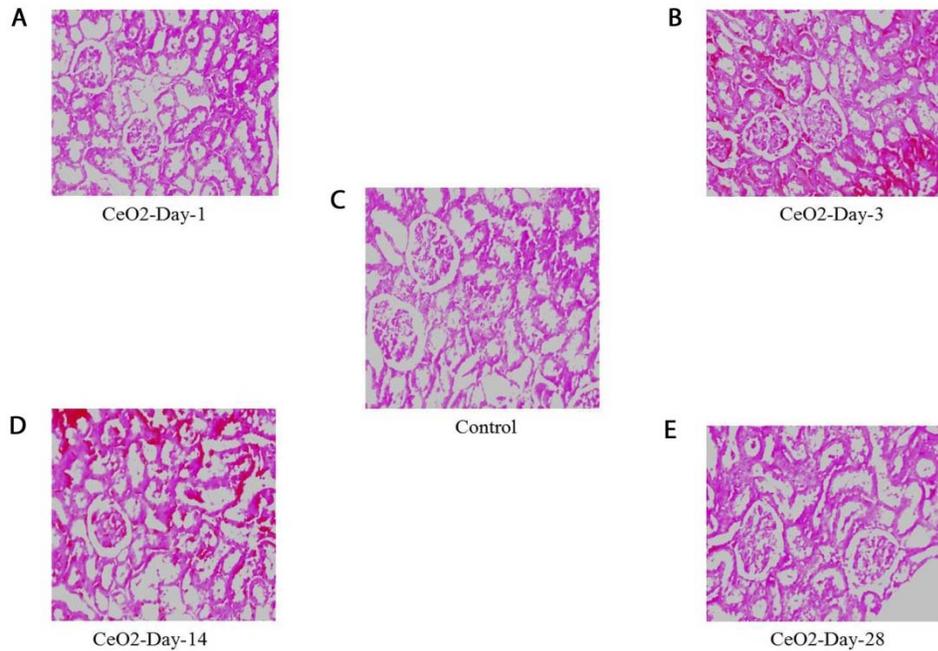**Values are expressed as mean ± SEM**

Figure 1: Nanoceria exposed Kidney morphology as determined though H and E staining at 20x

Changes in nanoceria exposed kidneys morphology as determined through hematoxylin and eosin staining at 20x. Hematoxylin and eosin staining did not reveal any significant changes in morphology of kidneys after 1, 3, 14, or 28 days exposure to 7 mg / kg body weight of nanoceria as against controls.

Figure 2: Nanoceria exposed Kidney morphology as determined though H and E staining at 40x

Changes in nanoceria exposed kidneys morphology as determined through hematoxylin and eosin staining at 40x. Hematoxylin and eosin staining did not reveal any significant changes in morphology of kidneys after 1, 3, 14, or 28 days exposure to 7 mg / kg body weight of nanoceria as against controls.
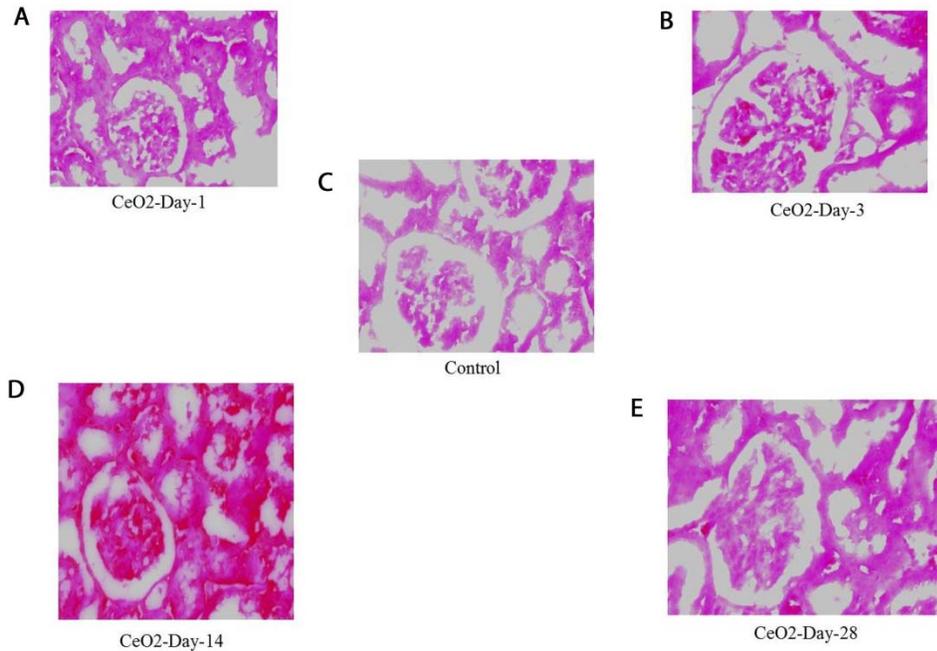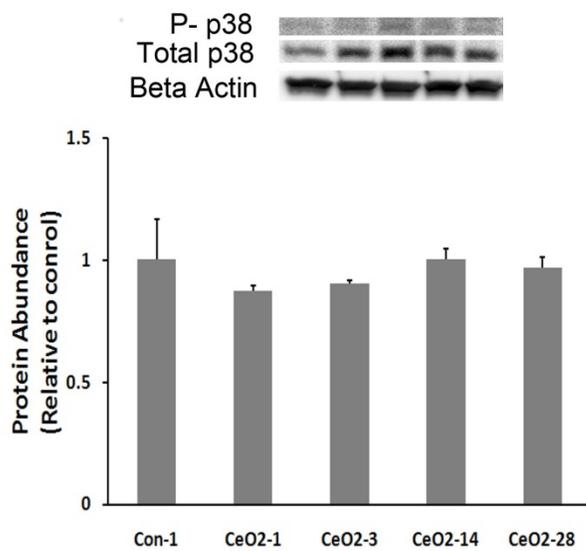
Figure 3: Effects of nanoceria exposure on p38 MAPK signaling pathway

Inhalation of cerium oxide nanoparticles does not activate p38 MAPK signaling. Results are expressed as arbitrary units for comparison.
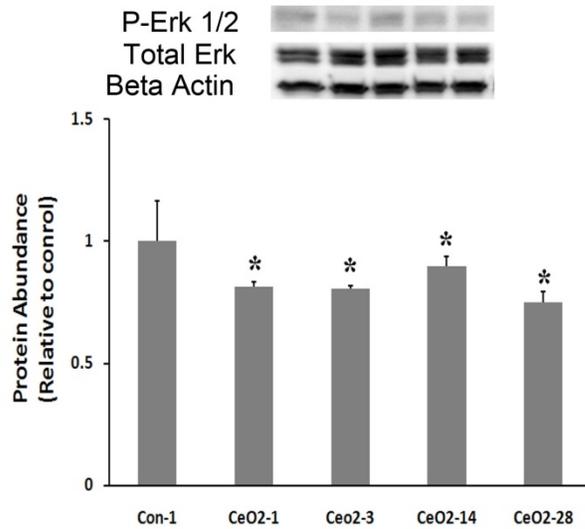
Figure 4: Effects of nanoceria exposure on p44/42 MAPK signaling pathway

Inhalation of cerium oxide nanoparticles is associated with diminished p44/42 MAPK activation. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in nanoceria exposed animals from the controls ($P < 0.05$).
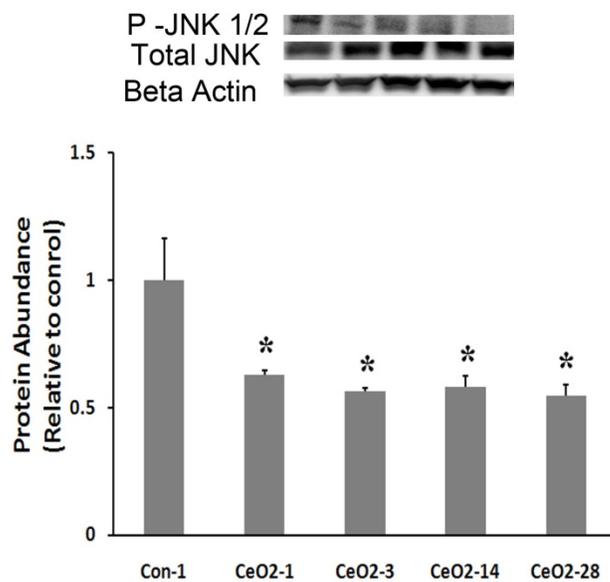
Figure 5: Effects of nanoceria exposure on JNK MAPK signaling pathway

Inhalation of cerium oxide nanoparticle is associated with diminished JNK MAPK activation. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in nanoceria exposed animals from the controls (P< 0.05).
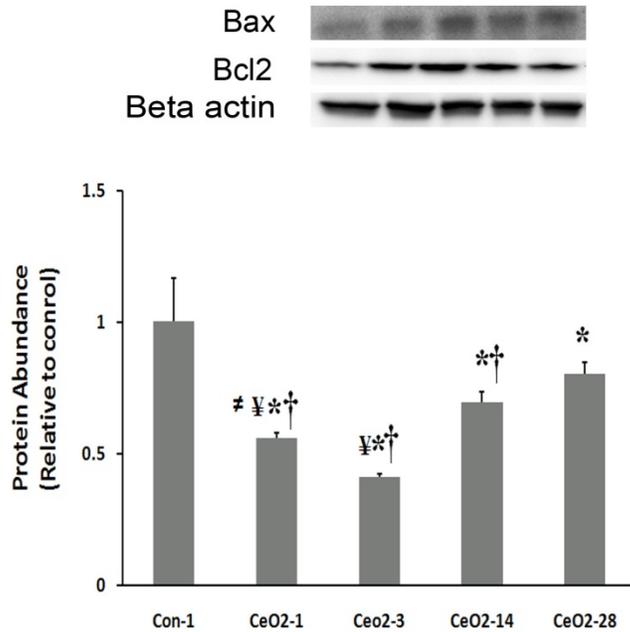
Figure 6: Effects of nanoceria exposure on Bax / Bcl-2 expression

Effect of cerium oxide nanoparticle inhalation on renal Bax / Bcl-2 expression. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in nanoceria exposed animals from the controls (P< 0.05). A dagger (†) indicates significant differences in Day 1, Day 3, Day 14, groups when compared to Day 28 group (P< 0.05). A yen (¥) indicates significant differences in Day 1, day 3 groups when compared to Day 14 group. (P< 0.05). A not equal to (≠) indicates significant differences in Day 1 when compared to Day 3 group exposed to nanoceria. (P< 0.05).

Figure 7: Effects of nanoceria exposure on caspase 9 levels

Effect of cerium oxide nanoparticle inhalation on renal caspase 9 levels. Results are expressed as arbitrary units for comparison.

Figure 8: Effects of nanoceria exposure on caspase 3 levels

Effect of cerium oxide nanoparticle inhalation on renal caspase 3 levels. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in nanoceria exposed animals from the controls (P< 0.05).
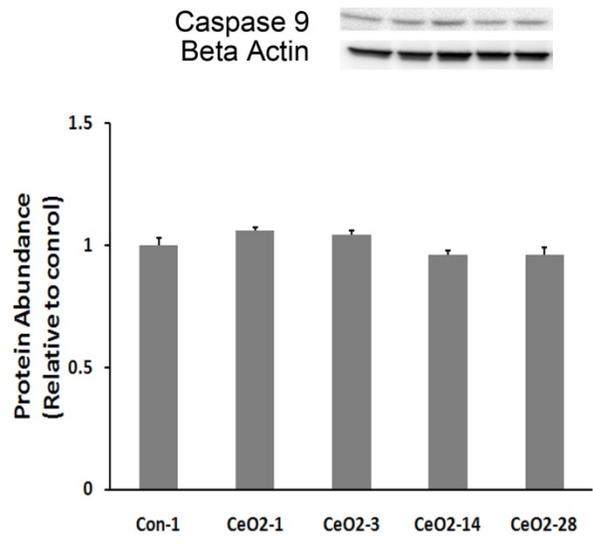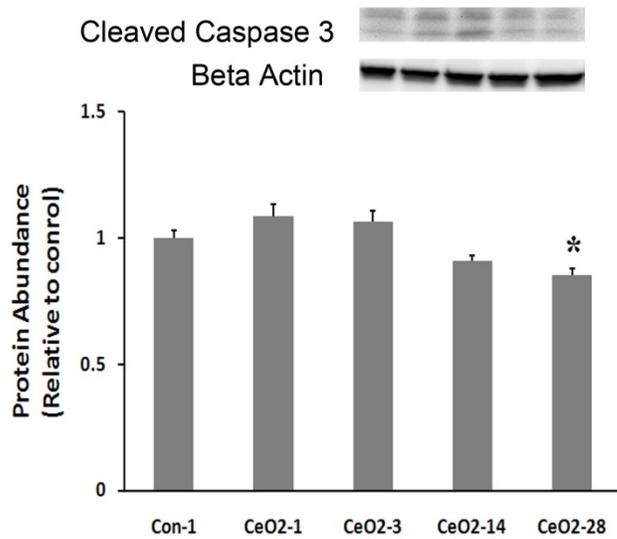
CHAPTER 4

Conclusions

Cerium oxide is a rare earth metal which is being exploited commercially for various industrial applications in its nano-particuluate form. Debate currently exists over its anti-inflammatory properties and whether it has application for therapeutic purposes or for the treatment of disease. Whereas some studies demonstrate that nanoceria is a potential ROS scavenger, other work has demonstrated that nanoceria at higher concentrations may be associated with increases in oxidative stress. Herein, we examined whether the inhalation of cerium oxide nanoparticles is associated with the development of oxidative stress in the kidneys of Sprague-Dawley rats. Our results indicate the following:

1. The intratracheal instillation of nanoceria does not alter the weights of kidneys compared to non-exposed animals.

2. The intratracheal instillation of nanoceria at a dosage of 7 mg / kg body weight does not alter kidney morphology for at least 28 days after exposure as assessed by histological staining.

3. The intratrachael instillation of nanoceria does not alter the serum biochemistry profile kidney function markers like BUN and Creatinine.

4. The intratracheal instillation of nanoceria is associated with diminished activation of p44/42-MAPK and JNK-MAPK.

5. The intratracheal instillation of nanoceria appears to reduce the ratio of Bax / Bcl2 and the expression of caspase 3.

Future Directions

Future directions based on this study could be centered on further studies to evaluate the toxicological potential of nanoceria on the kidneys. It is well known that particle size and charge can affect nanoparticle uptake into cells (6). Taking this into account, future research should evaluate the effect of nanoceria size and charge on kidney uptake and toxicity.

It is thought that nanoparticles remain resident once taken up by the cell. Other studies have suggested that the organs of the body tend to bioaccumulate nanomaterials over time (65). A limitation of the current study is that we only evaluated the potential toxicity of ceria for a relatively short time (28 days). Future studies could employ longer time points to evaluate if chronic exposure (6 months- 2 years) to cerium nanoparticles might induce different findings. Other work could examine different concentrations of nanoparticles to determine if there is a dose dependent effect on cellular function.

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