Published in final edited form as:

Neurobiol Dis. 2011 June ; 42(3): 514–523. doi:10.1016/j.nbd.2011.03.004.

Nanoceria extend photoreceptor cell lifespan in *tubby* mice by modulation of apoptosis/survival signaling pathways

Li Kong^{a,d,*}, Xue Cai^{a,*,#}, Xiaohong Zhou^{a,e}, Lily L. Wong^a, Ajay S. Karakoti^c, Sudipta Seal^c, and James F McGinnis^{a,b,#}

^aDepartment of Ophthalmology/Dean McGee Eye Institute, Oklahoma City, OK73104

^bDepartment of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK73104

^cAdvanced Materials Processing Analysis Center, Mechanical Materials Aerospace Eng., Nanosci. and Tech. Ctr., Univ of Central Florida, Orlando, FL32816

^dDepartment of Histoembryology of Dalian Medical University, 116044 Dalian, Liaoning, China

Abstract

Cerium oxide nanoparticles, nanoceria, are inorganic antioxidants that have catalytic activities which mimic those of the neuroprotective enzymes superoxide dismutase and catalase. We have previously shown that nanoceria preserve retinal morphology and prevent loss of retinal function in a rat light damage model. In this study, the homozygous *tubby* mutant mouse, which exhibits inherited early progressive cochlear and retinal degeneration, was used as a model to test the ability of nanoceria to slow the progression of retinal degeneration. *Tubby* mice were injected systemically, intracardially, with 20 µl of 1mM nanoceria in saline, at postnatal day 10 and subsequently at P20 and P30 whereas saline injected and uninjected wild type (or heterozygous *tubby*) served as injected and uninjected controls, respectively. Assays for retinal function, morphology and signaling pathway gene expression were performed on P34 mice. Our data demonstrate that nanoceria protect the retina by decreasing Reactive Oxygen Species (ROS), up-regulating the expression of neuroprotection-associated genes; down-regulating apoptosis signaling pathways and/or up-regulating survival signaling pathways to slow photoreceptor degeneration. These data suggest that nanoceria have significant potential as global agents for therapeutic treatment of inherited retinal degeneration and most types of ocular diseases.

© 2011 Elsevier Inc. All rights reserved.

[#]Corresponding authors: James F. McGinnis: Department of Ophthalmology/Dean McGee Eye Institute, Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK73104, james-mcginnis@ouhsc.edu, Tel: 405-271-3695, Fax: 405-271-3721, Xue Cai: Department of Ophthalmology/Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, xue-cai@ouhsc.edu. Tel: 405-271-6974, Fax: 405-271-3721.

^{*}These two authors made equivalent contributions to this work

^cCurrent address: Massachusetts Eye and Ear Infirmary, Howe Laboratory, Harvard Medical School. Boston, MA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure statement: Li Kong, none; Xue Cai, none; Xiaohong Zhou, none.

Potential conflict of interest: The University of Central Florida and the Oklahoma University Health Sciences Center own a patent [US patent: Inhibition of reactive oxygen species and protection of mammalian cells (7347987, March 25, 2008)] with SS, LLW and JFM listed as co-inventors.

Keywords

nanoceria; *tubby* mouse; inherited retinal degeneration; oxidative stress; apoptosis; neuroprotection

Introduction

Inherited retinal degeneration results in the apoptotic death of photoreceptors, and sometimes the retinal pigment epithelial cells (Chang et al., 1993; Portera-Cailliau et al., 1994), through caspase-dependent and -independent apoptotic pathways (Wenzel et al., 2005). It had been hypothesized, and experimentally demonstrated, that neurodegenerative ocular diseases can be directly linked with excessive oxidative stress (Dorrell et al., 2009; Komeima et al., 2006; Sanz et al., 2007) due to a rise in the level of reactive oxygen species (ROS) beyond the capacity of the cells' antioxidant defenses (Andersen, 2004; Cingolani et al., 2006). Documented data indicate that supplementation with agents such as sulforaphane (SF) (Gao and Talalay, 2004; Kong et al., 2009; Kong et al., 2007; Tanito et al., 2005), thioredoxin (Cohen et al., 2009) and others (Komeima et al., 2006; Sanz et al., 2007) can eliminate these pathological conditions. Similarly, neurotrophic proteins such as BDNF (brain-derived neurotrophic factor) and PEDF (pigment epithelium-derived factor) can protect against photoreceptor degeneration and preserve the retinal function in some animal models (Klocker et al., 2000; Schuettauf et al., 2004).

Investigations of cerium oxide nanoparticles (nanoceria) have revealed that its lattice constant increases with decreasing nanoparticle size (Deshpande et al., 2005). The surface area of nanoceria is dramatically enlarged in relation to its volume which increases oxygen exchange and redox reactions. This has been attributed to an increase in oxygen vacancies in the crystal structure (Patil et al., 2006). We hypothesized that nanoceria, owing to their chemical and physical structures, can protect cells from free-radical-induced damage. This is especially supported by the demonstration that nanoceria have catalytic activities like those of two major antioxidative enzymes, super oxide dismutase (Korsvik et al., 2007) and catalase (Trovarellis, 2002), and act as direct antioxidants and neuroprotective agents to limit the amount of intracellular ROS (Heckert et al., 2008; Korsvik et al., 2007; Singh et al., 2006), and protect neuronal cell and increase lifespan (Karakoti et al., 2008; Rzigalinski, 2005; Rzigalinski et al., 2006; Schubert et al., 2006; Singh et al., 2007). The nanoceria act as free-radical scavengers by switching between the +3 and +4 valence states via various surface chemical reactions (Patil et al., 2006) and one nanoceria may offer many sites of spin-trap activity, whereas current pharmacological agents offer only a few per molecule (Kotake, 1999). Additionally, the lattice defects in nanoceria possess the potential for regeneration (Trovarellis, 2002; Zhang et al., 2004) and do not require repetitive dosage as seen with the use of dietary supplements of antioxidants such as vitamins C and E. We previously reported that our formulated nanoceria possess the ability to protect photoreceptors from light damage and rescue the retinal function in wild type Sprague-Dawley albino rats (Chen et al., 2006). Recently nanoceria were shown had no toxicity in the murine cell line J774A.1 and no pathologic side effects in mouse tissue (Hirst et al., 2009), and without genotoxicity to cultured human lens epithelial cells (Pierscionek et al., 2010). Collectively, these data suggest that the nanoceria can safely scavenge ROS in the retina and thereby inhibit oxidative stress and inherited retinal degeneration.

Tubby mice are homozygous for a mutation in the *Tub* gene and exhibit inherited retinal and cochlear degeneration, major hallmarks of Usher's Syndrome in humans (Ohlemiller et al., 1995). We have shown that light accelerates the retinal degeneration of the *tubby* mouse (Kong et al., 2006) suggesting the involvement of ROS in the death of these cells.

Additional real time qRT-PCR and western experiments showed that the mRNA and protein expression of thioredoxin (Trx), thioredoxin reductase (TrxR) and NFE2-related factor-2 (Nrf2) are significantly reduced in the retinas of *tubby* mice even prior to photoreceptor cell degeneration (Kong et al., 2007). Furthermore, we reported that up-regulation of the “Trx system” in *tubby* mice by sulforaphane (SF) (Kong et al., 2009; Kong et al., 2007) or over expression of the human *Trx* gene in *tubby* mice (Kong et al., 2010), delayed photoreceptor degeneration. All of these data support the hypothesis that the nanoceria, which reduce oxidative stress, can slow the progression of retinal degeneration in the *tubby* mouse. The study of Bode and Wolfrum (2003) revealed that the apoptosis in *tubby* retina peaks at P19 (Bode and Wolfrum, 2003) and our previous work demonstrated that the rapid photoreceptor loss occurs between P14 to P34 with about 50% of photoreceptor cell loss by P28 (Kong et al., 2006). These data combined with the fact that nanoceria destroy ROS, led us to test the ability of nanoceria to inhibit retinal degeneration in the *tubby* mouse. As an initial study, P10 pups (before onset of photoreceptor degeneration) were injected systemically (intracardially) with 20 μ l of 1mM nanoceria in saline and with two subsequent injections performed at P20 and P30. Our data demonstrated that nanoceria protected the retina from oxidative stress, prevented cell death and ROS-mediated damage, increased the expression of neuroprotection-associated genes, down-regulated apoptosis signaling pathways while up-regulating cell survival pathways to slow the photoreceptor degeneration.

Materials and methods

Animals

Homozygous *tubby* (*tub/tub*) mice on a C57BL/6J background and C57BL/6J wild type (wt/wt) mice were used for this study. *Tubby* mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used to start the colony. Since *tub/tub* mice have reduced fertility, we mated heterozygote (*tub/wt*) mice or homozygotes $\times$ heterozygotes for producing the homozygous. All offspring were genotyped by PCR according to the protocol provided by the Jackson laboratories as we previously described (Kong et al., 2006). All animals were maintained in a 12h light-dark cycle with lighting intensity of 80 lux. Animals were cared for and handled according to the Association for Research in Vision and Ophthalmology statement for the use of animals in vision and ophthalmic research and with animal use protocols approved by Oklahoma Health Sciences Center (OUHSC) and Dean McGee Eye Institute Institutional Animal Care and Use Committees (IACUC).

Intracardial injection

Mice were divided into three groups. One cohort *tub/tub* was injected with nanoceria; one cohort *tub/tub* was injected with saline. Another cohort (either *tub/wt* or wt/wt) served as age-matched uninjected or saline injected controls. Pups at P10 were intraperitoneally anesthetized with ketamine-xylazine, and 20 μ l of either saline (0.9% NaCl) or 1mM nanoceria in saline was injected into the heart using chest palpitation. The same mice were subsequently injected at P20 and P30 with the same amount of nanoceria or saline.

Electroretinography (ERG)

Nanoceria or saline injected *tub/tub* and uninjected *tub/wt* (or wt/wt) at P34 were dark adapted overnight. After animals were anesthetized and pupils were dilated, full field ERG at a light intensity of 2000 cd. s/m² and serial intensity ERG at light intensities of 0.004, 0.04, 0.4, 4, 40, 400, 1000 and 2000 cd. s/m² were performed using an LKC Electroretinogram system (EPIC-2000, LKC Technologies, Inc., Gaithersburg, MD 20879) as previously described in detail (Kong et al., 2006).

Quantitative histology

Eyes at P34 were enucleated, fixed, embedded in paraffin, sectioned and stained with hematoxylin-eosin as previously described (Chen et al., 2006; Kong et al., 2007). In each of the superior and inferior hemispheres, outer nuclear layer (ONL) thickness was measured at nine defined points. Each point was centered on adjacent 220µm lengths of retina. The first point of measurement was taken at approximately 220µm from the optic nerve head, and subsequent points were located more peripherally. The data represent the average of 4 eyes from each group.

Measurement of ROS

Intracellular ROS generation was detected by multiple independent methods (Korystov et al., 2007; Takimoto et al., 2005). Dichlorofluorescein (DCF), the oxidation product of 2, 7-dichlorodihydro-fluorescein diacetate (DCFH-DA; Invitrogen, Carlsbad, CA) emits a green fluorescent signal localized primarily to mitochondria, and is a marker of cellular oxidation by hydrogen peroxide, hydroxyl radicals and peroxynitrite. Unfixed retinal cryosections at P18 were incubated with DCFH-DA (10 µM) for 60 minutes at 37°C. DCF detection at 505 nm was visualized by FV 500 Olympus confocal microscopy (Japan). Dihydroethidium (DHE) (Invitrogen, Carlsbad, CA) assesses O₂⁻ formation. DHE is oxidized on reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. Serial cryosections from fresh-frozen retinas at P18 were incubated with DHE (0.625 µM) at 37°C for 20 minutes, followed by confocal microscopy detection at 585 nm.

Quantitative real time RT-PCR (qRT-PCR) and semi-quantitative RT-PCR

RNA isolation and RT-PCR performance were carried out according to our previous report (Kong et al., 2009). Briefly, individual P34 retinas from 4 eyes of each group (injected with nanoceria or saline and non-injected controls) were dissected and collected under RNase free conditions. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA); 4 µg of total RNA was reverse transcribed to cDNA using first strand superscript III transcriptase and an Oligo dT primer according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). qRT-PCR was performed in triplicate for each cDNA sample in a 30 µl reaction volume containing SYBR green mix (Invitrogen) and 1 ng of cDNA as template using a single-color iCycler machine (Bio-Rad, Hercules, CA) for 40 cycles as described previously (Kong et al., 2009). Gene expression was calculated against the house-keeping gene GAPDH and data shown as fold change using the $2^{-\Delta\Delta C_t}$ method ($\Delta C_t = C_{t \text{ target gene}} - C_{t \text{ reference gene}}$ and $\Delta\Delta C_t = \Delta C_t \text{ treated group} - \Delta C_t \text{ control group}$).

Primers for target genes are: Caspase-8, forward primer, 5'-AGAAGAGGACCATGCTGGCAGAAA-3' and reverse primer, 5'-TGTTCTCTTGGCGAGTCACACAGT-3'; Bak 1, forward primer, 5'-ACGAACTCTTCACCAAGATCGCCT-3' and reverse primer, 5'-TCAAACCACGCTGGTAGACGTACA-3'. Primer sequence for GAPDH was the same as previously reported (Kong et al., 2007). Semi-quantitative RT-PCR was performed as described previously with GAPDH as an internal control (Kong et al., 2007). The PCR products were electrophoresed in a 1.2% agarose gel, ethidium bromide stained and photographed.

Caspase assay

The activities of caspase-3 and caspase-9 in retinas from each group P34 were measured using commercially available kits according to the manufacturer's protocol (BioSource International, Inc). Data came from three retina measurements which were repeated for a total of three times.

Western blot

Western blots were performed according to procedures reported previously [10]. Briefly, 4 retinas of each group at P34 were individually homogenized, protein was extracted and the concentrations were quantified. An equal amount of protein (30 µg) was loaded and separated on a 12% SDS-page gel, and electrophoretically transferred onto a PVDF membrane. The blots were blocked in 10% BSA and then were incubated in the following primary antibodies: rabbit anti-bFGF (1:200, Santa Cruz, CA) and anti-FGFR polyclonal antibody (1:500, Santa Cruz, CA), mouse anti-Ras (1:500, Millipore, Billerica, MA) and anti-actin monoclonal antibody (1:10000, Amersham Biosciences (GE Healthcare), Buckinghamshire, UK), rabbit anti-ERKs and anti-phospho ERKs polyclonal antibody (1:1000 and 1:500 respectively, Cell signaling technology, MA), rabbit anti-Nrf2 polyclonal antibody (1:200, Santa Cruz, CA), rabbit anti-Trx polyclonal antibody (1:2000, Redox Bioscience), rabbit anti-cytochrome *c* polyclonal antibody (1:200 Biovision, Mountain View, CA). Then blots were incubated in horseradish peroxidase-conjugated secondary antibody (1:5000, Amersham Biosciences (GE Healthcare), Buckinghamshire, UK) and developed by ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK). The amount of each protein was determined from the intensity of the band and standardized to that of actin. Data are expressed as the average of 4 samples within the same group and compared between groups.

TdT-mediated digoxigenin-dUTP nick-end labeling assay (TUNEL assay)

Detection of apoptosis by TUNEL assay was performed using a commercially available in situ apoptosis detection kit (Biovision, Mountain View, CA) with cryosections of P34 eyes according to the manufacturer's protocol. Observation of TUNEL-positive cells and imaging were conducted using a Nikon Eclipse 800 microscope on three eyes from each group and representative images are shown.

Statistical analysis

Each experiment was performed three times. Mean values from at least three eyes and two repeats were calculated and expressed as mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVA and Student's *t*-test with statistical differences considered significant at a *P* value of <0.05 .

Results

In this study, *tub/tub* mice were intracardially injected at P10, P20 and P30 successively with 20 µl of 1mM nanoceria in saline or with 20 µl of saline injection as control. Systemic delivery avoids even the small transient inflammatory response which occurs when the eye is simply injured with a needle puncture (Cao et al., 1997). Most importantly, the protective effects which occur from the systemic injection of nanoceria indicate that the nanoceria do not have to be delivered directly into the eye. Analysis of the eyes indicated that there are no differences between the uninjected and saline injected *tub/tub* animals (data not shown) and therefore only the data from the saline injected are shown. In addition, *tub/wt* animals exhibited no differences from *wt/wt*, so only the *tub/wt* data are shown as a control.

Nanoceria improve retinal responses to the light

To test the ability of nanoceria to preserve retinal function, retinal responses to the light stimulants were assessed by full field and serial intensity ERG at P34. As indicated in Fig. 1A (which shows the average of scotopic a-wave from 4 eyes), the amplitude of scotopic ERG was greatly increased (increased 85.7%) in the eyes of nanoceria injected mice ($105.185 \pm 97.027 \mu V$) compared to saline injected animals ($56.641 \pm 20.53 \mu V$). Serial

intensity ERG was also performed to evaluate the retinal responses to different light intensities. As shown in Fig. 1B and 1C, the amplitudes of scotopic a-wave and b-wave are significantly increased in nanoceria injected mice compared to those of saline injected mice ($P<0.05$). Interestingly the scotopic b-waves, which test the response of second-order neurons (inner neurons), are more significantly increased when the light intensity is elevated ($P<0.01$ & $P<0.001$).

Nanoceria slow photoreceptor degeneration

The thickness of the outer nuclear layer of the retina is directly proportional to the number of photoreceptor cells and by measuring its thickness across the retina in H&E stained sections, it is possible to document pan-retinal effects of degeneration and neuroprotection (Rapp & Williams, 1980). The results of this quantitative histology are shown in Fig. 2 and demonstrate that the systemic injection of nanoceria protects photoreceptor cells against the effects of the *tubby* mutation and slows retinal degeneration.

Nanoceria regulate the bFGF-RTK-Ras-ERK pathway

Normal cell development involves multiple signaling transduction pathways. Basic fibroblast growth factor (bFGF) has been reported to be expressed in almost all the tissues in the eye (Consigli et al., 1993) and its up-regulation protects photoreceptors against damage and enhances photoreceptor survival (Liu et al., 1998; Wen et al., 1998). Receptor tyrosine kinases (RTK) have been shown to be key regulators for normal cellular processes, including eye development, that signal through Ras (Firth et al., 2006). The FGF receptor family is an RTK class IV. Extracellular receptor kinase (ERK), a protein kinase downstream of Ras (Leevers and Marshall, 1992), is involved in the regulation of many activities in differentiated cells and can be activated by many stimuli. The RTK-Ras-ERK signaling cascade has been suggested to be essential for regulation of cell proliferation, differentiation, survival, cell cycle, cell movement and morphology (Hsiao et al., 2001; Isaksson et al., 1997; Seger and Krebs, 1995; Xie et al., 2006). So we performed western blots to determine if the expression levels of these effectors are regulated by nanoceria treatment. As shown in Fig. 3, the protein levels of bFGF, its receptor FGFR, and Ras were much lower in the saline injected *tubby* retina but were significantly increased after nanoceria treatment (Ras, $P<0.05$; bFGF and FGFR, $P<0.01$) to levels comparable to controls (Figs. 3A–D). The expression of ERK in nanoceria and saline injected *tubby* is similar to the *tub/wt* control levels (Fig. 3A) but the phosphorylated, activated form of ERK (p-ERK) was significantly elevated in the nanoceria treated mice compared to saline treatment ($P<0.05$) and comparable to the *tub/wt* control (Fig. 3E).

Nanoceria increase the expression of antioxidant-associated proteins

Trx, Nrf-2 and its translocation to the nuclear form, N-Nrf-2, are known to be involved in antioxidative effects and protection against oxidative stress-induced apoptosis signaling pathways (Cohen et al., 2009; Gao and Talalay, 2004; Kong et al., 2007; Kong et al., 2010; Mandal et al., 2009; Tanito et al., 2005). To confirm the antioxidative effect of nanoceria in the retina of *tubby* mutant mice, the expression of Nrf-2, N-Nrf-2 and Trx was examined on western blots. As shown in Fig. 4, the *tubby* mutant exhibits significantly lower expression levels of Nrf-2, N-Nrf-2 and Trx compared to *tub/wt*. After nanoceria treatment, Nrf-2, N-Nrf-2 and Trx in the mutant retina are all elevated compared to saline treatment, and similar to *tub/wt* levels (Fig. 4A). Statistical analysis indicated that the increases are significant (Nrf-2 and Trx, $P<0.05$; N-Nrf-2, $P<0.01$) (Fig. 4B, 4C, 4D).

Nanoceria reduce the ROS level in the retina

To determine if nanoceria treatment alters the level of ROS, we assayed the amount of ROS in the retina at P18 using oxidation sensitive dyes, DHE and DCFH-DA, which after oxidation produce red and green fluorescence respectively. Representative data in Fig. 5 show that the control retina (*tub/wt*) has detectable levels of ROS as visualized by both assays (Fig. 5C, 5F) whereas the *tubby* mutation results in detection of markedly higher levels of ROS (Fig. 5A, 5D). However, the retinas injected with nanoceria showed levels of ROS which were reduced almost to control levels (Fig. 5B, 5E).

Indicators of oxidative stress and apoptosis are increased in the *tubby* retina and decreased by nanoceria treatment

Apoptosis directly involves the activation of caspases which cleave intracellular proteins (Strasser et al., 2000; Wenzel et al., 2005). This process involves two major pathways. The first is the activation of the cleavage and maturation of effector caspases (Budihardjo et al., 1999) and the second is the caspase dependent release of cytochrome *c* from mitochondria (Green and Reed, 1998). In this study, the expression of caspase-8, Bak 1 and cytochrome *c* in the retina was examined after nanoceria treatment. The level of caspase-8 and Bak 1 mRNA (Fig. 6A) in the *tub/tub* retina is elevated. Quantitative measurement by qRT-PCR showed that mRNA level of caspase-8 in the *tub/tub* retina is two fold over that in the *tub/wt* control retina but injection of nanoceria reduces it to the control level (Fig. 6B). Similarly, the mRNA for Bak 1 is approximately eight fold higher in the *tub/tub* retina than in the *tub/wt* control retina and treatment with nanoceria reduces it to about two fold (Fig. 6C). Measurement of the activities of caspase-9 and caspase-3 shows that both are increased in the *tub/tub* retinas compared to the *tub/wt* control retinas and injection of nanoceria decreases both to control levels (Fig. 6D & 6E).

Total cytochrome *c* and cytoplasmic cytochrome *c* (Fig. 7A, 7B, 7C) were elevated in the *tub/tub* retina compared to the *tub/wt* control retina whereas nanoceria treatment of *tubby* reduced both to control levels. To determine the effects of the *tubby* mutation on apoptosis in the retina, the TUNEL assay was performed. Representative images are seen in FIG. 7D (a, b, c). The *tub/tub* retina had many more TUNEL positive cells than the *tub/wt* control retina and very few were detected in the nanoceria treated *tub/tub* mice.

Discussion

Traditional pharmacological antioxidant treatments require multiple daily dosing because the free radical scavenging of each antioxidant molecule is usually limited to one free radical. Another limitation of antioxidant treatment is that the distribution of antioxidants is often restricted to a specific site in the cell which may not be the site of free radical damage (Singh et al., 2007). In opposition, nanoceria can regeneratively scavenge free radicals (Trovarellis, 2002; Zhang et al., 2004) and one nanoceria may offer many sites of spin-trap activity. Their tiny size which is comparable to the atomic size makes them freely penetrate into the cell, and cell membrane and nuclear membrane do not appear to be barriers. The current work reports the use of nanoceria as direct *in vivo* antioxidants and neuroprotective agents to treat an inherited neurodegenerative mouse model. Nanoceria were intracardially injected into the *tubby* mutant mouse at a young age and the efficacy of protection in retinal degeneration was assessed at P34. A number of positive effects of nanoceria were observed: retinal function was preserved; more photoreceptor cells survived; ROS levels were significantly reduced; survival signaling pathways were up-regulated and the caspase-dependent apoptosis signaling pathway was inhibited along with the prevention of cytochrome *c* release from the mitochondria.

Nanoceria mediated Ras/Raf/MAPK pathway

The Ras/Raf/MAP kinase (or ERK) cascade is a central effector (cassette) for signaling cellular differentiation in development and proper growth in many tissues (Firth et al., 2006; Frebel and Wiese, 2006). It is activated by a variety of processes including the stimulation of growth factor receptors and it is known to be required for regulation of many cell activities of transcription factors involved in cell proliferation, differentiation, survival, cycling, movement and morphology (Seger and Krebs, 1995; Xie et al., 2006). It has been suggested that neuroprotective factors, such as BDNF, CNTF and bFGF, cause increased p-ERK expression in the retina (Akiyama et al., 2002; Klocker et al., 2000; O'Driscoll et al., 2008; Wahlin et al., 2000) and thereby protect the retina against injury (including light damage) and promote photoreceptor survival in albino and mutant rats and mice (Cao et al., 2001; Cayouette et al., 1999; Faktorovich et al., 1990; Faktorovich et al., 1992; LaVail et al., 1992; Levkovitch-Verbin et al., 2007; Liu et al., 1998; Miyazaki et al., 2003; Sakai et al., 2007; Schuettauf et al., 2004; Wen et al., 1996; Wen et al., 1998). In our current study, nanoceria treatment significantly elevated the expression of bFGF, RTK-FGFR, Ras and p-ERK, compared to the saline treatment, to levels comparable to the wild type. These data further confirm the neuroprotective properties of nanoceria and demonstrate that they can protect photoreceptor cells against degeneration by up-regulation of RTK-Ras-ERK cascade survival signal pathway.

Nanoceria upregulate Nrf-2 mediated Trx system

Numerous publications report that antioxidant Trx (Cohen et al., 2009) (Mitsui et al., 2002; Yamada et al., 2007; Zhou et al., 2009), can protect against oxidative stress-induced injury and other effects. Trx (Tanito et al., 2002a; Tanito et al., 2002b)) and other antioxidants such as DMTU (dimethylthiourea) (Organisciak et al., 1999; Ranchon et al., 1999), PBN (phenyl-N-tert-butyl-nitron) (Ranchon et al., 2001; Ranchon et al., 2003), curcumin (Mandal et al., 2009), flupirtine (Fawcett and Osborne, 2007), and others were reported to be able to protect photoreceptor or RPE cells against oxidative stress-induced damage including light-damage (Komeima et al., 2006). Sulforaphane (SF), an inducer of phase II detoxification enzymes and inhibitor of phase I enzymes (Gao and Talalay, 2004; Kong et al., 2009; Kong et al., 2007; Tanito et al., 2005), have also been shown to inhibit retinal degeneration. In addition, increased expression of Nrf-2, a transcription factor which binds the antioxidant-responsive element (ARE), also protect retinal cells from oxidative stress (Johnson et al., 2009; Uno et al., 2010).

Trx induction is known to be mediated by MAP kinase ERK (Andoh et al., 2005). Kong et al demonstrated that ERKs signal pathways are involved in SF-mediated up-regulation of Trx/TrxR/Nrf-2 expression *in vivo* (Kong et al., 2007). In this study, the administration of nanoceria significantly increased the expression of Trx, Nrf-2 and N-Nrf-2 over the saline injected controls to levels comparable to the *tubby*/wt. These data show that nanoceria have effects similar to antioxidant enzymes and they also induce the upregulation of the phase II enzymes, Trx and Nrf-2.

Nanoceria inhibit caspase-induced apoptosis

Apoptosis of neuronal cells is a mechanism common to all mutations in *tubby* family genes (Ikeda et al., 2002). As an active process, apoptosis requires protein synthesis and energy-containing substrates such as ATP to support the increasing transcription and translation changes of proapoptotic genes and proteins (Kerr et al., 1972; Nicotera, 2002; Sancho-Pelluz et al., 2008). Two major pathways are involved: one associated with the extracellular ligands which activate the cleavage and maturation of effector caspases (Budihardjo et al., 1999) and the other associated with the release of cytochrome *c* from the mitochondria (Kaufmann and Hengartner, 2001). Caspases have been classified into two groups with caspase-1, -4, -5,

-11, -12, and -14 participating in cytokine cleavage and maturation whereas caspase-2, -3, -6, -7, -8, -9 and -10 are directly involved in apoptosis (Kaufmann and Hengartner, 2001; Strasser et al., 2000). Involvement of caspases as the final step in inherited degeneration and light damage to the retina has been reported (Grimm et al., 2000). Caspase-3 is a major cell death effector and its cleavage and enzymatic activity play an important role in the regulation of photoreceptor cell degeneration. In the *rd1* mouse, caspase-3 was activated (Jomary et al., 2001; Kim et al., 2002). Inhibition of caspase-3 delayed cell death (Sharma and Rohrer, 2004) and the morphology of the retina was preserved as well (Zeiss et al., 2004). A similar observation was also reported in the S334ter rat (Liu et al., 1998) and *tubby* mouse (Bode and Wolfrum, 2003). BDNF suppresses cleavage and enzymatic activity of caspase-3 (Klocker et al., 2000). We previously demonstrated that the expression of cytochrome *c*, the activities of caspase-3 and -9 are increased in *tubby* mutant mice (Kong et al., 2006). In our current study, supplementation with nanoceria by intracardial injection effectively and significantly down-regulated the expression of caspase-3, -8, -9 and Bak1. As a consequence, the downstream effect of caspase inhibition, cytochrome *c* release from the mitochondria to the cytosol was blocked.

Taken together, it is obvious that nanoceria are effective in up-regulating the expression of neuroprotection and antioxidant-associated genes, while down-regulating apoptosis signaling pathways. However, nanoceria do not completely protect photoreceptors from degeneration although the expression of these positive-effect genes is increased to the wild type level, while the expression of apoptosis genes is reduced to the wild type level. Since multiple signaling pathways are affected by the *tubby* mutation itself, and nanoceria apparently do not affect the “basal level” of ROS and are not providing a “gain of function” effect.

We hypothesize that nanoceria, by their ROS scavenging activity reverse changes in multiple signal transduction pathways (Fig. 8) affected by mutation-induced increases in ROS. By decreasing ROS, nanoceria cause the upregulation of the expression of bFGF and its binding to its receptor tyrosine kinase, FGFR in the membrane. This event induces RTK dimerization and activation leading to the Ras- mediated phosphorylation of ERK to its activated form p-ERK. Phase II enzymes, including Trx, are up-regulated by Ras-raf-ERK signaling through increased Nrf-2. As a direct consequence of this system, the amount of ROS in the cytoplasm is reduced even further. As a downstream effect of ROS inhibition, sequential activation of apoptosis-associated cytokines, caspase-8 and Bak1 is suppressed and cytochrome *c* release from the mitochondria to the cytoplasm is inhibited. Activation of caspase-9 is prevented, and in turn inhibits the activation of the effector caspase-3. As a final result, caspase-3 cleavage and maturation are inhibited, thereby preventing photoreceptor cell death and preserving their morphology and function.

Our data demonstrate that nanoceria inhibit the rate of progression of inherited retinal degeneration in the *tubby* mouse and support our overarching hypothesis that oxidative stress is common to retinal degenerative diseases and, as such, is an “Achilles’ heel” for these diseases. This also specifically suggests that nanoceria are potentially very useful for the therapeutic treatment of more prevalent ocular diseases including diabetic retinopathy, age related macular degeneration and retinopathy of prematurity.

Acknowledgments

The authors thank the personnel at the Animal, Imaging, and Molecular modules of the Vision Research Core Facility at the University of Oklahoma Health Sciences Center. This work was supported by grants from: NIH (P30-EY12190, COBRE-P20 RR017703, R21EY018306, R01EY018724); FFB (C-NP-0707-0404-UOK08; NSF: CBET-0708172; OCAST: HR06-075), and unrestricted funds from Presbyterian Health Foundation and Research to Prevent Blindness (RPB). JFM is a recipient of an RPB Senior Scientific Investigator Award.

References

- Akiyama H, et al. Presence of mitogen-activated protein kinase in retinal Muller cells and its neuroprotective effect ischemia-reperfusion injury. *Neuroreport*. 2002; 13:2103–2107. [PubMed: 12438934]
- Andersen JK. Oxidative stress in neurodegeneration: cause or consequence? *Nat Med*. 2004; (10 Suppl):S18–S25. [PubMed: 15298006]
- Andoh T, et al. Role of the redox protein thioredoxin in cytoprotective mechanism evoked by (-)-deprenyl. *Mol Pharmacol*. 2005; 68:1408–1414. [PubMed: 16099847]
- Bode C, Wolfrum U. Caspase-3 inhibitor reduces apoptotic photoreceptor cell death during inherited retinal degeneration in tubby mice. *Mol Vis*. 2003; 9:144–150. [PubMed: 12724642]
- Budihardjo I, et al. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol*. 1999; 15:269–290. [PubMed: 10611963]
- Cao W, et al. Development of normal and injury-induced gene expression of aFGF, bFGF, CNTF, BDNF, GFAP and IGF-I in the rat retina. *Exp Eye Res*. 2001; 72:591–604. [PubMed: 11311051]
- Cao W, et al. Mechanical injury increases bFGF and CNTF mRNA expression in the mouse retina. *Exp Eye Res*. 1997; 65:241–248. [PubMed: 9268592]
- Cayouette M, et al. Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degenerations. *Neurobiol Dis*. 1999; 6:523–532. [PubMed: 10600408]
- Chang GQ, et al. Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. *Neuron*. 1993; 11:595–605. [PubMed: 8398150]
- Chen J, et al. Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. *Nat Nanotechnol*. 2006; 1:142–150. [PubMed: 18654167]
- Cingolani C, et al. Retinal degeneration from oxidative damage. *Free Radic Biol Med*. 2006; 40:660–669. [PubMed: 16458197]
- Cohen JJ, et al. Exogenous thioredoxin prevents ethanol-induced oxidative damage and apoptosis in mouse liver. *Hepatology*. 2009; 49:1709–1717. [PubMed: 19205032]
- Consigli SA, et al. The temporal and spatial expression of basic fibroblast growth factor during ocular development in the chicken. *Invest Ophthalmol Vis Sci*. 1993; 34:559–566. [PubMed: 8449676]
- Deshpande S, et al. Size dependency variation in lattice parameter and valency states in nanocrystalline cerium oxide. *Applied Physics Letters*. 2005:87.
- Dorrell MI, et al. Antioxidant or neurotrophic factor treatment preserves function in a mouse model of neovascularization-associated oxidative stress. *J Clin Invest*. 2009; 119:611–623. [PubMed: 19188685]
- Faktorovich EG, et al. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature*. 1990; 347:83–86. [PubMed: 2168521]
- Faktorovich EG, et al. Basic fibroblast growth factor and local injury protect photoreceptors from light damage in the rat. *J Neurosci*. 1992; 12:3554–3567. [PubMed: 1527595]
- Fawcett RJ, Osborne NN. Flupirtine attenuates sodium nitroprusside-induced damage to retinal photoreceptors, in situ. *Brain Res Bull*. 2007; 73:278–288. [PubMed: 17562394]
- Firth LC, et al. Analyses of RAS regulation of eye development in *Drosophila melanogaster*. *Methods Enzymol*. 2006; 407:711–721. [PubMed: 16757364]
- Frebel K, Wiese S, et al. Signalling molecules essential for neuronal survival and differentiation. *Biochem Soc Trans*. 2006; 34:1287–1290. [PubMed: 17073803]
- Gao X, Talalay P, et al. Induction of phase 2 genes by sulforaphane protects retinal pigment epithelial cells against photooxidative damage. *Proc Natl Acad Sci U S A*. 2004; 101:10446–10451. [PubMed: 15229324]
- Green DR, Reed JC. Mitochondria and apoptosis. *Science*. 1998; 281:1309–1312. [PubMed: 9721092]
- Grimm C, et al. Gene expression in the mouse retina: the effect of damaging light. *Mol Vis*. 2000; 6:252–260. [PubMed: 11134582]
- Heckert EG, et al. The role of cerium redox state in the SOD mimetic activity of nanoceria. *Biomaterials*. 2008; 29:2705–2709. [PubMed: 18395249]

- Hirst SM, et al. Anti-inflammatory properties of cerium oxide nanoparticles. *Small*. 2009; 5:2848–2856. [PubMed: 19802857]
- Hsiao FC, et al. Eyes absent mediates cross-talk between retinal determination genes and the receptor tyrosine kinase signaling pathway. *Dev Cell*. 2001; 1:51–61. [PubMed: 11703923]
- Ikeda A, et al. The tubby-like proteins, a family with roles in neuronal development and function. *J Cell Sci*. 2002; 115:9–14. [PubMed: 11801719]
- Isaksson A, et al. The deubiquitination enzyme fat facets negatively regulates RTK/Ras/MAPK signalling during *Drosophila* eye development. *Mech Dev*. 1997; 68:59–67. [PubMed: 9431804]
- Johnson J, et al. The flavonoid, eriodictyol, induces long-term protection in ARPE-19 cells through its effects on Nrf2 activation and phase 2 gene expression. *Invest Ophthalmol Vis Sci*. 2009; 50:2398–2406. [PubMed: 19117929]
- Jomary C, et al. Characterization of cell death pathways in murine retinal neurodegeneration implicates cytochrome c release, caspase activation, and bid cleavage. *Mol Cell Neurosci*. 2001; 18:335–346. [PubMed: 11640892]
- Karakoti AS, et al. Nanoceria as antioxidant: Synthesis and biomedical applications. *JOM*. 2008; 60:33–37. [PubMed: 20617106]
- Kaufmann SH, Hengartner MO. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol*. 2001; 11:526–534. [PubMed: 11719060]
- Kerr JF, et al. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 1972; 26:239–257. [PubMed: 4561027]
- Kim DH, et al. Activation of caspase-3 during degeneration of the outer nuclear layer in the rd mouse retina. *Ophthalmic Res*. 2002; 34:150–157. [PubMed: 12097798]
- Klocker N, et al. Brain-derived neurotrophic factor-mediated neuroprotection of adult rat retinal ganglion cells in vivo does not exclusively depend on phosphatidylinositol-3'-kinase/protein kinase B signaling. *J Neurosci*. 2000; 20:6962–6967. [PubMed: 10995840]
- Komeima K, et al. Antioxidants reduce cone cell death in a model of retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 2006; 103:11300–11305. [PubMed: 16849425]
- Kong L, et al. Molecular mechanisms underlying cochlear degeneration in the tubby mouse and the therapeutic effect of sulforaphane. *Neurochem Int*. 2009; 54:172–179. [PubMed: 19114066]
- Kong L, et al. Bright cyclic light accelerates photoreceptor cell degeneration in tubby mice. *Neurobiol Dis*. 2006; 21:468–477. [PubMed: 16216520]
- Kong L, et al. Delay of photoreceptor degeneration in tubby mouse by sulforaphane. *J Neurochem*. 2007; 101:1041–1052. [PubMed: 17394579]
- Kong L, et al. Neuroprotective effect of overexpression of thioredoxin on photoreceptor degeneration in Tubby mice. *Neurobiol Dis*. 2010; 38:446–455. [PubMed: 20298786]
- Korsvik C, et al. Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles. *Chem Commun (Camb)*. 2007:1056–1058. [PubMed: 17325804]
- Korystov YN, et al. Detection of reactive oxygen species induced by radiation in cells using the dichlorofluorescein assay. *Radiat Res*. 2007; 168:226–232. [PubMed: 17638409]
- Kotake Y. Pharmacologic properties of phenyl N-tert-butyl nitron. *Antioxid Redox Signal*. 1999; 1:481–499. [PubMed: 11233146]
- LaVail MM, et al. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc Natl Acad Sci U S A*. 1992; 89:11249–11253. [PubMed: 1454803]
- Leevers SJ, Marshall CJ. MAP kinase regulation--the oncogene connection. *Trends Cell Biol*. 1992; 2:283–286. [PubMed: 14731911]
- Levkovitch-Verbin H, et al. Regulation of cell death and survival pathways in experimental glaucoma. *Exp Eye Res*. 2007; 85:250–258. [PubMed: 17586494]
- Liu C, et al. Preconditioning with bright light evokes a protective response against light damage in the rat retina. *J Neurosci*. 1998; 18:1337–1344. [PubMed: 9454843]
- Mandal MN, et al. Curcumin protects retinal cells from light-and oxidant stress-induced cell death. *Free Radic Biol Med*. 2009; 46:672–679. [PubMed: 19121385]

- Mitsui A, et al. Overexpression of human thioredoxin in transgenic mice controls oxidative stress and life span. *Antioxid Redox Signal*. 2002; 4:693–696. [PubMed: 12230882]
- Miyazaki M, et al. Simian lentiviral vector-mediated retinal gene transfer of pigment epithelium-derived factor protects retinal degeneration and electrical defect in Royal College of Surgeons rats. *Gene Ther*. 2003; 10:1503–1511. [PubMed: 12900766]
- Nicotera P, et al. Apoptosis and age-related disorders: role of caspase-dependent and caspase-independent pathways. *Toxicol Lett*. 2002; 127:189–195. [PubMed: 12052658]
- O'Driscoll C, et al. Basic fibroblast growth factor-induced protection from light damage in the mouse retina in vivo. *J Neurochem*. 2008; 105:524–536. [PubMed: 18088352]
- Ohlemiller KK, et al. Cochlear and retinal degeneration in the tubby mouse. *Neuroreport*. 1995; 6:845–849. [PubMed: 7612867]
- Organisciak DT, et al. Light-induced damage in the retina: differential effects of dimethylthiourea on photoreceptor survival, apoptosis and DNA oxidation. *Photochem Photobiol*. 1999; 70:261–268. [PubMed: 10461466]
- Patil S, et al. Role of trivalent La and Nd dopants in lattice distortion and oxygen vacancy generation in cerium oxide nanoparticles. *Applied Physics Letters*. 2006:88.
- Pierscionek BK, et al. Nanoceria have no genotoxic effect on human lens epithelial cells. *Nanotechnology*. 2010; 21:035102. [PubMed: 19966402]
- Portera-Cailliau C, et al. Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 1994; 91:974–978. [PubMed: 8302876]
- Ranchon I, et al. Systemic administration of phenyl-N-tert-butyl nitrone protects the retina from light damage. *Invest Ophthalmol Vis Sci*. 2001; 42:1375–1379. [PubMed: 11328754]
- Ranchon I, et al. Functional protection of photoreceptors from light-induced damage by dimethylthiourea and Ginkgo biloba extract. *Invest Ophthalmol Vis Sci*. 1999; 40:1191–1199. [PubMed: 10235553]
- Ranchon I, et al. Free radical trap phenyl-N-tert-butyl nitrone protects against light damage but does not rescue P23H and S334ter rhodopsin transgenic rats from inherited retinal degeneration. *J Neurosci*. 2003; 23:6050–6057. [PubMed: 12853423]
- Rzagalinski BA. Nanoparticles and cell longevity. *Technol Cancer Res Treat*. 2005; 4:651–659. [PubMed: 16292885]
- Rzagalinski BA, et al. Radical nanomedicine. *Nanomedicine (Lond)*. 2006; 1:399–412. [PubMed: 17716143]
- Sakai T, et al. Prolonged protective effect of basic fibroblast growth factor-impregnated nanoparticles in royal college of surgeons rats. *Invest Ophthalmol Vis Sci*. 2007; 48:3381–3387. [PubMed: 17591912]
- Sancho-Pelluz J, et al. Photoreceptor cell death mechanisms in inherited retinal degeneration. *Mol Neurobiol*. 2008; 38:253–269. [PubMed: 18982459]
- Sanz MM, et al. Significant photoreceptor rescue by treatment with a combination of antioxidants in an animal model for retinal degeneration. *Neuroscience*. 2007; 145:1120–1129. [PubMed: 17293057]
- Schubert D, et al. Cerium and yttrium oxide nanoparticles are neuroprotective. *Biochem Biophys Res Commun*. 2006; 342:86–91. [PubMed: 16480682]
- Schuettauf F, et al. Adeno-associated viruses containing bFGF or BDNF are neuroprotective against excitotoxicity. *Curr Eye Res*. 2004; 29:379–386. [PubMed: 15764082]
- Seger R, Krebs EG. The MAPK signaling cascade. *Faseb J*. 1995; 9:726–735. [PubMed: 7601337]
- Sharma AK, Rohrer B, et al. Calcium-induced calpain mediates apoptosis via caspase-3 in a mouse photoreceptor cell line. *J Biol Chem*. 2004; 279:35564–3572. [PubMed: 15208318]
- Singh N, et al. Treatment of neurodegenerative disorders with radical nanomedicine. *Ann N Y Acad Sci*. 2007; 1122:219–230. [PubMed: 18077575]
- Singh N, et al. Antioxidant properties of cerium oxide nanoparticles. *Free Rad. Biol. Med*. 2006; 41:s152.
- Strasser A, et al. Apoptosis signaling. *Annu Rev Biochem*. 2000; 69:217–245. [PubMed: 10966458]

- Takimoto E, et al. Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load. *J Clin Invest.* 2005; 115:1221–1231. [PubMed: 15841206]
- Tanito M, et al. Sulforaphane induces thioredoxin through the antioxidant-responsive element and attenuates retinal light damage in mice. *Invest Ophthalmol Vis Sci.* 2005; 46:979–987. [PubMed: 15728556]
- Tanito M, et al. Cytoprotective effect of thioredoxin against retinal photic injury in mice. *Invest Ophthalmol Vis Sci.* 2002a; 43:1162–1167. [PubMed: 11923261]
- Tanito M, et al. Attenuation of retinal photooxidative damage in thioredoxin transgenic mice. *Neurosci Lett.* 2002b; 326:142–146. [PubMed: 12057848]
- Trovarellis, A., et al. Catalysis by ceria and other related materials. London, UK: Imperial College Press; 2002.
- Uno K, et al. Role of Nrf2 in retinal vascular development and the vasoobliterative phase of oxygen-induced retinopathy. *Exp Eye Res.* 2010; 90:493–500. [PubMed: 20064509]
- Wahlin KJ, et al. Neurotrophic factors cause activation of intracellular signaling pathways in Muller cells and other cells of the inner retina, but not photoreceptors. *Invest Ophthalmol Vis Sci.* 2000; 41:927–936. [PubMed: 10711715]
- Wen R, et al. Alpha 2-adrenergic agonists induce basic fibroblast growth factor expression in photoreceptors in vivo and ameliorate light damage. *J Neurosci.* 1996; 16:5986–5992. [PubMed: 8815881]
- Wen R, et al. Continuous exposure to bright light upregulates bFGF and CNTF expression in the rat retina. *Curr Eye Res.* 1998; 17:494–500. [PubMed: 9617544]
- Wenzel A, et al. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog Retin Eye Res.* 2005; 24:275–306. [PubMed: 15610977]
- Xie L, et al. Ras signaling is essential for lens cell proliferation and lens growth during development. *Dev Biol.* 2006; 298:403–414. [PubMed: 16889766]
- Yamada T, et al. Thioredoxin-1 protects against hyperoxia-induced apoptosis in cells of the alveolar walls. *Pulm Pharmacol Ther.* 2007; 20:650–659. [PubMed: 17045827]
- Zeiss CJ, et al. Caspase-3 in postnatal retinal development and degeneration. *Invest Ophthalmol Vis Sci.* 2004; 45:964–970. [PubMed: 14985318]
- Zhang F, et al. Cerium oxidation state in ceria nanoparticles studied with X-ray photoelectron spectroscopy and absorption near edge spectroscopy. *Surf. Sci.* 2004; 563:74–82.
- Zhou F, et al. Attenuation of neuronal degeneration in thioredoxin-1 overexpressing mice after mild focal ischemia. *Brain Res.* 2009; 1272:62–70. [PubMed: 19328186]

Research Highlights

- There are currently no successful treatments for inherited retinal degeneration.
- Reactive oxygen species are common to all neurodegenerative diseases.
- Nanoceria destroy ROS and slow inherited retinal degeneration in tubby mice.
- Nanoceria may slow progression of macular degeneration & diabetic retinopathy.
- Nanoceria may also be therapeutically beneficial in many ocular diseases.

Figure 1.

Nanoceria inhibit loss of function in the *tubby* mouse retina as determined by electroretinography (ERG) at P34. Full field ERG assessment demonstrated that nanoceria treatment (*tub/tub-CeO₂*) improved rod function in *tub/tub* mice when compared to saline treatment (*tub/tub-saline*) (A). The image shown is the average amplitude of 4 eyes from each group. Serial intensity ERG of scotopic a-wave (B) and b-wave (C) demonstrated that both amplitudes were significantly increased in nanoceria treated *tub/tub* eyes compared to the saline injection group. N=4 in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Figure 2.

Nanoceria provide pan-retinal protection of *tubby* photoreceptor cells. Photomicrographs of H&E stained retinas in superior from *tub/tub* treated with saline (*tub/tub*-saline), or with CeO₂ (*tub/tub*-CeO₂) and *tub/wt* injected with saline (*tub/wt*-saline) (A). Quantitative histology (B) shows that the outer nuclear layer (ONL) thickness was greatly reduced in the *tub/tub* mice (*tub/tub*-saline) compared to the *tub/wt* but was less reduced in nanoceria treated *tub/tub* eyes (*tub/tub*-CeO₂). Data shown in (C) is the average of all the measurements in the same group. N=54 measurements in each group. Scale bar, 100μm. *** P<0.001

Figure 3.

Nanocerium increase the expression of bFGF, FGFR, Ras, p-ERK in the *tubby* retina. Western blots (A) were used to evaluate expression levels of survival signaling pathway components. Densitometric analysis showed that the elevation of bFGF (B), FGFR (C), Ras (D) and p-ERK (E) are statistically significant and comparable to the *tub/wt* levels when standardized to actin. The level of ERK protein was unaffected by the *tubby* mutation and nanocerium treatment (A). Data shown are representative of 4 western blots (A) and the average (mean $\pm$ SEM) of 4 retinas for quantitation (B-E). * $P < 0.05$; ** $P < 0.01$

Figure 4.

Nanoceria increase the expression of antioxidant associated proteins in the *tubby* retina. Western blot data (A) and densitometric analysis showed that nanoceria treatment increased Nrf-2 (B), N-Nrf-2 (C) and Trx (D) expression in the *tubby* retina to levels equivalent to the *tub/wt* controls. Data shown are representatives of 4 western blots (A) and the average (mean $\pm$ SEM) of 4 retinas for quantitation (B–D). *P<0.05; **P<0.01

Figure 5.

ROS are elevated in the *tubby* retina and decreased by nanoceria treatment. Intracellular ROS levels were detected with dihydroethidium (DHE) for superoxide (O_2^-) (A, B, C) and 2', 7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) for hydrogen peroxide (H_2O_2) (D, E, F) on fresh cryosections at P18. Both assays show increased levels of ROS in the *tubby* retina treated with saline (A, D) compared to the control retina (C, F) and decreased ROS in the retinas of nanoceria treated *tubby* mice (B, E). Data shown are representative of 4 retinas from each group. ONL, outer nuclear layer. Scale bar, 100 μ m, Nuclei are stained with DAPI.

Figure 6.

Nanoceria decrease mRNA expression of caspase-8 and Bak 1 and the activity of caspases-9 and -3 in the *tubby* retina. Semi-quantitative RT-PCR (A) shows increased caspase-8 and Bak 1 mRNA levels with saline treatment and a decreased mRNA levels in the retinas of nanoceria treated *tubby* mice. Quantitative measurements by qRT-PCR showed that the reductions of the gene expression were significant (B & C). Assays for caspases-9 (D) & -3 (E) show that they are elevated in saline treated *tubby* retinas and decreased in the nanoceria treated mice. Data are from 4 retinas from each group and representative data are shown in (A) and the averages (mean $\pm$ SEM) in (B-E). * $P < 0.05$; ** $P < 0.01$

Figure 7. Nanoceria decrease indicators of apoptosis in the *tubby* retina. Levels of total and cytoplasmic cytochrome *c* are increased in the *tubby* retina (A, B, C) compared to the *tub/wt* control retina and nanoceria treatment decrease both. The TUNEL assay (D) shows similar changes with *tub/tub* (a) having many more TUNEL positive nuclei than the *tub/wt* retina (c) whereas treatment of *tubby* with nanoceria reduces these numbers. Data shown (A, D) are representative of those from 4 retinas from each group and their average (mean $\pm$ SEM) (B, C). * $P < 0.05$, Scale bar, 40µm.

Figure 8.

Documented changes in signal pathways in the *tubby* retina induced by nanoceria. The arrows within the title box indicate the changes in *tubby* retinas compared to wild type. The arrows in the colored boxes indicate nanoceria induced changes, either up or down, in the *tubby* retina. Neuroprotective genes are up regulated and pro-apoptosis genes are down-regulated by nanoceria. We think all of these nanoceria-induced changes are due to ROS downregulation and are indirectly regulated by nanoceria.