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MINIREVIEWS

Cerium oxide nanoparticles as promising ophthalmic therapeutics for the treatment of retinal diseases

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Abstract

Nanotechnology offers exciting new approaches for biology and medicine. In recent years, nanoparticles, particularly those of the rare metal cerium, are showing potential for a wide range of applications in medicine. Cerium oxide nanoparticles or nanoceria are antioxidants and possess catalytic activities that mimic those of super oxide dismutase and catalase, thereby protecting cells from oxidative stress. The retina is highly susceptible to oxidative stress because of its high oxygen consumption and high metabolic activity associated with exposure to light. Many retinal diseases progress through oxidative stress as a result of a chronic or acute rise in reactive oxygen species. Diseases of the retina are the leading causes of blindness throughout the world. Although some treatments may delay or slow the development of retinal diseases, there are no cures for most forms of blinding diseases. In this review is summarized evidence that cerium oxide nanoparticles can function as catalytic antioxidants *in vivo* in rodent models of age-related macular degeneration and inherited retinal degeneration and may represent a novel therapeutic strategy for the treatment of human eye diseases. This may shift current research and clinical practice towards the use of nanoceria, alone or in combination with other therapeutics.

Key words: Nanoceria; Age-related macular degeneration; Inherited retinal degeneration; Oxidative stress; Antioxidant

Core tip: This review outlines the recent findings that cerium oxide nanoparticles (nanoceria) may represent novel and broad spectrum therapeutic agents to treat retinal diseases including age-related macular degeneration, retinal angiomatous, inherited retinal degeneration, and fight inflammation and pathologies associated with oxidative stress.

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INTRODUCTION

Many retinal diseases including retinopathy of prematurity, inherited retinal degeneration, diabetic retinopathy, retinitis pigmentosa, glaucoma, and age-related macular degeneration are the leading causes of blindness in infants, adults, and the elderly, respectively. The etiology or development of many retinal diseases involves oxidative stress[1-4]. An imbalance between the production of reactive oxygen species (ROS) and the detoxification of their reactive intermediates causes oxidative stress[5]. Excessive ROS levels can damage lipids, proteins, and nucleic acids. This process subsequently leads to cell death unless it is neutralized by the oxidant defense system. The retina possesses the highest rate of oxygen metabolism and therefore is at higher risk of oxidative damage due to redox imbalance.

Besides traditional antioxidant agents, in recent years special attention has been given to cerium oxide nanoparticles or nanoceria as antioxidants in biological systems[6,7]. Cerium (Ce) is a rare earth element in the lanthanide series of the periodic table. Cerium oxide (CeO_2) nanoparticles are used extensively in a variety of applications such as oxygen sensors[8,9]. The underlying molecular mechanism for the action of cerium oxide nanoparticles is generally thought to be their dual oxidation state, depending on the reaction conditions[10,11]. Nanoceria switch between Ce^{4+} and Ce^{3+} states creating an oxygen vacancy. This capability of these nanoparticles is similar to that of biological antioxidants[12]. Because of these unique antioxidant properties nanoceria act as free-radical scavenger. Free radical scavenging by nanoceria functions by decreasing ROS and has potential uses in various biological applications[7]. It has been recently reported that cerium oxide nanoparticles possess neuroprotective[13,14], radioprotective[15], cardioprotective[16], anti-inflammatory[17], anti-invasive[18], pro-oxidative and antioxidative[19-23], anti-angiogenic[24], pro-apoptotic and anti-apoptotic[21,12] properties. During the past few years, much attention and efforts has been made at addressing the potential use of nanoceria as therapeutic antioxidants for the treatment of oxidative stress related diseases[25-27]. Due to their smaller particle size at about 5 nm in diameter, which allows for easier passage through cell membranes, non-toxic nature and excellent biocompatibility, cerium oxide nanoparticles also have the potential to be used as drug carriers and delivery agents.

In the last few years, our group is involved in developing cerium oxide nanoparticles as therapeutic agents for treatment of retinal diseases. We demonstrated for the first time that these nanoparticles are able to prevent the increases of intracellular ROS concentrations *in vitro* using primary cell cultures of rat retina and could protect retinal morphology and function *in vivo* using an albino rat light-damage model[28]. Next, in the homozygous *tubby* mutant mouse, which displays inherited early progressive cochlear and retinal degeneration that are similar to those of human Usher syndrome, we showed that cerium oxide nanoparticles preserve the retina by decreasing the concentrations of ROS, up-regulating the neuroprotection-associated genes expression; down-regulating apoptosis signaling pathways and/or up-regulating survival signaling pathways[29]. Furthermore, in an age-related macular degeneration (AMD) model and in particular for retinal angiomatous proliferation (RAP), the very low-density lipoprotein receptor knockout mouse (*vldlr*$^{-/-}$), we have reported that cerium oxide nanoparticles stopped the development and regression of pathological neovascularization[30]. Our data also demonstrated that nanoceria inhibited the expression of genes associated with inflammation, angiogenesis, and down-regulated MAP kinases, Akt, ASK1 and NF-κB signaling pathways[31,32]. This review aims to provide the recent findings and potential applications of nanoceria for the treatment of retinal diseases.

OXIDATIVE STRESS AND RETINAL DISEASES

Oxidative stress is defined as a disturbance in the balance between the production of ROS, which include hydrogen peroxide, superoxide anion, and hydroxyl radicals, and antioxidant defenses. Although ROS have important roles in regulating signal transduction and cellular function[33], their overproduction can damage lipids, proteins, and DNA, thus affecting many cellular and physiological mechanisms. Numerous pathological conditions have an oxidative stress component, including cardiovascular diseases[34], neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases[35-37], and cancer[38]. Oxidative stress has also been implicated in retinal diseases such as AMD, inherited retinal degeneration, diabetic retinopathy, retinitis pigmentosa, glaucoma and uveitis[1-4,39]. The retina is extremely vulnerable to ROS damage[40]. ROS can be formed in many ways including as a product of the respiratory chain in mitochondria, photochemical and enzymatic reactions as a result of the exposure to ultraviolet light, ionizing radiation, or heavy metal ions[41-47]. Retinal cells have the highest rate of oxygen metabolism of any cells and are frequently exposed to the damaging effects of oxidative stress due to the the excessive exposure to light.

AMD is the leading cause of severe and irreversible loss of vision in the elderly in the world. AMD is divided into two broad types: "dry" and "wet" that account for about 85% and 15% of cases, respectively. "Wet" or exudative AMD, is the most severe form of AMD and is associated with subretinal neovascularization. By contrast, "dry" also known as atrophic or non-exudative AMD, tends to exhibit a slow progression of the disease. This complex disease has both genetic and environmental risk factors with a number of gene polymorphisms being identified which increase susceptibility to environmental risk factors such as smoking, hypertension, diet, obesity, prolonged sun exposure, and oxidative stress[4,48,49]. While there is currently no cure for AMD, some treatments can prevent severe vision loss or decrease the progression of the disease considerably. AMD treatments include anti-

vascular endothelial growth factor (VEGF) therapy, laser surgery, photodynamic therapy, vitamins and nutritional supplements[50-55]. The abundance and complex interactions between the risk factors for AMD limit the effectiveness of therapeutic options. Therefore, new therapeutics is needed to target multiple pathophysiological aspects that contribute to development of AMD, most importantly oxidative stress.

There are other inherited and acquired diseases or disorders that may affect the retina. Retinitis pigmentosa (RP) is a heterogeneous group of inherited ocular diseases that result in a progressive retinal degeneration. RP is the largest Mendelian genetic cause of blindness affecting 1 in 3000 to 5000 people worldwide[54]. This disease exhibits abnormalities in the photoreceptors or in the retinal pigment epithelium of the retina, which lead to progressive visual loss. RP can be inherited in an autosomal dominant, autosomal recessive or X-linked manner[55]. RP may also occur as part of Usher syndrome and Bardet-Biedl syndrome[56]. Usher syndrome is the most common hereditary form of combined deafness and blindness in humans[55]. The oxidative stress hypothesis is supported by several lines of evidence in experimental models of Retinitis pigmentosa[57-60]. In addition, it has been found that Retinitis pigmentosa patients have reduced ocular antioxidant status and antioxidant imbalance in the peripheral blood[60]. Although there is no cure for RP, treatments are available for managing some aspects of its clinical manifestations[61].

CERIUM OXIDE NANOPARTICLES

Cerium belongs to the lanthanide series of rare earth elements. Although most of the rare earth elements of the periodic table exist in the trivalent state, cerium in an oxide nanoparticle can occur in either a 3+ (fully reduced) or 4+ (fully oxidized) state and may flip-flop between the two in a redox reaction. As a result of this, cerium oxides form oxygen vacancies or defects in the lattice structure[11,62]. It is these defects or reactive sites on the cerium oxide nanoparticles that serve as sites for free radical scavenging. Cerium oxide nanoparticles react catalytically with ROS, including hydroxyl radical, superoxide radical and hydrogen peroxide, providing antioxidant properties[12,63]. It has been demonstrated that cerium oxide nanoparticles act as a catalyst that mimics enzymatic antioxidants including superoxide dismutase (most apparent when cerium is in the 4+ state)[64] and catalase (most apparent when cerium is in the 3+ state)[65]. Various techniques including frame spray pyrolysis[66] and wet chemical methods[12,17] have been reported to synthesize cerium oxide nanoparticles. The radical scavenging activities of cerium oxide are even further increased when synthesized as a nanoparticle. Moreover, as the size of the cerium oxide nanoparticle decreases, there is a concurent increase of cerium in the +3 state, which may further enhance reducing power[67]. Smaller diameter nanocrystals containing more cerium (+3) were found to be more reactive toward hydrogen peroxide[68]. In addition, the presence of a surface coating did not prevent the reaction between the nanocrystal surface cerium (3+) and hydrogen peroxide[68]. Therefore, the most reactive nanoparticles are at about 5-10 nm diameter with the thinnest surface coating (*e.g.*, oleic acid). The radical scavenging properties of cerium oxide can be drastically increased during the reduction to the nanoscale.

Cerium oxide nanoparticles used in our studies were synthesized using wet chemical method as described previously[12]. Briefly, cerium nitrate hexahydrate was dissolved in distilled water and the solution was oxidized using excess of hydrogen peroxide. To maintain the synthesized nanoparticles in suspension, the pH of the solution was kept below 3.0. These cerium oxide nanoparticles contain individual crystallites of 3-5 nm and can be diluted in aqueous and cellular media. The size and shape of the particles was characterized using transmission electron microscope, zeta potential of the suspension was monitoring using dynamic light scattering and X-ray photoelectron spectroscopy was used to determine the surface oxidation state of the nanoparticles as reported previously by us[69].

BIOLOGICAL PROPERTIES OF CERIUM OXIDE NANOPARTICLES

Although cerium oxide nanoparticles have been widely used as oxygen sensors[9] and automotive catalytic converters[70], they have recently begun to be used in biological systems[6,7]. The ability of these nanoparticles to switch oxidation states and their antioxidant activity has a unique advantage for therapeutic implications. The biological properties using *in vivo* mice models of AMD and inherited retinal degeneration and potential applications of cerium oxide nanoparticles as ophthalmic therapeutics are discussed below.

Antioxidant properties

The antioxidant properties of nanoceria were investigated first in primary cell cultures of dissociated rat retinas. Chen *et al*[28] demonstrated by flow cytometric analysis of dichlorofluorescein (DCF) stained retinal cells that nanoceria particles (1, 3, 5, 10 or 20 nmol/L) can effectively inhibit hydrogen peroxide-induced rise of intracellular ROS. Next, we showed that cerium oxide nanoparticles possessed radical scavenging activity *in vivo* by preventing increases in retinal ROS in an albino rat light-damage model[28]. Furthermore, we explored the *Vldlr* knockout mouse, which carries a loss-of-function mutation in the *Vldlr* gene[71]. Studies have revealed that the *Vldlr*$^{-/-}$ mouse recapitulates many key characteristics in patients with AMD who have Retinal Angiomatous Proliferation, a form of wet AMD, and can serve as a unique mouse model of neovascularization-associated oxidative stress[72-74]. Our studies have revealed that a single intravitreal injection of 1 μL of 1 mmol/L (172 ng) nanoceria suspended in saline at postnatal day (P)7 greatly reduced the amount of ROS, measured by

two independent methods, DCF and dihydroethidium (DHE), in the *Vldlr*$^{-/-}$ retinas three weeks later at P28[28]. Similar results were obtained with three other biomarkers of oxidative damage, NADPH oxidase (p47phox), nitrotyrosine and 8-hydroxy-2-deoxyguanosine (8-OHdG). We further confirmed our previous observation by demonstrating that acrolein, a commonly used oxidative stress marker for detecting lipid peroxidation, is higher in *Vldlr*$^{-/-}$ retinas and nanoceria greatly reduced the level of acrolein[32].

Key mediators of the biological effects downstream of ROS include several signaling pathways such as MAP kinases, ASK1, and PI3K/Akt[75,76]. We hypothesized that if ROS were destroyed by cerium oxide nanoparticles, the downstream effects should be decreased. Therefore, we determined whether MAP kinases and Akt are elevated in the retinas of *Vldlr*$^{-/-}$ mice and whether nanoceria can inhibit their activation. Both kinases are elevated in *Vldlr*$^{-/-}$ retinas and a single intravitreal injection of cerium oxide nanoparticles for 1 wk inhibits the phosphorylation of ERK, JNK, and the p38 MAPKs, as well as Akt almost to control wild type (WT) mice treated with nanoceria[31]. We further examined the long-term therapeutic effects of cerium oxide nanoparticles in *Vldlr*$^{-/-}$ retinas and showed that phosphorylated ASK1, JNK and p38, as well as NF-κB are remarkably reduced by nanoceria treatment up to 6 wk post injection[32].

In another experimental paradigm, the *tubby* mouse was used as a model of inherited retinal degeneration to test the ability of cerium oxide nanoparticles to act as direct *in vivo* antioxidants. *Tubby* mice are homozygous for a mutation in the *Tub* gene and have hearing loss and retinal degenerations, major hallmarks of Usher syndrome[77]. To examine the ability of nanoceria to alter ROS, we determine the amounts of ROS by DCF and DHE methods in the retina of *tubby* mice at P18 injected intracardially with 20 μL of 1 mmol/L cerium oxide nanoparticles[29]. The levels of ROS in injected with nanoceria retinas were decreased to control levels. Moreover, we demonstrated that the expression of antioxidant-associated proteins, thioredoxin (Trx) and nuclear factor erythroid 2-related factor (Nrf2) is increased after nanoceria treatment. These results clearly suggest that cerium oxide nanoparticles can scavenge ROS in the retina and thereby inhibit oxidative stress in mice models of AMD and inherited retinal degeneration.

Anti-angiogenic properties

Angiogenesis is a process of forming new blood vessels that is a hallmark in the pathology of many diseases including AMD, diabetic retinopathy, and retinopathy of prematurity. Activators of angiogenesis include the VEGF, angiopoietins and members of the fibroblast growth factor (FGF) family. There is considerable evidence that increased production of ROS in the retina participates in retinal angiogenesis. We have shown that upregulation of retinal VEGF can be detected as early as P14 in *Vldlr*-/- mice[30]. To examine if nanoceria treatment could reduced angiogenesis by inhibiting VEGF, we determined the effect of nanoceria on VEGF protein expression in *Vldlr*$^{-/-}$ retinas at P14 and P28. We observed a significant decreased of VEGF in retinas of *Vldlr*$^{-/-}$ mice after a single injection of nanoceria at P7. We examined the localization of VEGF and found that cerium oxide nanoparticles inhibit the ectopic expression of VEGF in the outer nuclear cell layer (ONL) of the *Vldlr*$^{-/-}$ retina. Furthermore, using real-time PCR we demonstrated that cerium oxide nanoparticles dramatically decreased the levels of *Vegfa* expression in *Vldlr*-/- retinas[31]. Our PCR array results also showed that the expression of most of the *Fgf* genes, including *Fgf* 1, 2, 3, 5, 7, 9, 11, 21, and 22, are increased in the retina of *Vldlr*$^{-/-}$ mice and cerium oxide nanoparticles were able to decrease significantly their expression. These results clearly support our hypothesis that the rise in retinal VEGF in *Vldlr*$^{-/-}$ mice can be prevented by the scavenging activity of cerium oxide nanoparticles.

Anti-inflammatory properties

Oxidative stress is well known to increase not only angiogenesis, but to drive the onset of inflammation. There is substantial evidence to show that inflammation play a role in AMD[78]. Although some reports have shown that several inflammatory cytokines are elevated in *Vldlr*$^{-/-}$ retinas[72,79] the expression pattern of cytokines and their functions in the *Vldlr*$^{-/-}$ mice have not been thoroughly determined. Therefore, we examined the cytokine expression in the *Vldlr*$^{-/-}$ retina using a mouse cytokine PCR array that profiles 88 key cytokine genes[31]. We found that 37 cytokines were up-regulated and after one week of nanoceria injection 23 cytokines were down-regulated. Nanoceria markedly reduced the overexpression of Tlsp, Lif, IL-3, IL-7, IL-9, IL-12b, Lep, Ifn1, and others. This study suggests that cerium oxide nanoparticles have significant potential as anti-inflammatory agents.

Anti-apoptotic properties

Excessive production of ROS is the key event leading to cell death or apoptosis. The principle mechanism underlying retinal cell death and consequent blindness in several diseases is apoptosis. Apoptosis of neuronal cells is common to all mutations in *tubby* gene family members[80]. To determine the effect of cerium oxide nanoparticles on apoptosis in the retina of *tubby* mouse, the TUNEL assay was conducted[29]. The *tubby* retina demonstrated many more TUNEL positive cells that control retina. In this study, we also demonstrated that intracardial injection with cerium oxide nanoparticles significantly down-regulated caspase-3, 8, 9 and Bak1 expression. Likewise, we found that nanoceria markedly reduced the levels of caspase-3 in the retina of the *Vldlr*$^{-/-}$ mouse[32]. Taken together, it is obvious that cerium oxide nanoparticles down-regulate caspase-induced apoptosis in the retina of mouse models of AMD and inherited retinal degeneration.

Protection of retinal function

To examine the ability of cerium oxide nanoparticles to protect retinal function, retinal responses to the

light stimulus were determined by full field and serial intensity electroretinography (ERG) in tubby mice at P34[29]. Full field ERG showed that injections with cerium oxide nanoparticles improved rod function in *tubby* mice compared to control, saline injected group. Serial intensity ERG of scotopic a- and b-waves showed that both amplitudes were significantly increased in nanoceria injected *tubby* eyes. Moreover, no changes in retinal functions was detected in nanoceria or saline injected rats for 9 d and even after 4 mo post injection[69]. There were no changes in scotopic a- and b-waves, photopic b-wave, and flicker. These data suggest that cerium oxide nanoparticles did not have side effects in the healthy retina.

Toxicity

There is always a concern regarding the potential toxicity of nanomaterials for biological applications. Several reports have shown that cerium oxide nanoparticles (< 10 nm) are well tolerated by animals and are not toxic[25,81], while others provide conflicting data about toxicity[82,83]. Most likely this discrepancy could be due to variation in methods of synthesis or due to differences in physiochemical properties of nanoparticles, surface charge, aggregation of the particles. Nanoceria used in our studies were small in size (3-5 nm) and well dispersed. To determine the safety of cerium oxide nanoparticles for therapeutic use, the cytotoxic effects of the particles intravitreally injected in rat retina after 9, 60 and 120 d was examined[69]. We performed quantitative analyses on superior and inferior central retina, superior and inferior peripheral retina and we did not determine any reduction in thickness in the layers examined for injected with cerium oxide nanoparticles eyes. As mentioned above there were no changes in retinal function between nanoceria or saline injected rats. These results indicate that cerium oxide nanoparticles synthesized according to our procedure[12,69], are not toxic to the rat retina as evaluated by morphology and function up to 12 mo post injection.

Bio-distribution

We determined nanoceria distribution and clearance in the eye using inductively coupled plasma mass spectrometry[12,69]. We observed the highest concentration of cerium oxide nanoparticles in retinal portion of the eye. A small amount of cerium oxide nanoparticles 1 h post injection were detected in the lens and the rest of the eye cup. We determined that approximately 70% of injected cerium oxide nanoparticles were retained in the rat retina more than 120 d and the elimination half-life is calculated to be 414 d. Only trace amounts of cerium oxide nanoparticles were detected in the liver and kidney from 120 d injected rats. These results strongly suggest that cerium oxide nanoparticles are rapidly and preferentially taken up by retinal cells and the rate of elimination is very slow. It is not yet known the mechanism of uptake of nanoceria in retinal cells. Three possible endocytosis pathways may be involved in uptake of nanoparticles into cells, including caveolae-, clathrin-mediated endocytosis, and macropinocytosis. It has been reported that fluorescein-conjugated nanoceria were taken up by keratinocytes *via* clathrin- and calveolae-mediated endocytic pathways[84]. Recently another study indicated that nanoceria could be also taken up into cells through caveolae- and clathrin-mediated endocytosis. Nanoceria were distributed throughout the cytoplasm but not into nucleus[85].

CONCLUSION

Cerium oxide nanoparticles extended the life of photoreceptor cells and preserved vision for up to 4 mo in a mouse with inherited retinal degeneration. Nanoceria prevent development of pathological neovascularizations in the *Vldlr*$^{-/-}$ mouse (a model for Wet AMD) and also regress vascular lesions existing at the time of injection. Nanoceria have a half-life in the retina of 417 d and had no toxic effect on retinal structure and function when present for over a year. Nanoceria affect multiple signal transduction pathways by upregulating neuroprotective genes and downregulating pro-apoptotic and pro-inflammatory genes. Most recently, we showed that cerium oxide nanoparticles inhibit the growth of inherited retinoblastoma malignancies *in vivo* and shrink the volume of tumors present at the time of injection. Collectively, these data suggest that nanoceria are global antioxidants, which have "pan-disease" effectiveness against a number of degenerative eye diseases in multiple animal models and may be just as effective in the therapeutic treatment of many human eye diseases.

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