Ceria Nanoparticles Reduce Disease Severity in a Mouse Model of Multiple Sclerosis

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ABSTRACT

Cerium oxide nanoparticles (nanoceria) are widely used as catalysts in industrial applications due to their potent free radical scavenging properties. Given that free radicals play a prominent role in the pathology of many neurological diseases, we explored the use of nanoceria as potential therapeutic agent for oxidative injury. Using a mouse hippocampal brain slice model of cerebral ischemia, we have recently shown that ceria nanoparticles reduce ischemic cell death by approximately 50% and decrease the accumulation a wide range of free radicals in brain tissue including peroxynitrite, which was reduced by ~70% (1) . To extend these findings, we have recently investigated the neuroprotective effects of ceria using the murine experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis. Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the CNS that affects more than 2 million people worldwide. Many of the pathological features of the onset of MS are modeled by EAE, an inflammatory disorder induced by immunization with myelin antigens in rodents. EAE is characterized by blood-brain barrier (BBB) breakdown, perivascular infiltration of immune cells, microglia activation, and demyelination. The EAE model has been critical in the development of current therapies used in the treatment of MS.

In the this study, 39 female SJL-EAE mice were treated with either vehicle (n = 17) or vehicle + cerium oxide nanoparticles (custom synthesized, 2.5 nm), 1 day prior to disease induction, the day of induction (both 15 mg/kg) and days 3, 7, 14 and 21days post-induction (6 mg/kg) for the preventative treatment group (n =8). The therapeutic treatment group had the same dosing schedule as the preventative group less the first two injections. Testing included daily clinical scoring and three motor tests to evaluate coordination (rotarod), forelimb strength (hanging wire) and cerebellar function (balance beam). Ceria treatment significantly decreased the onset of the disease for all clinical and motor measures except the balance beam. The onset of clinical symptoms and motor deficits was delayed ~20% in ceria treated groups. Moreover, ceria treatment significantly decreased the severity of clinical symptoms and improved motor performance on all motor tests. Clinical measures were reduced ~40% in ceria treated groups and rotarod performance was improved ~50%, hanging wire performance improved ~60% and balance beam improved ~40%. Taken together, these data show that ceria nanoparticles can ameliorate the severity and motor deficits associated with EAE.

INTRODUCTION

Multiple sclerosis (MS) has long been considered an immune mediated inflammatory disease leading to the degeneration of the myelin sheath surrounding nerve cells and ultimately their death. There are approximately 300,000 patients with the disease with an additional 400 cases diagnosed per week. The inflammatory cascade leading to neuronal death in MS is believed to be responsible for both the acute and progressive motor deficits associated with the disease. Clinical data has revealed that the severity of inflammation, and the symptoms it produces, waxes and wanes over time suggesting that MS is a biphasic disease divided into an inflammatory relapsing-remitting phase and a degenerative chronic-progressive phase. Recent evidence suggests that the activation of different populations of immune surveillance cells are responsible for the distinct phases of the disease. The most common disease course, termed relapsing and remitting MS, is characterized by clearly defined attacks of worsening neurologic and motor function. These relapses or 'flare-ups', are followed by partial or complete recovery periods (remissions), during which symptoms improve and there is no apparent worsening or progression of disease. Approximately 85% of people with MS are initially diagnosed with relapsing-remitting MS (RRMS). Chronic, Progressive Multiple Sclerosis (CPMS) affects approximately 15-30% of the MS patients and is characterized by a steady progression of clinical neurological damage superimposed with relapses and minor remissions [1, 2]. People who develop CPMS often will have previously experienced a period RRMS the duration of which may have lasted between 2 -40 years or more. During the progressive phase of the disease, disability starts advancing much more quickly than it did during the previous RRMS phase. The majority of people with the RRMS will eventually develop CPMS. A patient with RRMS for 10 years will have a 50% chance developing CPMS and approximately 25 years after the onset of RRMS, 90% of the patients will have CPMS.

The inflammatory and neurodegenerative cascades that occur with MS are closely related. At the nexus of these two cellular processes is the generation of free radicals associated with the potent, activation of the immune system. Free radicals are highly reactive chemicals

that are capable of damaging proteins and lipids associated with cell structures that are required for normal cell function. They are formed both during normal and pathological processes although the extent of their accumulation varies greatly during the two conditions. For example, mitochondria generate free radicals as part of a normal series of steps in which carbon based fuels (glucose, fats and proteins) are oxidized by oxygen to generate fuel for cells. The production of these free radicals are normally offset by naturally occurring, antioxidant systems in the cell that serve to neutralize free radicals associated with normal cell metabolism, thus minimizing the damage associated with these highly reactive compounds. In disease states, the accumulation of free radicals far exceeds the capacity of the body to neutralize them leading to cellular damage. Accumulating data indicate that accumulation of free radicals plays a major role in the pathogenesis of multiple sclerosis (MS) [1, 3]. The increase in free radicals in multiple sclerosis is generated primarily by activated immune cells (macrophages and microglia) that mediate the destruction of myelin, the protective sheath covering the axons of nerve cells. The principal free radical species implicated in the majority of damage in MS is peroxynitrite. Peroxynitrite is formed very early during the course of MS and correlates with disease progression in immune-mediated inflammatory diseases in general [4-6]. Oligodendrocytes, the cell type responsible for myelinating nerve axons in the brain, are killed by very low levels of peroxynitrite and result in what is termed 'sclerotic lesions'. Both the presence of nitric oxide and peroxynitrite has been consistently demonstrated in acute and chronic active MS lesions but not in inactive lesions [5, 6] suggesting that this free radical is a key mediator of tissue damage in the brain with MS.

DEVELOPMENT OF CERIA FOR THE TREATMENT OF MULTIPLE SCLEROSIS

Over the past 3 years, we have developed exceedingly small, ceria nanoparticles (1-2.5 nm) that function as very potent, regenerative, antioxidants. The underlying mechanism of action of ceria nanoparticles originates from their high redox potential (1.55 V) and their ability to bind oxygen containing molecules and shift reversibly between Ce^{4+} and Ce^{3+} states:

$$CeO_2 \leftrightarrow Ce_2O_3 + ½\, O_2$$

The loss of oxygen and the reduction of Ce^{4+} to Ce^{3+} are accompanied by the creation of oxygen vacancies in the nanoparticles lattice. These oxygen vacancies provide binding sites for the free radicals which then can be neutralized through electron transfer by the ceria[9]. We have found that the number of free radical binding sites on the nanoparticles and the rate at which they neutralize these radicals can be controlled in by altering the synthesis steps in their fabrication. Technologically, this is a major step forward since the chemistry at the nanoscale for many elements such as ceria is not entirely predictable from traditional chemical reactions with larger, macroscale particles (e.g. 100 nm>). Furthermore, the application of surface coatings to ceria nanoparticles alters the surface charge and can prevent aggregation, minimize protein adsorption, improve biocompatibility and increase cellular uptake and circulation time *in vivo* [7,9]. We have developed a ceria-based nanoparticle product with potent antioxidant properties that can be 'tuned' by altering the surface properties and composition of the particle. The particles consist of biocompatible ceria that are capable of reducing the excess accumulation of peroxynitrite at ~75% in our in vitro animal models as well as neutralizing other biological relevant free radicals [8].

ANIMAL MODELS TO EXPLORE THE NEUROPROTECTIVE EFFECTS OF CERIA

More recently we began testing our nanoparticles *in vivo* in the murine, experimental autoimmune encephalomyelitis model (EAE) of MS which have been instrumental in the development of drugs currently used to treat the disease. The relapsing-remitting form of the disease is modeled well by the $PLP_{139-151}$ peptide-induced EAE Model which shows many of the cardinal features of RRMS. Following disease induction mice develop the first episode of paralysis 11-14 days after immunization and, similar to most MS patients, they fully or almost fully recover from this first wave of paralysis. $PLP_{139-151}$ is extensively expressed in the brain and this mouse EAE model results in sclerotic lesions that resemble many of the histological features present in MS in humans. Using this model we have shown that administration of cerium nanoparticles by an intravenous route (IV) either before (preventative model) or after disease induction (therapeutic model; Table 1) results in significant improvement in the severity of disease, based on clinical scoring, and functional motor testing (Figure 1, n = 40 animals). For these experiments, we used the rotorod as a measure of motor coordination (latency to fall; seconds), the balance beam as an index of balance (higher scores reflect better performances) and fine motor control and the hanging wire as a measure of forelimb grip strength (latency to fall; seconds). These data show ceria treatment significantly reduces disease severity and improves all test of motor function testing (Table 2). Importantly, our data show that delivery of the particle **after the onset** of the disease can attenuate motor deficits and disease severity.

Using induction-coupled mass spectroscopy experiments (ICP-MS) we have shown that our particles penetrated and accumulate in the brain of healthy mice or EAE induced animals similarly suggesting that the degradation of the blood brain barrier associated with the EAE disease did little to improve brain deposition. This finding indicates

that the flux of particles from the capillaries to the brain tissue was *independent* of blood-brain barrier integrity. Given the small size of these particles (~2 nm) relative to endothelial tight junctions (4 nm), the particles likely pass freely through this barrier without the need of carrier-mediated transport although it is possible that they may also be carried by endogenous transporters into the brain parenchyma similar to nano-gold. Compared to other ceria nanoparticles either commercially or custom synthesized, the brain deposition of our particles is 150- 450x higher than other ceria nanoparticles reported in the literature for a similar loading dose. We believe the key to our technology in penetrating the blood-brain barrier is the small, uniform size of the particles and the biocompatible stabilizer/zeta potential associated with the particles.

USE OF CERIA IN THE TREATMENT OF MS

A variety of studies indicate that endogenous antioxidants like glutathione are decreased in MS and damage to mitochondria induced by lipid peroxidation can lead to additional ROS generation. This increase in oxidative load occurs concurrently with the up-regulation of antioxidants in some cells near the active MS lesions, however these endogenous compensatory increases in antioxidant activity is not sufficient to counteract the damaging effects of increased ROS production and activation of inflammatory cascades in MS.

Table 1 Dosing Paradigm Treatment Groups

Design	Day Prior to Induction	Induction Day	Day 3 Post Induction	Day 7 Post Induction	Day 14 Post Induction	Day 21 Post Induction
Preventative	IV (10 mg/Kg)	IV (10 mg/Kg)	IV (6mg/Kg)	IV (6mg/Kg)	IV (6mg/Kg)	IV (6mg/Kg)
Therapeutic	none	none	IV (6mg/Kg)	IV (6mg/Kg)	IV (6mg/Kg)	IV (6mg/Kg)

Figure 1 PLP Model

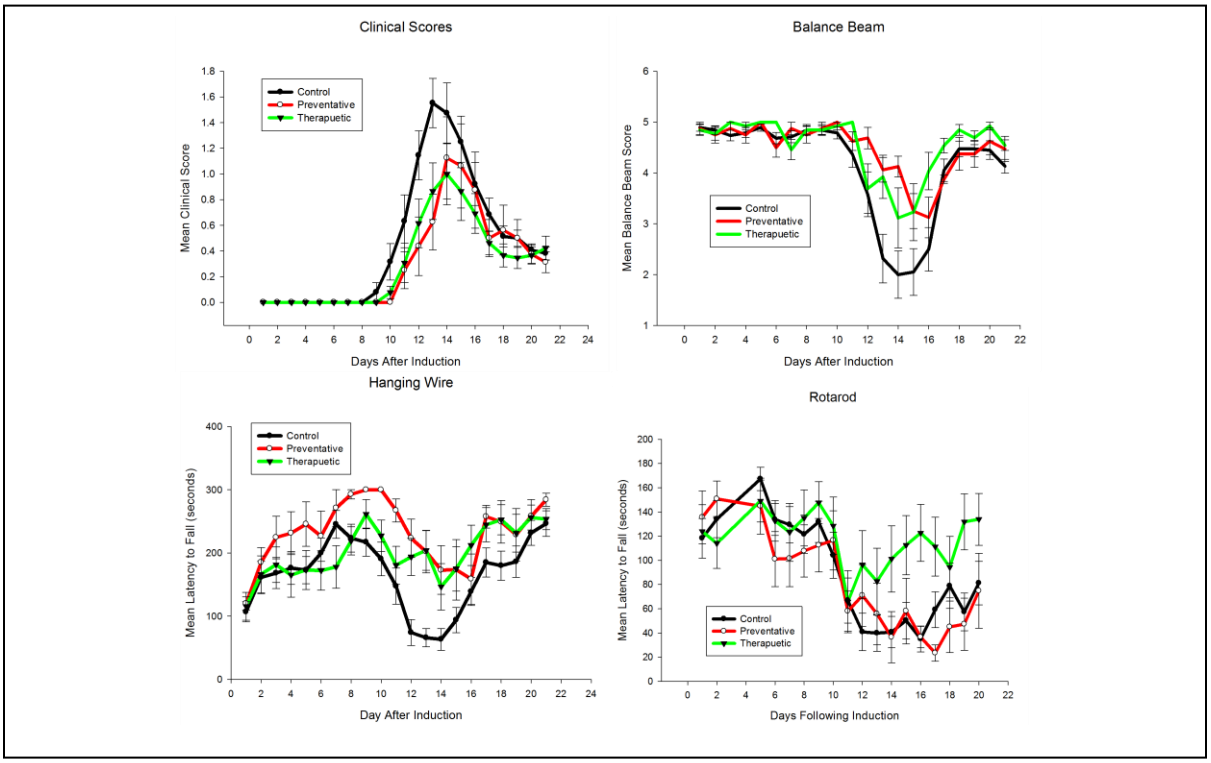

Table 2

PLP Model	Peak Clinical Scores	Rotorod Max Latency to Fall	Hanging Wire Max Latency to Fall	Balance Beam Score
Control (n= 20) vs Preventative (n=8)	$\bar{\times}$ 1.7±0.2 SE $\bar{\times}$ 0.9±0.3 SE p = 0.048	$\bar{\times}$ 33.8±10 SE $\bar{\times}$ 27.1±5.6 SE p = 0.799	$\bar{\times}$ 63.3±17 SE $\bar{\times}$ 158.7±39 SE p = 0.001*	$\bar{\times}$ 1.8±0.4 SE $\bar{\times}$ 4.1±0.58 SE p = 0.005*
Control (n=20) vs Therapeutic (n=12)	$\bar{\times}$ 1.7±0.2 SE $\bar{\times}$ 0.9±0.25 SE p = 0.038*	$\bar{\times}$ 33.8±10 SE $\bar{\times}$ 100.8±16 SD p = 0.003*	$\bar{\times}$ 63.3±17 SE $\bar{\times}$ 146.6±37 SE p = 0.001*	$\bar{\times}$ 1.8±0.4 SE $\bar{\times}$ 3.1±0.63 SE p= 0.005*

Modulating gene expression alone by more recent therapies is not enough to fully compensate for the increased free radical load generated by the disease. Since ceria appears to target only free radical accumulation and importantly has less of an effect on baseline ROS levels, thus we expect that normal redox signaling will be largely unaffected while concurrently attenuating the damaging effects associated with free radical accumulation. Consistent with this is our finding that in vitro measurement of ROS accumulation in several different models has shown that our particles decrease baseline free radical levels approximately 20-30% [8].

We view our technology as complimenting the actions of newly developed drugs by providing additional, needed antioxidant power that cannot be achieved by endogenous mechanisms alone. Previous attempts at using antioxidants to mitigate free radical damage have met with variable results for two principle reasons. First, other antioxidant technologies have poor penetration in the brain and have a finite capacity to neutralize reactive oxygen species. The unique redox potential of ceria allows the particle to continually neutralize ROS as long as it is present in the tissue and its small size permits it to readily cross the blood-brain barrier even in the absence of disease. Second, the catalytic activity of ceria nanoparticles in participating in coupled redox reactions involved in free radical neutralization is orders of magnitude higher in our nanoparticles compared to traditional, dietary antioxidants or other synthetic, free radical spin-trap agents [10].

Taken together, our finding suggest that the development of ceria as potential therapy for oxidative injury may be warranted given their ability to easily traverse biological membranes, their tissue retention and high catalytic activity.

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