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Mechanisms of Yttrium Oxide Toxicity in HEK293 Cells

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By

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ABSTRACT

As a non-metal oxide, yttrium oxide (Y_2O_3) nanoparticles have numerous applications in chemical synthesis, mechanical polishing and as additives to drugs, cosmetics, varnishes and food. Recent data have suggested that these particles are capable of inducing oxidative stress and cytotoxicity in human endothelial cell lines. To examine the potential mechanisms of yttrium oxide toxicity, human embryonic kidney (HEK293) cells were exposed to 1, 5, 10, 50 and 100 µM of Y_2O_3 nanoparticles for 12, 24, 36 or 48 hr. We hypothesized that exposure of HEK293 kidney cells to Y_2O_3 nanoparticles would be associated with increased evidence of intracellular oxidative stress and cell death. Our data suggested that exposure to Y_2O_3 nanoparticles was associated with time and dose dependent decrease in cell viability and evidence of increased cellular superoxide levels. Immunoblotting of protein isolates demonstrated that changes in cell viability were associated with alterations in protein kinase B (Akt), Bax / Bcl-2 and caspase-3 expression. Taken together, these data suggest that Y_2O_3 nanoparticle exposure may be associated with diminished cell viability and that these alterations are associated with increased oxidative stress and alterations in cellular signaling.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
ECL	Enhanced chemiluminiscence
FBS	Fetal bovine serum
HE	Hydroethidium
HEK	Human Embryonic Kidney
MTT	3-4, 5-Dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.5% tween-20
PKB	Protein kinase B
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST	Tris buffered saline with 0.5% tween-20
TUNEL	TdT-mediated dUTP nick end labeling

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Mechanisms of yttrium oxide toxicity in HEK293 cells

CHAPTER 1

Introduction

Nanoparticles have structural features with at least one dimension in the 1–100 nm range [1]. Nanoparticles often times possess unique chemical, mechanical, electrical, optical, magnetic, and biological properties that make them ideal candidates for a variety of novel commercial and medical applications. Engineered nanoparticles are commonly produced in a wide variety of types, including metal and metal oxide particles, polymer nanoparticles, and quantum dots [2]. Current nanoparticle applications include the use of these materials for drug delivery systems, energy conservation, and consumer products [2]. Although exhibiting tremendous potential for the development of new materials and processes, it is currently unclear whether nanomaterials may be toxic to the environment. This gap in our understanding is important given the rapidly developing commercialization of nanoparticles both here in the United States and abroad [3].

Exposure to nanoparticles could occur through oral, dermal, inhalation, and injection routes, all of which could potentially initiate adverse biological reactions [4]. The unique physiochemical properties of nanoparticles (i.e., shape, particle size, agglomeration state, crystal structure, chemical composition, surface area, surface chemistry, surface charge, and porosity) are most likely responsible for the biological activity that occurs in response to nanoparticle exposure [2]. Specifically, nanoparticles of smaller size have a greater potential for biological interaction because these particles have a greater surface area per unit mass [5].

As a non-metal oxide, yttrium oxide (Y_2O_3) nanoparticles have numerous applications in chemical synthesis, mechanical polishing and as additives to drugs, cosmetics, varnishes and food. In recent years, the use of Y_2O_3 nanoparticles has been extended to biomedical and biotechnological fields where these particles have been used for the quantification of different biomolecules and as potential delivery platforms for drug delivery[5].

The effects of Y_2O_3 nanoparticles on biological systems are not well understood.Y_2O_3 nanoparticles are able to rescue cells from oxidative stress because of antioxidant properties in neuron cells. Whether Y_2O_3 nanoparticles exhibit similar effects in other cell types is not well understood. Given the possibility that inhaled nanoparticles may penetrate the lungs and become deposited in extra-pulmonary tissues [6] and the role that the kidneys play in detoxification of the body, we sought to determine whether exposure to Y_2O_3 nanoparticle elicits a toxicological effect on the human embryonic kidney cell line (HEK293). This cell line was selected for study on the basis of previous reports demonstrating that these cells can be used to model toxicity in the human kidney [7, 8].

Purpose and hypothesis

The purpose of this study is to assess the toxic effects of yttrium oxide on kidney cells. We hypothesized that exposure of HEK293 kidney cells to Y_2O_3 nanoparticles would be associated with increased evidence of intracellular oxidative stress and cell death. To test this hypothesis the following two specific aims were pursued:

Specific Aim 1: To determine the toxic dose and time course of HEK293 cell death following exposure to Y_2O_3 nanoparticles.

Specific Aim 2: To determine if yttrium oxide induced cell death in the HEK293 cell line is associated with elevations in oxidative stress.

CHAPTER 2

Review of literature

Introduction

A review of the relevant literature concerning this study will be presented. The following areas of interest will be addressed: 1) What is nanotechnology? 2) Potential impact of nanotechnology, 3) Potential effects of nanoparticles on cellular structure and function, 4) Yttrium oxide nanoparticles, 5) Regulation of cellular toxicity and apoptosis and 6) Yttrium oxide toxicity and apoptosis.

What is nanotechnology?

Nanotechnology is the study of manipulating matter on an atomic and molecular scale. Generally, nanotechnology deals with developing materials or other structures possessing at least one dimension sized from 1 to 100 nanometers [1]. Nanotechnology is diverse, ranging from extensions of conventional device physics to completely novel approaches based upon molecular self-assembly, to developing new materials with dimensions on the nano scale to investigating whether we can directly control matter on the atomic scale. Nanotechnology requires the application of fields of science as diverse as surface science, organic chemistry, molecular biology, and semi-conductor physics. There is much debate on the future implications of nanotechnology. Nanotechnology may be able to create many new materials

with a vast range of applications, such as in medicine, biomaterials and energy production. On the other hand, nanotechnology raises many of the same issues as any new technology, including concerns about the toxicity and environmental impact of nanomaterials [1].

Potential impact of nanotechnology

There are possible hazards that arise with the development of nanotechnology. The Center for Responsible Nanotechnology suggests that novel developments could result, among other things, in untraceable weapons of mass destruction, networked cameras for use by the government, and weapons developments fast enough to destabilize arms races [9]. One area of concern regarding the application of nanomaterials is the effect that industrial-scale manufacturing and use of nanomaterials would have on human health and the environment [10]. Some groups such as the Center for Responsible Nanotechnology have advocated that nanotechnology should be specially regulated by governments for these reasons [11]. Others counter that overregulation would stifle scientific research and the development of innovations that could greatly benefit mankind [12].

Manufactured nanoparticles display physicochemical characteristics and coatings that impart upon them unique electrical, thermal, mechanical, and imaging properties that are highly desirable for applications within the commercial, medical and environmental sectors. Potential occupational and public exposure to manufactured nanoparticles is expected to increase dramatically in the near future as more and more consumer products begin to incorporate the use of nanomaterials [13].

Potential effects of nanoparticles on cellular structure and function

It is well known that nanoparticles can enter cells and react with subcellular structures [14]. Whether the cells retains the particles, or whether the particles trigger or catalyze chemical reactions, depends on the chemical composition and size of the particle [14]. In many cases, it is thought that the particles are able to freely reside in the cell where they may interact with different cellular organelles. For example, C60-fullerene particles have been shown to distribute throughout the cell including inside the nucleus [15]. Experimentally, nanoparticles have been shown to segregate to the cell membrane, cytoplasm [16, 17], mitochondria [18, 19], in lipid vesicles [16, 19], on the nuclear membrane [16] and within the nucleus [16, 18]. The mechanism(s) that control nanoparticle localization within the cell are currently not well understood.

Additional studies have shown that free radical formation can be triggered by nanoparticles such as fullerenes, carbon nanotubes, quantum dots, or exhaust emission particles [20]. The resulting overproduction or the chronic production of reactive oxygen species (ROS) can damage DNA, proteins and lipids, which then influence cellular processes [20]. Nanoparticles can also affect the fundamental cell functions and cell physiological processes like cell proliferation, cell metabolism and cell death [21]. It has been shown that carbon nanotubes can alter cell proliferation, apoptosis and other aspects of cellular homeostasis [22]. Interestingly, it appears that these changes are not only concentration dependent but they also depend on the size and purity of the nanomaterials used [23]. Other work has indicated that cellular response may also be cell type dependent [21] suggesting that nanoparticle toxicity is dependent upon a variety of different factors.

Yttrium oxide nanoparticles

Recent data have suggested that Y_2O_3 nanoparticles are able to rescue cells from oxidative stress-induced cell death in a manner that appears to be dependent upon the structure of the particle and one that is largely dependent of particle size [24]. It is thought that this effect may be due to the ability of yttrium oxide particles to protect the cell from oxidative stress. How this may occur is not well understood as it is possible that the Y_2O_3 nanoparticles may act directly as an antioxidant, or, alternatively, they could also inhibit the activity of enzymes that function to generate reactive oxygen species [24, 25].

Regulation of cellular toxicity and apoptosis

Phosphoinositide signaling is an important signal transduction system in the last decade or so [26]. It is well accepted that lipid molecules can function as second messengers and that these molecules may be involved in regulating a wide range of cellular responses. Recent data suggest that the phosphatidylinositol 3-kinase (PI3-K) plays a key role in cell signaling and that it is involved in the regulation of cell proliferation, survival, motility and secretion [27, 28]. One product of the PI3-K molecule is PIP3. PIP3 is an important lipid second messenger, which is involved in the regulation of downstream PI3-K signaling through its ability to activate other protein kinases. A diverse group of Ser/Thr kinases are regulated downstream of PI3-K, including protein kinase C (PKC) and protein kinase B / Akt [28]. Akt is important in the maintenance of cell survival as it is involved in the regulation of the anti-apoptotic molecules Bcl-2 and Foxo-1 [29, 30]. The activity of Akt has been shown to be regulated by

phosphorylation [31]. How yttrium oxide exposure may regulate Akt signaling has, to our knowledge, not been investigated.

Apoptosis is a mechanism wherein cells die gradually in an orderly fashion. Apoptosis is regulated by both intrinsic and extrinsic signaling pathway [32]. It is thought that apoptosis is regulated, at least in part, by members of Bcl-2 family with Bcl-2 functioning to prevent apoptosis whereas Bax serves to promote apoptosis[33].

The extrinsic apoptosis pathway is activated when cytokines such as TNF-α bind to the receptors on cell surface [34]. The binding of TNF-α to its receptor results in the recruitment procaspase-8 to the cell membrane, which leads to the formation of a death inducing signaling complex. Conversely, activation of intrinsic apoptosis pathway is under the control of mitochondria and is regulated by elevations in oxidative stress, cell starvation, or changes in the amount of growth factors [35]. During stressful conditions mitochondria initiate apoptosis by releasing cytochrome c, which then causes the release of apoptotic protease - activating factor (Apaf-1) [18]. Apaf-1, in turn, causes the release of caspase-9, which initiates the activation of other caspases and eventually cell death.

Previous studies have shown that nanoparticles can both induce and prevent apoptosis. For examples, ceria nanoparticles are able to decrease the amount of reactive oxygen species seen after glutamate exposure, which has been shown to have a protective effect to cultured HT22 rodent nerve cells [24]. It is thought that this protective effect is due to the ability of the ceria nanoparticles to act as free radical scavengers [36]. It has also been demonstrated that ceria nanoparticles are neuroprotective to adult rat spinal cord neurons. Other work has shown

that ceria nanoparticles protect against the progression of cardiac dysfunction and remodeling by attenuation of myocardial oxidative stress [37].

Unlike ceria nanoparticles, carbon nanotubes, iron oxide and titanium oxide nanoparticles have been shown to cause cellular death [38]. Carbon nanotubes (CNTs) are allotropes of carbon with a cylindrical nanostructure. Nanotubes have been constructed with length-to-diameter ratio of up to 132,000,000:1 [39]. These cylindrical carbon molecules have unusual properties that may be of significant value for use in the electronics, optics and other fields of materials science and technology. The toxicity of carbon nanotubes has been an important question in nanotechnology. It has been shown that nanotubes can cross membrane barriers, and that these materials can induce harmful effects such as inflammatory and fibrotic reactions [40]. Numerous reports have examined the general toxicity of oxide nanomaterials; however, little data exist on how these nanomaterials may cause toxicity from a mechanistic perspective [41].

Yttrium oxide toxicity and apoptosis

Recent data suggest that exposure to Y_2O_3 nanoparticles can function to rescue cells from various forms of lethal stress [42]. Whether Y_2O_3 nanoparticles exhibit toxic or protective effect appears to depend on particle size, dose, and exposure time [43]. Other work has demonstrated yttrium oxide-induced apoptosis is governed, at least in part, by particle size. In this study our main focus is on how different concentrations of similar size yttrium oxide nanoparticles particles might affect the cellular response in HEK293 cells.

CHAPTER 3

Mechanisms of yttrium oxide toxicity in HEK 293 cells

Abstract

As a non-metal oxide, yttrium oxide (Y_2O_3) nanoparticles have numerous applications in chemical synthesis, mechanical polishing and as additives to drugs, cosmetics, varnishes and food. Recent data have suggested that these particles are capable of inducing oxidative stress and cytotoxicity in human endothelial cell lines. To examine the potential mechanisms of yttrium oxide toxicity, human embryonic kidney (HEK293) cells were exposed to 1, 5, 10, 50 and 100 µM of Y_2O_3 nanoparticles for 12, 24, 36 or 48 hr. We hypothesized that exposure of HEK293 kidney cells to Y_2O_3 nanoparticles would be associated with increased evidence of intracellular oxidative stress and cell death. Our data suggested that exposure to Y_2O_3 nanoparticles was associated with time and dose dependent decreases in cell viability and evidence of increased cellular superoxide levels. Immunoblotting of protein isolates demonstrated that changes in cell viability were associated with alterations in protein kinase B (Akt), Bax / Bcl-2 and caspase-3 expression. Taken together, these data suggest that Y_2O_3 nanoparticle exposure of similar size with different concentrations may be associated with diminished cell viability and that these alterations are associated with increased oxidative stress and alterations in cellular signaling.

Key words: Cyto toxicity, nanoparticles, AKT, MTT, oxidative stress, apoptosis.

Introduction

Research in the nanosciences is growing at a breathtaking pace. It is anticipated that the unique properties of nanomaterials and nanoparticles will allow the development of new products that will be of value in almost every field of human activity [28]. Although very promising, recent studies examining the biological effects of nanoparticles have demonstrated that some may exhibit an unexpected degree of toxicity to living organisms and humans [44-46].

As a non-metal oxide, Y_2O_3 nanoparticles have numerous applications in chemical synthesis, mechanical polishing and as additives to drugs, cosmetics, varnishes and food [47]. In recent years, the use of Y_2O_3 nanoparticles has been extended to biomedical and biotechnological fields where these particles have been used for the quantification of different biomolecules and as potential delivery platforms for drug delivery [5]. The effects of Y_2O_3 nanoparticles on biological systems and the effects these nanoparticles may have on different cell types are not well understood. Given the possibility that inhaled nanoparticles may penetrate the lungs and become deposited in extra-pulmonary tissues [6] and the role that the kidneys play in detoxification of the body we sought to determine whether exposure to Y_2O_3 nanoparticle can cause toxicological effect on the human embryonic kidney cell line (HEK293). This cell line was selected for study on the basis of previous reports demonstrating that these cells can be used to model toxicity in the human kidney [7]. To investigate the possible mechanisms of cytotoxicity, a variety of surrogate parameters were examined including the analysis of cellular morphology, the generation of reactive oxygen species (ROS), cell viability, and intracellular signaling. Our data suggest that Y_2O_3 nanoparticles induce cytotoxicity in

HEK293 cells in a dose and time dependent manner. This cytotoxicity appears to be associated with elevation in oxidative stress and activation of AKT, Caspase-3 and up regulation of Bax / Bcl-2 ratio in dose- dependent manner.

Materials and methods

Materials

HEK293 cells were obtained from American Type Cell Culture (ATCC). DMEM modified high glucose medium, fetal bovine serum, penicillin streptomycin antibiotics, trypsin, dimethyl sulfoxide (DMSO), MTT (3-4, 5-Dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide), and Y_2O_3 nanoparticles were purchased from Sigma-Aldrich (St. Louis, MO, USA). Akt (#9272), phospho-Akt (Ser473) (#9271), anti-rabbit secondary antibody (#7074) were from Cell Signaling Technology, Inc., Danvers, MA, USA. Enhanced chemiluminiscence (ECL) western blotting detection reagent was obtained from Amersham Biosciences (Piscataway, NJ). Restore western blot stripping buffer was purchased from Pierce (Rockford, IL). All other chemicals were purchased from Fisher Scientific (Hanover, IL).

Methods

Cell culture and nanoparticles treatment

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% heat-inactivated fetal bovine serum, penicillin (100 IU / ml), and streptomycin

(10 µg / ml). Cells were grown and maintained in 150 cm^2 cell culture flasks at 37 °C in a 5% CO2 humidified incubator.

Y_2O_3 nanoparticles (10 wt. % in water, average diameter 40 nm) were added to the culture media and dispersed by sonication (100 W x 20 min) before treatment. Potential agglomeration was assessed by dynamic light scattering using a Horiba LB-55 as outlined by the manufacturer.

Cell viability assay

Cell viability was measured by the MTT (3-(4-5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) assay as outlined elsewhere [48]. Briefly, cells (2×10^4) were seeded in 96-well tissue culture plates in 200 µl of media per well for 24 hr. At the end of the stabilization period, the medium was removed, and then replaced with serum free media containing either 1, 5, 10, 50 or 100 µM Y_2O_3 nanoparticles for either 12, 24, 36, or 48 hr. Experiments were performed in quadruplicate. Negative (untreated) and positive (50 µM paraquat) controls were included in all experiments. At the appropriate time period, 20 µl of MTT solution (2 mg / ml) was added to the media and the cells were incubated for 4 hr at 37 °C. Cells were treated with 200 µl of DMSO and the absorbance was quantified at 570 nm using a micro plate spectrophotometer (VersaMax, Molecular Devices, and Sunnyvale, CA, USA). Viability after treatment was expressed relative to the negative controls.

Dihydroethidium staining

Confluent HEK293 cells were treated with 1, 10 or 100 µM of Y_2O_3 nanoparticles for 24 hrs, washed with PBS, and 5µM dihydroethidium was added to the medium for 30 min. Superoxide levels were examined by fluorescent microscopy using an EVOS fluorescent microscope (Fisher scientific, IL, and USA).

TUNEL staining

Confluent HEK293 cells were treated with 1, 10 or 100 µM of Y_2O_3 nanoparticles for 24 hrs, washed with PBS, and then fixed with 4% paraformaldehyde. After washing with PBS (pH 7.4), cells were permeabilized with 0.1% sodium citrate and 0.1% Triton X. DNA fragmentation was determined by TdT-mediated dUTP nick end labeling (TUNEL) as outlined by the manufacturer. Fluorescent images were obtained using an EVOS fluorescent microscope (Fisher scientific, IL, USA)

Preparation of protein isolates and immunoblotting

Confluent HEK293 cells were treated with 1, 10 or 100 µM of Y_2O_3 nanoparticles for 24 hr, washed with PBS and trypsinized as outlined elsewhere [49]. Cells were collected and pelleted (500 x g for 3 min) and homogenized in TPER (10µl/mg) containing protease and phosphatase inhibitors for 5 min at 100 W using a PowerGen homogenizer (Fisher Scientific, IL, USA,). Samples were placed on ice 30 min and vortexed frequently before centrifugation (2,000 x g for 5 min at 4 °C). Protein concentration was determined in triplicate by the Bradford procedure [50].

Forty micrograms of total protein from each sample were separated using 15% SDS-PAGE and then transferred onto a nitrocellulose membrane using standard conditions [51]. Membranes were stained with Ponceau S, and the amount of protein quantified by densitometry to confirm successful transfer of proteins and equal loading of lanes. For immunodetection, membranes were blocked for 1 hr at room temperature in blocking buffer (5% non-fat dry milk in TBS-T (20 mM Tris-base, 150 mM NaCl, 0.05% Tween-20), pH 7.6), serially washed in TBS-T at room temperature and then probed with antibodies overnight at 4 ^{0} C in primary antibody buffer (5% BSA in TBS-T, pH 7.6, primary antibody diluted 1:1000). After washing in TBS-T (3 X 5 min each) membranes were incubated with an HRP conjugated secondary antibody in blocking buffer for 1 hr. After removal of the secondary antibody, membranes were washed (3 X 5 min each) in TBS-T and protein bands were visualized by ECL reagent. Target protein levels were quantified by AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Results are presented as mean ± SEM. Data were analyzed by using the Sigma Stat 3.0 Statistical program. Data were analyzed using a two-way ANOVA followed by the Student-Newman-Keuls post-hoc testing where appropriate. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Yttrium oxide exposure is associated with cytotoxicity and increased superoxide but not apoptosis.

Cell viability in response to Y_2O_3 nanoparticle exposure appeared to decrease in a time and concentration dependent manner (Figure 1). There is reduced cell viability with Y_2O_3 nanoparticle concentration of 100 µM at 48 hr. At 24 hr Y_2O_3 induced cytotoxicity is equal to that of paraquat. To examine whether exposure to Y_2O_3 nanoparticles was associated with changes in the amount of intracellular reactive oxygen species, we repeated the exposure experiments and then treated the cells with dihydroethidium, which is oxidized to ethidium and fluoresces in the presence of superoxide [52]. As expected, we observed what appeared to be a dose-dependent increase in the amount of ethidium reactivity (Figure 2). To determine if yttrium oxide exposure is associated with cellular apoptosis, we next examined cells that had been incubated with Y_2O_3 nanoparticles for the presence of double stranded DNA breakage using the TUNEL assay. Similar to our findings on cell viability, Y_2O_3 nanoparticle exposure appeared to be associated with a slight increase in TUNEL reactivity (Figure 3). To determine if elevation in oxidative stress is associated with AKT activation, which can be reflected in the form of cytoplasmic exportation of FOXO-3, we next examined cells that had been incubated with Y_2O_3 nanoparticles for the presence of FOXO-3 in cytoplasm. Corresponding to elevation in oxidative stress, there is translocation of FOXO-3 from the nucleus to the cytoplasm. Taken together these data suggest that yttrium oxide exposure in HEK293 cells is associated with diminished cellular viability that may be caused, at least in part, by elevations in superoxide.

Yttrium oxide exposure is associated with protein kinase B / Akt phosphorylation.

Compared to observations in the control (untreated cells) the amount of phosphorylated Akt levels was 17.0 ± 1.8%, 15.7 ± 0.8%, and 27.9 ± 0.8% higher in the HEK293 cells exposed to 1, 10, and 100 µM of Y_2O_3 nanoparticles, respectively ($P < 0.05$, Fig 5).

Yttrium oxide exposure is associated with increased caspase-3 cleavage and elevations in the ratio of Bax / Bcl-2.

Compared to control cells, the cleavage of caspase-3 was 11.52 ± 0.30 % in the HEK293 cells exposed to 10 µM of Y_2O_3 nanoparticles ($P <0.05$, Figure 6). Similarly, the ratio of Bax / Bcl-2 was 13.39 ± 0.28 %, 8.68 ±0.42% and 11.0 ± 0.36% higher in HEK293 cells exposed to 1, 10, and 100 µM of yttrium oxide nanoparticles, respectively ($P <0.05$, Figure 7).

Discussion

The potentially hazardous effects of nanoparticles are an emerging area of research to regulatory authorities [53-55]. Although Y_2O_3 nanoparticles exhibit the potential to act as antioxidants, whether these particles are cytotoxic is not well understood. Here we examine if different concentrations of Y_2O_3 nanoparticles are cytotoxic to HEK293 cells and the potential mechanism(s) of toxicity, if present. Our data suggest that exposure to Y_2O_3 nanoparticles results in diminished cell viability and elevations in cellular oxidative stress.

The results of viability tests showed that cell viability diminished in a concentration and time dependent fashion. Cytotoxicity appeared to be maximal at a concentration of 100 μM and after 48 hr (Figure 1). It has been known that the toxicity of nanoparticles is closely related to the concentration of the particles [56]. Whether a similar cytotoxicity to yttrium oxide exposure would be observed in other cell types is currently unknown but is worthy of future investigation.

Similar to what we observed for cell viability, it also appears that increased exposure to Y_2O_3 nanoparticles is associated with increases in cellular superoxide levels (Figure 2). It is thought that superoxide levels are an important regulator of cellular death [57] as excess superoxide levels have been shown to induce changes in mitochondrial membrane permeability, which, if allowed to proceed unchecked, can trigger the apoptotic process [58, 59]. How exposure to Y_2O_3 nanoparticles may cause an increase in cellular superoxide levels is currently unclear.

Given that elevations in oxidative stress can lead to cellular death [60] we next examined whether exposure of HEK293 cells to Y_2O_3 nanoparticles is associated with increases

in cellular apoptosis using the TUNEL assay. Consistent with our cell viability assays, we found that yttrium oxide exposure was associated with evidence of HEK293 apoptosis (Figure 3).

In an effort to understand the potential mechanism(s) responsible for these findings we next examined how exposure to Y_2O_3 nanoparticles affected the phosphorylation status of protein kinase B / Akt. The Akt is serine / threonine kinase, which is involved in the regulation of cell metabolism and cell death [61]. For these experiments we employed a two - pronged approach. In the first set of experiments we used HEK293 cells, which stabely expressed a GFP labeled FOXO-3 protein. Given that the cellular location of FOXO-3 is regulated by Akt activity, we hypothesized that this cell line would allow us to examine the functionality of Akt signaling in live cells under real world condition. After treatment of the HEK293 cells with different concentrations of Y_2O_3 nanoparticles, we observed what appeared to be an increase in amount of FOXO-3: GFP that was localized in the cytoplasm (Figure 4). This finding suggests that Y_2O_3 nanoparticle exposure is associated with increased activation (phosphorylation) of Akt. To confirm these findings, immunoblotting was performed. As expected, our data suggested that increased yttrium oxide exposure was associated with increased Akt phosphorylation (activation) (Figure 5). Given that Akt activation is strongly anti-apoptotic, we interpret these data to suggest that the phosphorylation of Akt is a compensatory response that is initiated to stave off the possibility of yttrium oxide-induced apoptosis.

A critical step in the execution of the apoptotic program is the fragmentation of caspase-3 into its 19- and 17- kDa fragments [62]. Caspases are cysteine-dependent aspartate specific proteases that function as endonucleases in execution of cell death [62, 63]. Compared to that observed in the control cells, we found that exposure of HEK293 cells to 10 μM of Y_2O_3

nanoparticles was associated with increases in the cleavage of caspase-3 whereas exposure to 1 or 100 μM was not (Figure 6). In an effort to explain this rather curious finding further, we next examined the ratio of Bax / Bcl-2 following Y_2O_3 nanoparticle exposure. It has been shown that cellular function is diminished as the Bax / Bcl-2 ratio increases [64]. Consistent with our cell viability experiments, we found that exposure to Y_2O_3 nanoparticles was associated with an elevation in the ratio of Bax / Bcl-2 (Figure 7).

In conclusion, this study suggests that exposure to yttrium oxide nanoparticles is associated with diminished cellular viability and increases in cellular superoxide levels and that these findings may be associated with increased cell death that may be dependent, at least in part, due to elevations in the Bax / Bcl-2 ratio. Future research examining other pathways or molecules is needed to better ascertain the exact mechanism(s) by which yttrium oxide exposure may affect cell function. Given our preliminary findings, additional studies may be warranted.

FIGURES

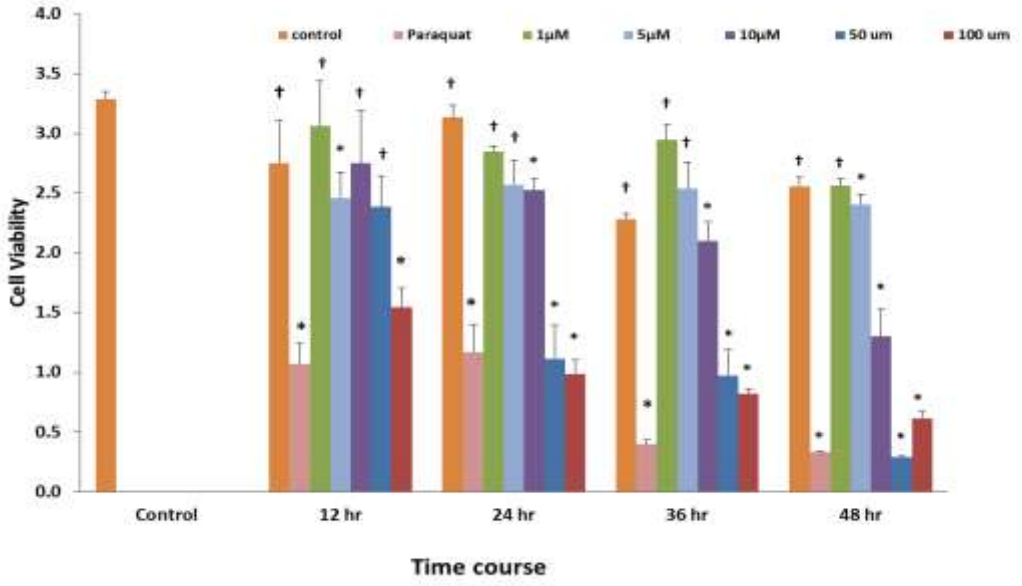

Figure 1. Effect of yttrium oxide nanoparticles on HEK293 viability.

Cell viability was assessed by the MTT assays after exposure to different concentrations of Y_2O_3 nanoparticles for 12, 24, 36 or 48 hr. * significantly different from the untreated negative controls. † Significantly different from paraquat control.

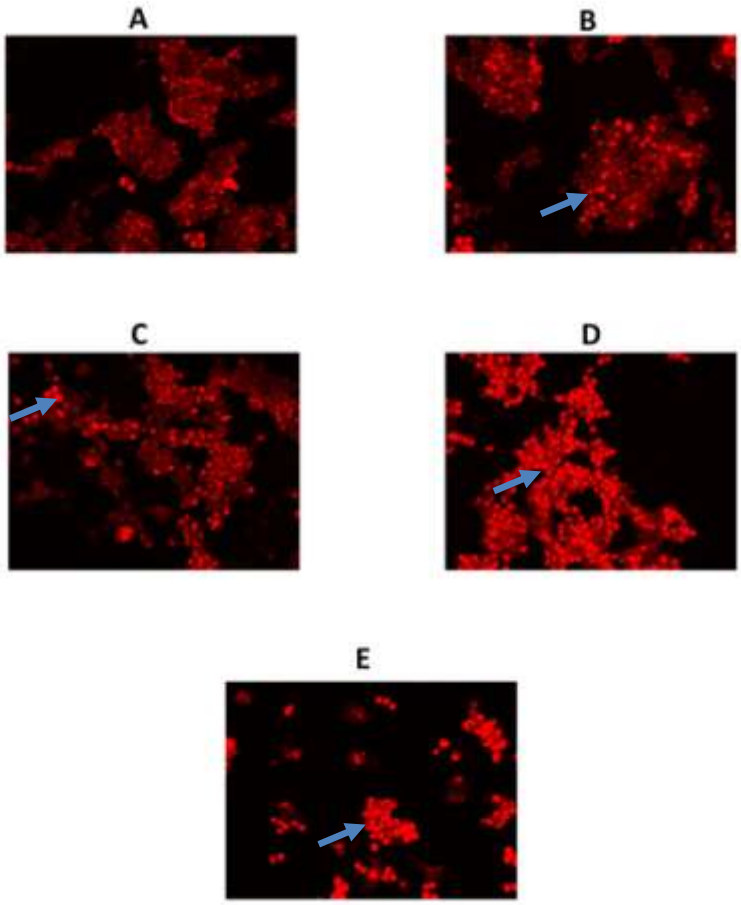

Figure 2. Exposure to yttrium oxide nanoparticles is associated with elevations in cellular superoxide levels. Cells were exposed to different concentrations of Y_2O_3 nanoparticles for 24 hrs prior to incubation with dihydroethidium and visualization under fluorescence (Indicated by arrow). An increase in the amount of fluorescence is proportional to increase in superoxide levels. Panel A: Control (no Y_2O_3), B: 1 µM Y_2O_3 , C: 10 µM Y_2O_3, D: 100 µM Y_2O_3, and E: Paraquat 50 µM. Cells were imaged at 200 X magnification.

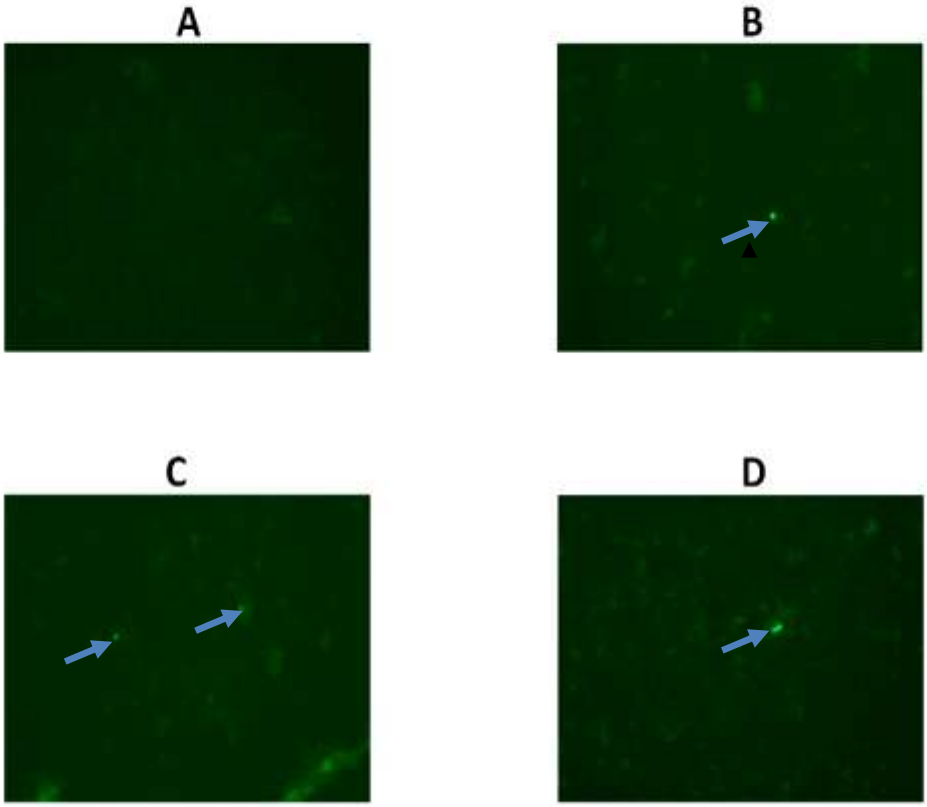

Figure 3. Exposure to yttrium oxide nanoparticles is associated with increased cellular apoptosis in HEK293 cells. Cells were exposed to different concentrations of Y_2O_3 nanoparticles for 24 hrs prior to TUNEL staining. An Increase in the amount of fluorescence in the cells indicates increased cellular apoptosis (Indicated by arrow). Panel A: Control (no Y_2O_3), B: 1 µM Y_2O_3 , C: 10 µM Y_2O_3, and D: 100 µM Y2O3. Cells were imaged at 200 X magnification.

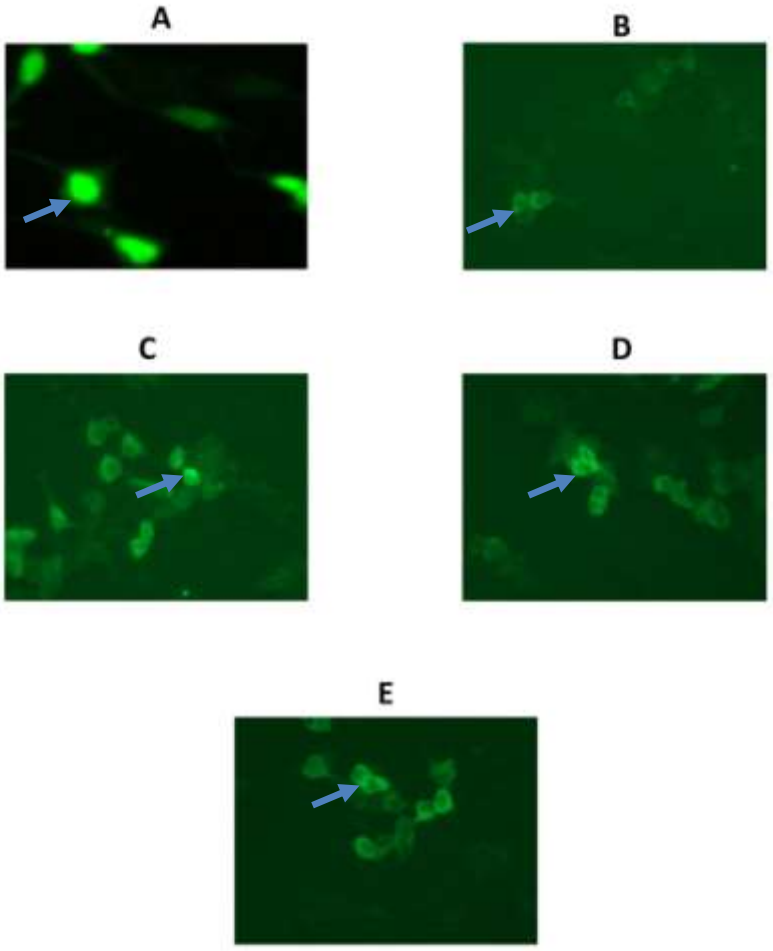

Figure 4. Exposure to yttrium oxide nanoparticles is associated with nuclear exportation of FOXO-3 gene. Cells were exposed to different concentrations of Y_2O_3 nanoparticles for 24 hrs prior to visualization of FOXO-3: GFP under fluorescence (Indicated by arrow). Panel A: Control (no Y_2O_3), B: 1 µM Y_2O_3 , C: 10 µM Y_2O_3, D: 100 µM Y_2O_3, and E: Paraquat 50 µM. Cells were imaged at 400 X magnification.

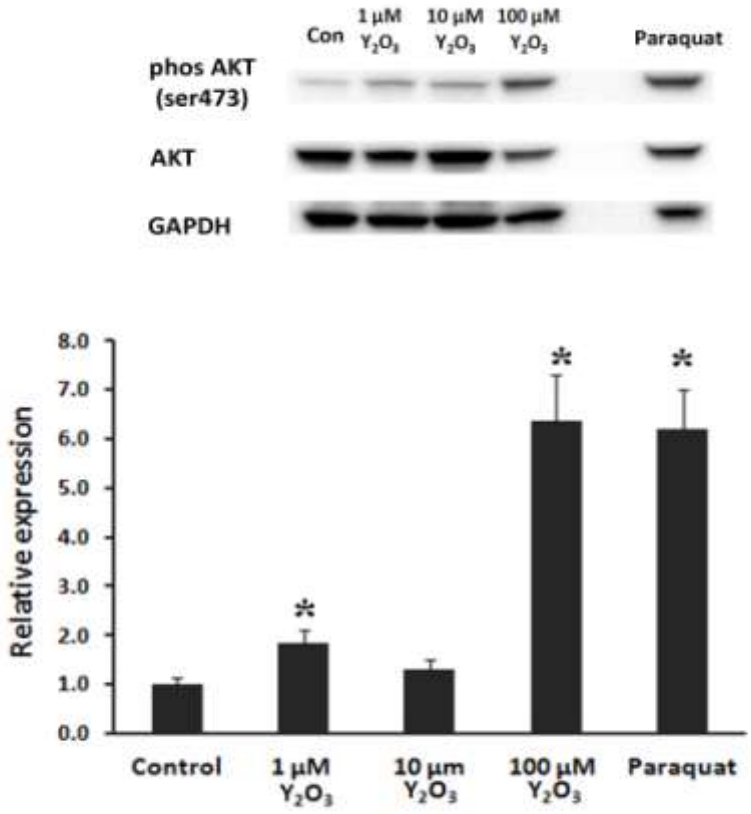

Figure 5. Yttrium oxide nanoparticle exposure is associated with the phosphorylation of Akt. Protein isolates obtained from control and Y_2O_3 treated cells were analyzed by immunoblotting for alterations in Akt expression and phosphorylation (Ser473). * Significantly different from untreated controls.

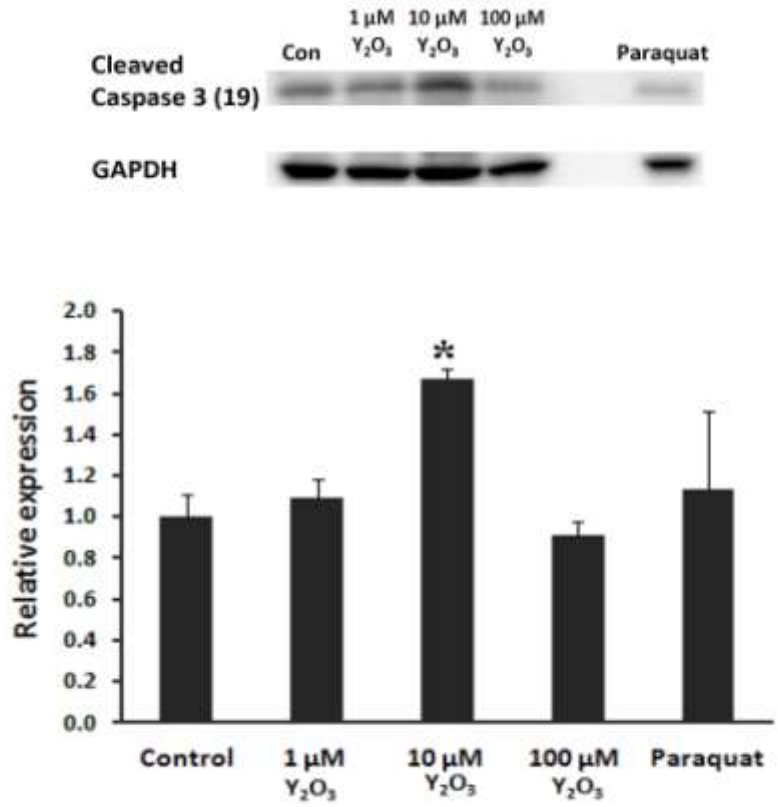

Figure 6. Cleaved caspase-3 is activated in HEK293 cells treated with Y_2O_3.

Protein isolates obtained from control and Y_2O_3 treated cells were analyzed by immunoblotting for caspase-3 cleavage. * Significantly different from untreated controls.

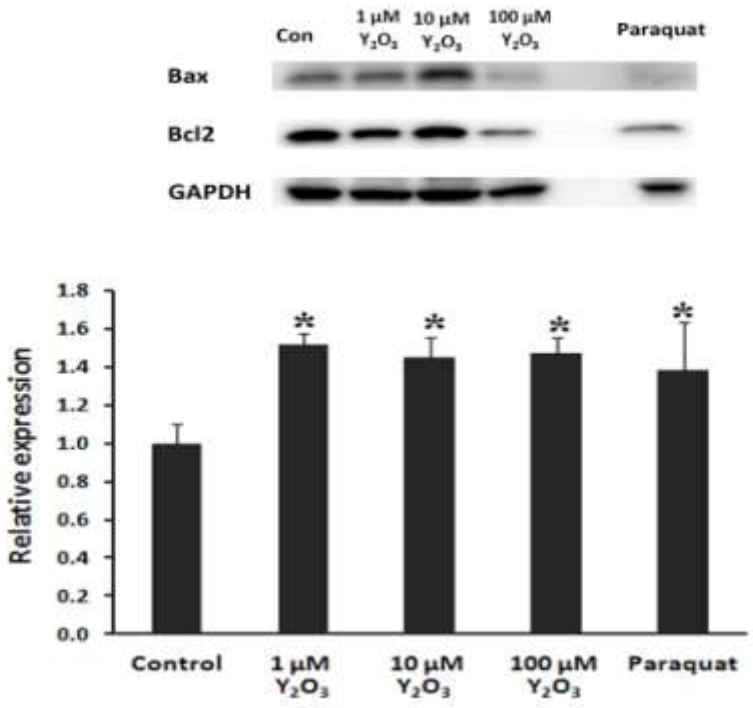

Figure 7. Upregulation of Bax/Bcl-2 in HEK293 cells treated with Y_2O_3.

Protein isolates obtained from control and Y_2O_3 treated cells were analyzed by immunoblotting for alterations in the ratio of Bax/Bcl-2. * Significantly different from untreated controls.

CHAPTER 4

Conclusions

1. Yttrium oxide nanoparticles induce cytotoxicity in HEK293 cells in a dose and time dependent manner.
2. Yttrium oxide nanoparticles increase cellular superoxide levels in HEK293 cells in a dose dependent manner.
3. Yttrium oxide nanoparticle exposure (100 µM) is associated with protein kinase B / Akt phoshorylation (activation) in HEK293 cells.
4. Yttrium oxide nanoparticle exposure (10 µM) is associated with increased cleavage of caspase-3 in HEK293 cells.
5. Yttrium oxide nanoparticle exposure is associated with elevations in the Bax / Bcl-2 ratio in HEK293 cells.

Taken together these data suggest that yttrium oxide exposure induces cytotoxicity in HEK293 cells in time and dose dependent manner that is associated with elevations in oxidative stress and changes in the regulation of Akt, caspase-3 and Bax / Bcl-2 ratio.

Future directions

Previous work has suggested that nanoparticle size is an important determinant of whether a particle may be toxic or not [45]. Based on this report and the data obtained in this

study, future research directions could be aimed at understanding the potential interactive effects that Y_2O_3 nanoparticle size and concentration may have on cell death.

How Y_2O_3 nanoparticles may increase cellular ROS levels and the effects that elevations in ROS may have on cellular structure are not well understood. To address this gap in the literature, future work employing electron microscopy could be undertaken to examine where yttrium oxide nanoparticles may be located in the cell. Concurrent with these experiments, other work could pursue how yttrium oxide exposure may affect cell size, mitochondrial vacuolization, and whether these materials may affect the structure of the endoplasmic reticulum. Similarly, other studies might investigate whether Y_2O_3 nanoparticle exposure is associated with changes in the amount of lipid or protein oxidation.

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