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Cerium oxide nanoparticles with antioxidant properties ameliorate strength and prolong life in mouse model of amyotrophic lateral sclerosis

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Abstract

Cerium oxide nanoparticles (CeNPs) neutralize reactive oxygen and nitrogen species. Since oxidative stress plays a role in amyotrophic lateral sclerosis (ALS) in humans and in the $SOD1^{G93A}$ mouse model of ALS, we tested whether administration of CeNPs would improve survival and reduce disease severity in $SOD1^{G93A}$ transgenic mice. Twice a week intravenous treatment of $SOD1^{G93A}$ mice with CeNPs started at the onset of muscle weakness preserved muscle function and increased longevity in males and females. Median survival after the onset of CeNP treatment was 33.0 ± 3.7 days (N = 20), and only 22.0 ± 2.5 days in mice treated with vehicle, control injections (N = 27; $P = 0.022$). Since these citrate–EDTA stabilized CeNPs exhibited catalase and oxidase activity in cell-free systems and in *in vitro* models of ischemic oxidative stress, we hypothesize that antioxidant activity is the protective mechanism prolonging survival in the $SOD1^{G93A}$ mice.

Key words: Oxidative stress; Cerium oxide nanoparticles; Amyotrophic lateral sclerosis

We have been investigating the therapeutic potential of antioxidant, cerium oxide nanoparticles (CeNPs). Cerium oxide nanoparticles are antioxidant on the basis of superoxide dismutase and catalase mimetic activity,[1–3] unlike many other antioxidant therapies (glutathione, ascorbate, vitamin E, etc.), CeNPs are antioxidant on the basis of catalytic activity, and the nanoparticles are not consumed in the redox reaction. The small size of the custom CeNPs (~3 nm diameter; see Supplementary Data for physico-chemical characterization of the CeNPs) gives these nanoparticles unusual access to the central nervous system.[4] These nanoparticles have a relatively long tissue half-life and persist in brain tissue in mice for weeks where they remain biologically active.[4] CeNPs have shown benefit in a variety of disease models in which oxidative stress plays a prominent role: ischemic brain injury,[5–7] Experimental Autoimmune Encephalomyelitis (EAE),[4] heart failure[8] and light-induced damage of the retina.[9]

Given the efficacy of the CeNPs in disease models in which oxidative stress plays a prominent role, we hypothesized that CeNPs might provide similar therapeutic benefit in a murine model of amyotrophic lateral sclerosis (ALS). The majority of patients with ALS have no family history of the disease. However, 5-10% of patients have an inherited form of ALS, and of the familial forms of ALS, approximately 20% are related to inherited mutations of the copper/zinc superoxide dismutase enzyme (SOD1).[10] Transgenic mice that overexpress an $SOD1^{G93A}$ point mutation, which replicates a common mutation in familial ALS, have a toxic gain of function related to

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expression of the mutant SOD1 gene.[11,12] The SOD1^{G93A} mice display many of the biochemical, clinical and pathological features of both familial and sporadic ALS in humans, and the transgenic SOD1^{G93A} animals are often the first model system in which potential ALS therapies are tested.[13,14]

Oxidative stress and oxidative damage have received persistent attention as causes of ALS. First, evidence of excess reactive oxygen species (ROS) and reactive nitrogen species (RNS) and their attendant damage to DNA, RNA, lipids and proteins are abundant in the SOD1^{G93A} mice.[15–19] Evidence of oxidative and nitrative stress is also detectable in tissue from patients with ALS, though the particular manifestations of oxidative stress detected are variable among studies.[16,17,20–22] Second, antioxidant drugs have shown efficacy in animal studies of ALS,[23–26] and edaravone, a drug approved for ALS in humans, is a redox active agent.[27–29] Furthermore, drugs with anti-inflammatory and antioxidant mechanisms of action emerged as the most promising candidates for further investigation in ALS in a meta-analysis of multiple, preclinical drug trials.[30] Finally, ALS shares many pathological and biochemical features with other neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, and evidence of oxidative stress and trials of antioxidant therapies in these diseases also fuel the persistent enthusiasm for antioxidant therapies in ALS.[17,31–33] Antioxidant drugs have not, however, actually been effective in clinical trials in ALS in humans thus far, though this may be changing.[27,28] This failure may reflect more about the pharmacology of particular antioxidants tried rather than the general benefit of antioxidants in the presence of oxidative stress.

There is no genuinely effective treatment for ALS.[10,34] The only widely approved therapy is riluzole, which is expensive and extends life for only two-three months.[35] There have been numerous tantalizingly effective drugs in animal models of ALS, but to date, all of these drugs, except riluzole, have failed in clinical trials in humans.[30,34] As a result, a consensus statement was developed to try to improve the usefulness and predictive power of preclinical, animal studies in ALS.[36] In the current study, we tried to adhere to the consensus guidelines for preclinical testing in ALS, and we tested the hypothesis that treatment of SOD1^{G93A} transgenic mice with CeNPs would ameliorate muscle weakness and prolong survival when CeNP treatment was begun at the onset of motor weakness.

Methods

All protocols involving animals were approved by the St. Lawrence University Institutional Animal Care and Use Committee.

Murine model of ALS

High copy number male transgenic B6SJL-Tg(SOD1*G93A)1Gur/J mice and B6SJLF1/J female mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred. Offspring were genotyped by Mouse Genotype (Carlsbad, CA) using standard PCR primers, and only heterozygotes of both sexes were included in these studies.

CeNP trial in SOD1^{G93A} mice

The onset of muscle weakness was measured using hanging wire testing. SOD1^{G93A} mice were trained on this task for 6 days when males were 83 days old or females were 100 days old to establish a baseline value. Thereafter, each animal was tested on the hanging wire apparatus and weighed twice per week. The onset of muscle weakness was defined by the occurrence of two consecutive decreases in hanging wire performance, and both hanging wire test times had to be less than the average baseline value. A schematic outline of the experiment is shown in Figure 1.

We used a clinical score algorithm to define the onset of clinical disease (Table 1). Both positive and negative attributes of behaviors associated with specific scores were defined, and the date of clinical disease onset was defined by the first occurrence of a clinical score < 5. The surrogate endpoint for death was the righting reflex, a standard test in ALS.[37] In addition, mice were euthanized by an isoflurane overdose for any of the following reasons: prolapse of the anus/uterus, autosarcophagy, loss of ≥20% of body weight or any clinical score ≤2.

After the onset of muscle weakness, the clinical disease and body weight assessment were conducted daily, and the hanging wire testing was conducted two times per week. Once the clinical score was <5, each mouse was weighed daily, the clinical score was assessed twice per day, and the hanging wire test was performed three times per week. Investigators weighing the animals, performing the behavioral scoring and measuring the hanging wire times were all blinded to the treatment each animal received.

Drug therapy with CeNPs started once muscle weakness was apparent on the hanging wire test. We used custom made CeNPs that were synthesized with equal amounts of ethylenediaminetetraacetic acid (EDTA) and citrate as a surfactant (see Supplementary Data). As each mouse developed muscle weakness, it was assigned randomly to one of the treatment groups, either CeNPs or vehicle, control injections. Randomization was stratified by gender. The treatment group received bi-weekly intravenous (I.V.) tail injections via 30 ga needle of CeNPs (20 mg/kg) diluted in 100 μL vehicle (136 mM NaCl buffered with 10 mM Na–HEPES and titrated to pH 7.4). Control animals received identical, bi-weekly, 100 μL, I.V. tail vein injections with vehicle alone. Tail vein injections were given under brief isoflurane anesthesia.

Biodistribution of cerium in SOD1^{G93A} mice

Mice were euthanized with an isoflurane overdose and transcardially perfused with phosphate buffered saline, and internal organs were removed to analyze tissue-specific cerium content. Specimens were digested in nitric acid and prepared for inductively coupled plasma mass spectrometry (ICP-MS) at the Trace Metal and Analytics Facility at Dartmouth College to assess the level of cerium in individual tissues after CeNP treatment.

Statistical analysis

For ordinal data (clinical scores), Freidman's test for nonparametric, multiple comparisons was used to evaluate main effects, and Dunn's post-hoc test (one-tailed) was used to compare treatment groups to control. Day-by-day comparisons

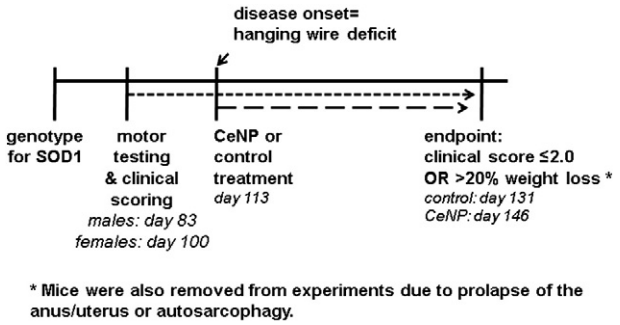

Figure 1. Experimental design used to study the SOD1^{G93A} mouse model of ALS. The time line of the experiment is shown in which the effect of CeNP treatment on motor function (measured by the hanging wire test) and longevity was assessed. Days noted represented the mean for each indicated group. CeNP treatment was initiated at the onset of motor weakness in each animal (indicated by an arrow), which was assessed using the hanging wire test. Clinical onset was defined by a clinical score < 5 (clumsy gait progressing toward hind limb paralysis during disease development).

and other comparisons used Wilcoxon's signed rank test (one-tailed). Hanging wire data were analyzed using a two-way, repeated measures ANOVA to test for main effects, and Dunn's test (one-tailed) was used for post-hoc tests when the ANOVA indicated that significant differences existed among treatment groups. A repeated measures design to test for main effects was used to assess drug effects on clinical scores and hanging wire testing, and the Holm–Sidak method was used for post-hoc analysis. Both Kaplan–Meier survival analysis and Cox proportional hazards analysis were performed to determine whether there were significant differences in survival between the treated and control animals. Any animal that developed symptoms outside of the primary criteria (clinical scoring and body weight), which would indicate a non-neuromuscular cause of death, was censored in the survival analysis.[38] Survival times are reported as medians ± SEMs, and other variables are reported as means ± SEMs unless stated otherwise in the legend.

Results

CeNPs remain monodispersed in physiological solution and exhibit antioxidant function

The CeNPs synthesized for this study had a negative zeta potential (−22.94 ± 1.07 mV), were small (mean hydrodynamic diameter = 3.3 nm;) and uniform in size (polydispersity = 0.176; Supplementary Data, Figure 1). Inclusion of ethylenediaminetetraacetic acid (EDTA) and citric acid (CA) in the synthetic process seemed to be important for surface stabilization, as the EDTA/citric acid stabilized CeNPs did not aggregate after more than 4 weeks in saline solutions (Supplementary Data, Figure 1, *D*). The CeNPs exhibited catalase-mimetic activity similar to equimolar concentrations of the antioxidant, *N*-acetyl cysteine (NAC), but with a much lower oxidase activity than NAC in a cell-free system (Supplementary Data, Figure 2). Moreover, both CeNPs and NAC significantly improved cell viability in an *in vitro* model of ischemic oxidative stress (Supplementary Data, Figure 3) compared to vehicle-treatment ($P < 0.01$ for both comparisons), though a much higher concentration of NAC (1700 fold higher than the CeNPs; 10 mM vs. 5.8 μM, respectively) was required to achieve similar neuroprotection. In addition, CeNPs prevented an oxidative shift in the redox balance of brain tissue *in vitro* after addition of peroxide or induction of tissue oxidation following ischemic stress (Supplementary Data, Figure 4). These data demonstrate that CeNPs have the capacity to resist multiple forms of oxidative stress in brain tissue.

CeNPs ameliorate deficits in motor function

Motor function was assessed serially in SOD1^{G93A} mice by measuring performance on the hanging wire test in order to determine when motor function first diminished. Motor weakness was detected in females (n = 28) at age 117.5 ± 1.5 days and in males (n = 19) at age 94.5 ± 1.5 days. Body weight fell only a small, albeit significant amount ($P \leq 0.05$), throughout the time when muscle strength was declining dramatically (Figure 2).

The onset of muscle weakness was identical in the treatment (11 females, 9 males) and control groups (17 females, 10 males); randomization occurred only after weakness developed. After treatment began, the latency to fall during the hanging wire test continued to decline in the control animals (Figure 3). However, the rate of decline in the CeNP treated animals was significantly slower in both males and females (Figure 3), and the mean latency to fall over the first 25 days of treatment was significantly greater in CeNP treated males and females compared to control animals ($P = 0.019$). Thus, CeNP therapy was associated with better preserved muscle strength (Figure 3).

Effect of CeNP treatment on longevity

Kaplan–Meier survival analysis and Cox proportional hazards regression analysis were performed to determine the effect of CeNP treatment on survival duration. The combined longevity of male and female mice after initiation of treatment or vehicle control is shown in Figure 4, *A*. Male and female mice that received CeNP treatment (beginning at onset of motor weakness) survived significantly longer than the control animals (Figure 4, *A*; $P = 0.022$ log rank test; the Cox proportional hazards analysis revealed identical findings; Table 2). Moreover, regardless of treatment group, the duration of illness was longer in male compared to female mice, though this failed to reach statistical significance (Figure 4, *B*; Table 2; $P = 0.061$). Nevertheless, female mice lived longer than male mice across both treatment groups ($P = 0.017$; Table 2).

The increased longevity of the CeNP treated animals occurred because the onset of clinical disease (clinical score < 5) was delayed in these animals. Clinical disease developed 23.0 ± 1.5 days after the initiation of CeNP treatment in male mice and 16.0 ± 3.1 days in female mice compared to 11.0 ± 2.3 days in control male mice and 12.0 ± 1.9 days in female control mice. The duration of muscle weakness before the onset of clinical disease in CeNP treated versus control animals, pooled across sexes, failed to achieve statistical significance ($P = 0.061$). We also analyzed survival from the date of birth (DOB) of each

Table 1
Clinical score parameters.

5	Deficits: None Abilities: Test on flat surface. Normal gait and movement. Animal walks on its toes.
4.5	Deficits: Test on flat surface. Clumsy gait. Animal does not transfer weight on toes, but walks flat-footed. When picked by the tail, animals often do not splay their legs out. Abnormal rotational movement in the hip when walking Abilities: Test on flat surface. No dragging of toes, as the foot is dorsiflexed and brought under the body during walking movement. Timing in flexion/extension of limbs during walking movement normal
4	Deficits: Test on flat surface. Limping or dragging of at least 1 hind limb. Toes drag during dorsiflexion. Delay in flexion/extension of leg of either limb during walking movement Abilities: Animal is able to stand on hind legs and bear weight. Forelimb fully functional based on ability to bear weight
3	Deficits: One hind limb drags (extended) and is not spontaneously flexed by the animal. Animal is unable to stand and bear weight on one hind limb. Abilities: Forelimbs are able to bear weight, and animal has a well-groomed face. Animal can right itself from either side when tested in the home cage. Contralateral limb flexes and extends during walking movement; movement may occur slowly.
2.5	Deficits: Both hind limbs are extended and there is no period of flexion evident. Loss of weight bearing and inability to stand on both hind limbs. Abilities: Animal can right itself within 30 seconds from either side in the home cage. Animal forelimb function is preserved based on movement in cage, and animal has a well-groomed face.
2	Deficits: Bilateral hind limb failure and ANY forelimb paralysis, rotational movement in the cage, single forelimb movement and/or a poorly groomed face. Animal is unable to right itself on the same side as the affected forelimb.
1	Deficits: Animal cannot right itself from one side within 30 s.
0	Deficits: Animal cannot right itself from either side within 30 s.

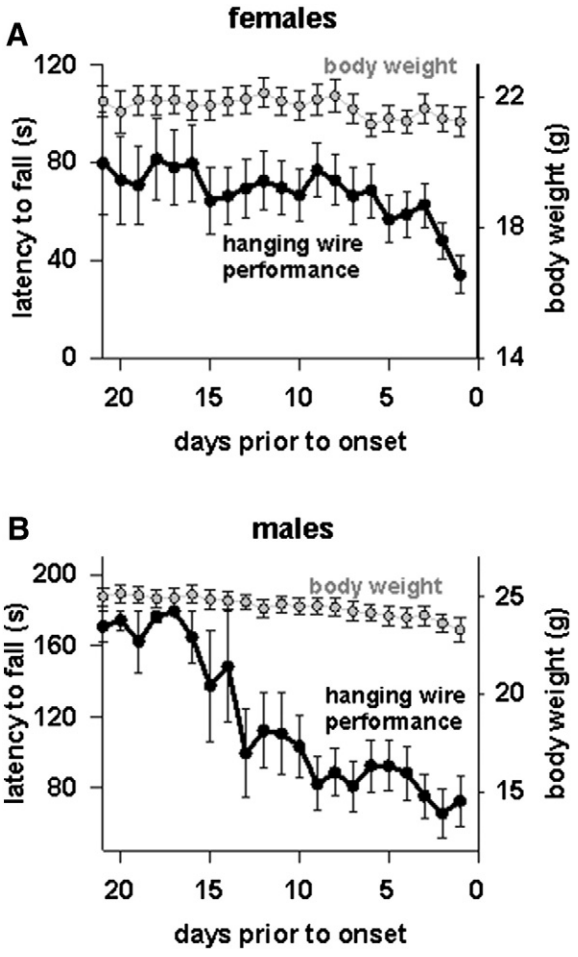

Figure 2. Hanging wire performance was used to identify progression of motor weakness in SOD1^{G93A} mice. Two consecutive days of reduced hanging wire performance (black line) below mean baseline levels indicated the onset of motor weakness in each female (n = 28) (**A**) and male mouse (n = 19) (**B**). Mean body weight is shown by the gray line in each panel. Note that the change in body weight was subtle until muscle weakness was quite advanced. "Onset" refers to onset of clinical disease defined by clinical score < 5.

animal, and the median estimated survival of control animals was 134.0 ± 4.1 days for females and 119.0 ± 3.9 days in males, compared to 146 ± 2.7 days and 132 ± 3.9 days in CeNP-treated females and males, respectively (these changes were not statistically significant; $P = 0.084$; Table 2).

The onset of clinical disease, defined by a clinical score < 5, was also delayed in the treated animals (Table 2). Once the clinical score was less than 5, the disease progression was not different between treated and untreated animals. The median survival after the onset of clinically defined disease ranged between 7 and 11 days. Thus, the effect of CeNP treatment seemed to be related to extension of the interval between the onset of muscle weakness and the onset of clinical disease, which is consistent with the better preservation of muscle strength in the CeNP treated animals shown in Figure 3.

The animals that lived longer received more injections. Female control mice received 4.9 ± 0.5 injections and males received an average of 7.2 ± 1.2 injections, whereas the CeNP treated animals received on average of 7.1 ± 1.1 injections for the females and 9.1 ± 1.1 injections for the males. Thus, the treated animals, pooled across sex, received significantly more injections ($P = 0.044$).

Based on the survival analysis from the onset of treatment, median survival times increased ~13 days in CeNP treated males and ~8 days in CeNP treated females. The duration of disease seemed to be longer in male mice: male mice developed evidence of muscle weakness at an earlier age than female mice, survived slightly longer with the disease, but still died before the female mice since the onset of disease (no matter whether motor deficit onset or clinical onset) was delayed in female mice.

At the initiation of treatment, the following weights were recorded: control males 22.4 ± 0.68 grams (g), control females 18.7 ± 0.14 g, treatment males 22.8 ± 0.7 g, and treatment females 17.8 ± 0.4 g. Over the course of treatment, body weight fell in both the CeNP treated and control animals, and the body weights at the time of death in the two groups were similar, but because the CeNP treated animals lived longer, the rate of decline of body weight was slower in the CeNP treated group.

Previous preclinical studies in the SOD1^{G93A} transgenic animals have been criticized because animals that died of other causes were included in the mortality statistics.[30,38] In this study,

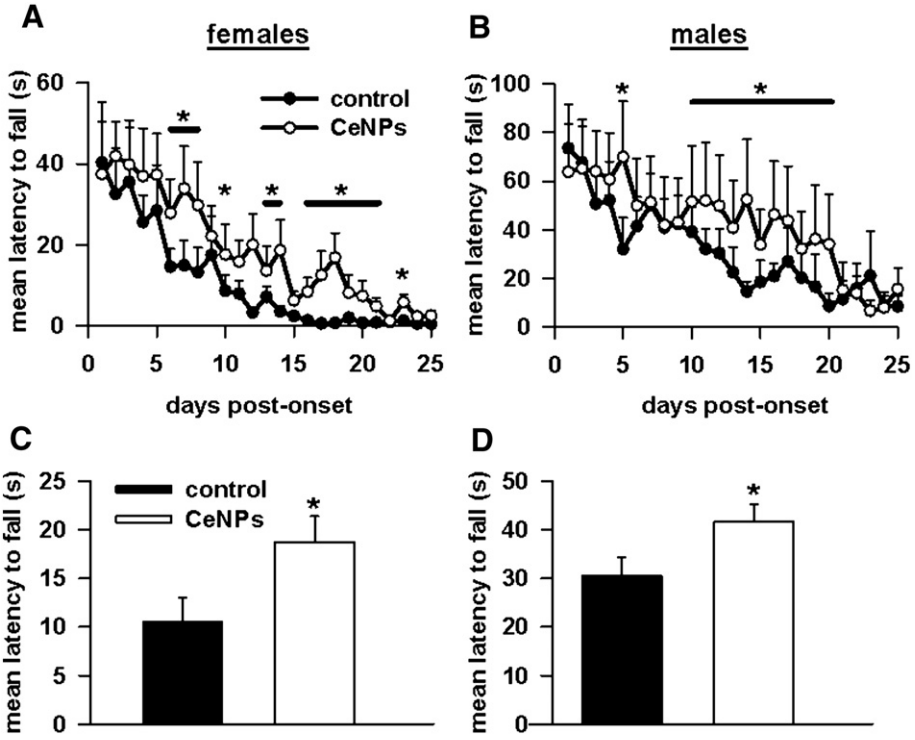

Figure 3. CeNP treatment preserved motor performance of SOD1^{G93A} mice. Treatment was begun at the onset of muscle weakness as defined by decreased hanging wire performance in each mouse. CeNPs (20 mg/kg) or control treatment was delivered intravenously at onset and twice weekly thereafter. Hanging wire performance, indicated by latency to fall from the apparatus, of females (**A**) and males (**B**) was assessed daily. Significant differences between control and CeNP treated animals are indicated by the asterisks for females (days 6-8, 10, 13-14, 16-21, 23) and males (days 5 and 10-20). Mean hanging wire performance during the time period shown in (**C**) and (**D**). The CeNP treatment groups were significantly stronger in both females and males ($P = 0.008$; panel **C** and $P = 0.019$; panel **D** for females and males, respectively).

a similar number of male and female animals were censored from inclusion in the longevity analysis for the following reasons: three animals (2 control and 1 treated) developed autophagy before reaching the clinical disease endpoint; two animals (both treated) developed eye problems that required premature euthanasia; four animals (2 control and 2 treated) lacked complete motor testing data required to define the onset of muscle weakness; one animal (treated) was removed because of a transcription error; and two animals (1 control and 1 treated) never showed any evidence of motor disease which was likely related to a genotyping error.

Cerium levels in treated animals

The concentration of cerium in the brain was correlated with disease protection in our previous study of EAE in mice.[4] Therefore, we assessed tissue levels of cerium in the animals that received CeNP treatment (Figure 5). Tissue concentrations of cerium were highest in the spleen and liver, as seen by others.[4,39–41] Cerium was also detected in the brain and in the kidney. The brain levels of cerium are in the range that one would expect from twice weekly dosing with 20 mg/kg CeNPs.[4] Animals that received no CeNP treatment have brain tissue cerium levels of 4.4 ± 1.5 ng cerium/g wet tissue weight, which is attributable to trace amounts of cerium in mouse chow.

Discussion

Properties of CeNPs and their catalase and oxidase activity

There are several key physical and chemical characteristics of the CeNPs used in this study. The particles are among the smallest synthesized (3.3 nm diameter); they possess a moderately negative zeta potential; they remain monodispered in physiological salt solutions for extended periods of time (the combination of CA/EDTA in the stabilization process reduces the propensity of CeNPs to aggregate, even as the calcium concentration is increased). The catalase activity is significantly higher than larger commercially available CeNPs (Estevez et al., unpublished observations); and the oxidase activity of these CeNPs was considerably lower than NAC. The high catalase and low oxidase activities of the CeNPs translated into significant neuroprotection of brain tissue during oxidative stress.

Translational efficacy of the CeNPs in the SOD1^{G93A} transgenic model of ALS

Despite great optimism about the utility of studies in transgenic animals that model human diseases, the predictive value of the SOD1^{G93A} transgenic animals in forecasting human responses to novel therapies has been poor.[30] Two methodological issues have been identified that reduce the predictive power of animal studies: poor experimental design and study

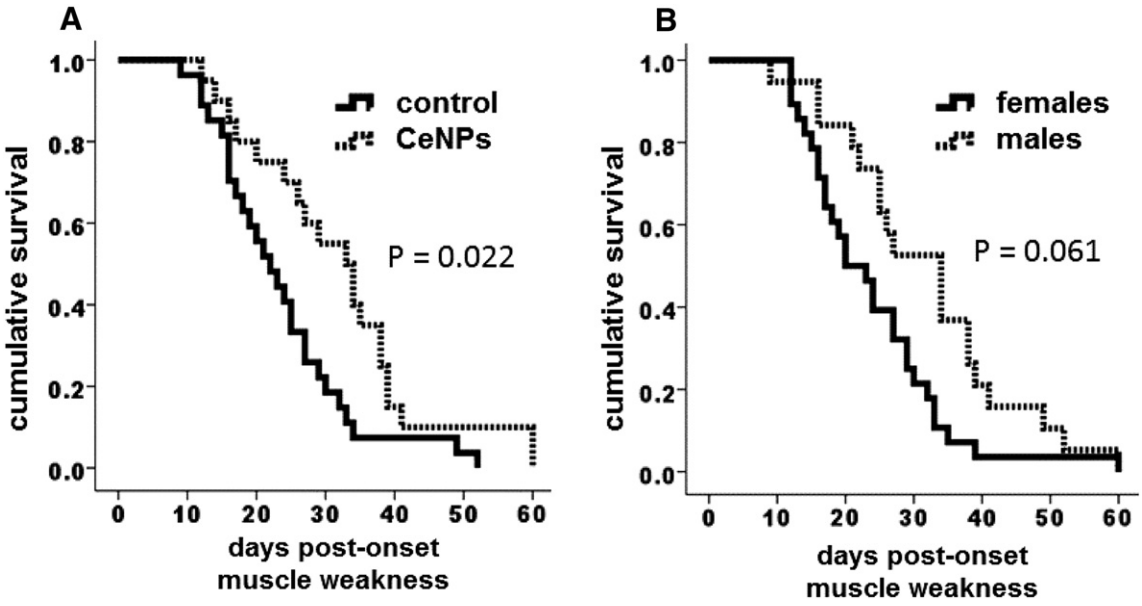

Figure 4. Kaplan–Meier analysis of the survival of SOD1^{G93A} mice. The survival interval of mice treated with CeNPs (n = 20) was significantly longer than control, vehicle treated animals (n = 27; $P = 0.022$ as shown in **A**). The pattern of improved longevity in CeNP treated animals was identical in male and female mice (**B**). After the onset of treatment, male mice (n = ;19) lived longer than female mice (n = 28; $P = 0.061$; panel **B**); this trend did not vary based upon the treatment each animal received. Despite living longer with muscle weakness, the onset of muscle weakness was earlier in male mice, and the longevity of male mice was less than the longevity of female mice (see Table 2).

execution, and treatment schedules that do not simulate the use of therapeutic agents in humans.[30,36,38] Therefore, we followed as closely as possible the recommendations of the consensus guidelines for preclinical testing in ALS.[36] We studied a relatively large number of animals (27 mice in the control group and 20 mice in the treatment group), and each litter of mice had equal representation in each treatment group. We censored animals that died of other causes from the mortality statistics, used explicitly defined endpoints as 'mortality' surrogates, started drug treatment at the onset of muscle weakness, maintained treatment-blinded clinical score assessments, and used appropriate statistical models to analyze mortality data. Moreover, we used the hanging wire test, an explicit assessment of muscle strength, to guide when we started CeNP treatment, and began CeNP treatment only after motor weakness developed in each animal, which simulates the reality of treating humans with ALS.

Effect of CeNP treatment—muscle strength

Motor function declines prior to presentation of clinical symptoms in SOD1^{G93A} mice, and the defined onset of muscle weakness preceded the onset of clinically detectable disease based on clinical disease scoring by ~15 days in males and ~12 days in females (Figure 2), which is similar to the premorbid periods described by others.[42–44] Neither body weight measurements nor clinical disease scoring provided a reliable early indication of the onset of muscle weakness in our study or others.[44–46]

The benefit of CeNPs seemed to be in the prolongation of the period of muscle weakness before the onset of clinical disease, since once the clinical score fell below 5, treated and untreated animals both died over the course of approximately 7–11 days. The prolongation of this pre-morbid period is consistent with the effect of CeNP treatment on the hanging wire scores, which were improved by CeNP treatment compared to vehicle treated control mice before there was any change in the clinical score (Figure 3). In a previous study of EAE in mice, levels of cerium tended to accumulate in the brain, and the higher the brain levels of cerium, the greater the benefit of the treatment.[4] Cerium levels in the brains of CeNP-treated mice in the current study were within the therapeutic range in our previous studies (50–200 ng Ce/g wet brain weight).[4] We believe that the therapeutic benefit of CeNPs is correlated with brain levels of cerium, and were we to repeat this study, it would be appropriate to give a loading dose of CeNPs to try to achieve higher brain tissue CeNP concentrations sooner after the onset of muscle weakness.

In the SOD1^{G93A} mice, significant vacuolization of motor neurons occurs before muscle weakness develops,[42,47] but neurons do not appear to die until late in the course of the illness—symptoms in mice correlate better with abnormal neuronal morphology than they do with the death of neurons.[48] Thus, the prolongation of the premorbid period in the CeNP treated mice is consistent with the idea derived from pathological examinations that neurons remain alive (albeit with diminished function) and susceptible to therapeutic interventions until late in the course of the illness when clinical signs of disease develop and neurons begin to die.

Effect of CeNP treatment—longevity

CeNP treatment significantly prolonged survival in SOD1^{G93A} mice in a study that complied, as closely as possible, with the stringent recommendations for preclinical evaluations of

Table 2
Summary time to event statistics using Kaplan–Meier or Cox regression model.

	Kaplan–Meier analysis				Cox proportional hazards analysis		
	Median estimated days (±SEM)		Log rank	Wilcoxon			
	Control (N = 27)	Drug (N = 20)	*P* values		Hazard ratio	SEM	*P* values
Age of motor onset	113.0 ± 1.5 Males: 95.0 ± 1.0 Females: 118.0 ± 1.5	113.0 ± 2.2 Males: 94.0 ± 1.5 Females: 117.0 ± 1.6	0.871	0.846	1.05	0.317	0.518
Age of clinical onset: treatment effect	126.0 ± 3.1 Males: 110.0 ± 3.9 Females: 128.0 ± 2.5	131.0 ± 2.2 Males: 126.0 ± 7.4 Females: 133.0 ± 2.9	0.245	0.228	1.7	0.319	0.083
Age at time of death: treatment effect	131.0 ± 4.1 Males:119.0 ± 3.9 Females:134.0 ± 4.1	146.0 ± 2.7 Males:132.0 ± 3.9 Females:146.0 ± 2.7	0.084	0.134	1.7	0.315	0.090
Motor deficits to death interval: treatment effect	22.0 ± 2.5 Males: 25.0 ± 2.3 Females: 19.0 ± 2.1	33.0 ± 3.7 Males: 38.0 ± 2.8 Females: 27.0 ± 4.9	0.022	0.014	1.76	0.162	0.026
Motor deficits to clinical score interval: treatment effect	12.0 ± 1.7 Males: 11.0 ± 2.3 Females: 12 ± 1.9	20.0 ± 2.2 Males: 23.0 ± 1.5 Females 16 ± 3.1	0.061	0.102	1.7	0.315	0.075
Clinical scores to death interval: treatment effect	10.0 ± 1.2 Males: 11.0 ± 0.5 Females: 7.0 ± 1.3	11.0 ± 0.5 Males: 10.0 ± 1.5 Females: 11.0 ± 0.5	0.913	0.277	1.032	0.309	0.919
	Males (N = 19)	**Females** (N = 28)					
Age at time of death: sex effect	129.0 ± 2.9 Control: 119 ± 3.9 Drug: 132 ± 2.8	142.0 ± 3.3 Control: 134.0 ± 4.1 Drug: 146.0 ± 2.7	0.017	0.027	1.9	0.308	0.033

potential drug therapies for ALS.[36,38] The difference in median survival between treated and untreated animals (pooled across gender) was ~11 days. The increased longevity associated with CeNP treatment is comparable to previous studies in which therapy started at the onset of motor weakness.[23,26] Moreover, the longevity of the control, vehicle treated animals is well aligned with survival times in vehicle treated high copy number SOD1^{G93A} transgenic animals.[38,43,44] The benefit of treatment did not depend on the sex of the animals—both male and female mice benefitted equally. Males lived a shorter period of time, which is frequently seen in studies of SOD1^{G93A} mice,[38,44] but male mice seem to have a longer disease interval, the period between the onset of muscle weakness and death (Figure 4, *B*). The explanation for the longer period of illness in males may be as simple as the fact that male mice weighed more and could not sustain a grip on the hanging wire as long[46] and so presented with motor weakness at an earlier age.

Comparison to other therapies

Oxidative stress is commonly cited in discussions of the pathogenesis of ALS in the SOD1^{G93A} mice and in humans.[15,17,49–51] Not surprisingly, a wide range of naturally occurring and synthetic antioxidant molecules have been tested to treat ALS. Three previous antioxidant drugs achieved beneficial effects in SOD1^{G93A} mice, yet none of these three drugs has demonstrated efficacy in ALS in humans. AEOL 10150, also a catalytic, SOD mimetic, failed in a Phase II clinical trial (though these results were never published). CDDO, a triterpenoid that triggered endogenous antioxidant processes through nuclear factor erythroid 2-related factor 2 (Nrf2) activation, also failed in human trials.[52] Edaravone—though it reduced levels of 3-nitrotyrosine—failed to show any survival benefit in humans with ALS.[27] Our results in the SOD1^{G93A} animals are comparable to the preclinical results of these previous studies of antioxidants, but an essential, prudent, next step will, nevertheless, entail replication of our study.

The failures of AEOL 10150 and the triterpenoids emphasize that any successful drug in humans must have effective pharmacological properties and must not be toxic or have adverse off target effects. Trials of antioxidants in humans have been hampered by the poor CNS penetration of most drugs and insufficient accumulation of the drug at the putative site of pathology. Moreover, many antioxidants are consumed in the process of neutralizing ROS. Adverse effects of some of these antioxidants have also been attributed to off-target effects of the drug.[52] The results of edaravone studies are most closely similar to our study of CeNPs. In this respect, both agents are redox active,[29] and both agents appear to penetrate the blood brain barrier. In preclinical studies of SOD1^{G93A} mice, both agents slowed the decline in muscle weakness even when the drugs were started late in the course of the disease, but edaravone did not improve longevity, whereas CeNPs significantly increase longevity from the date that treatment started. The greater apparent potency of CeNPs compared to edaravone, though they share a similar mechanism of action, may be attributable to the durable, regenerative catalytic activity of CeNPs.[4,6]

Despite the apparent advantages of CeNPs, there are concerns about their pharmacological properties. CeNPs have had variable tissue uptake depending on the surface treatment of the

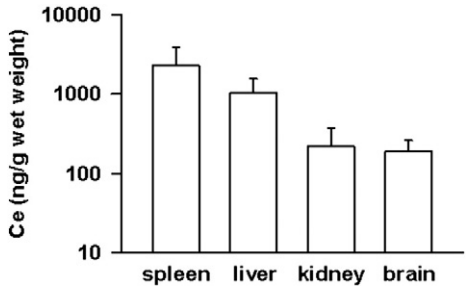

Figure 5. Tissue levels of cerium in the organs of SOD1^{G93A} mice. Data from four CeNP-treated mice were obtained following treatment with 5-8 doses of cerium oxide nanoparticles. Tissues were harvested immediately after each animal was euthanized, frozen and later analyzed by ICP-MS. Cerium content is expressed as nanograms Ce per gram of tissue wet weight. Cerium levels in the brains of untreated, control mice were 4.4 ± 1.5 ng cerium/g wet tissue weight (J.S. Erlichman, unpublished observations).

nanoparticles.[53–55] For example, citrate coated CeNPs rapidly aggregate in physiological solutions.[41,56] Aggregates of CeNPs are rapidly cleared by the liver and spleen which may, in part, explain the high concentrations of cerium found in these organs after administration of CeNPs.[4,39–41,56] Citrate coated particles do not seem to cross the blood brain barrier, but are captured in capillary endothelial cells.[56] In the current study, we did not separate brain parenchyma rich tissue from capillary rich tissue, and so we cannot say with certainty that the cerium levels reported in Figure 5 represent parenchymal cerium. However, transmission electron microscopy (TEM) and measures of tissue redox activity indicated that the CeNPs that we used, which are coated with citrate and EDTA, do cross the blood brain barrier and are broadly distributed throughout the brain tissue.[4] TEM analysis does have limitations when attempting to identify nanomaterial in biological matrices since engineered nanoparticles (ENPs) can be indistinguishable from cellular structures in the same size range.[57] While ICP-MS analysis does not allow localization, it does provide a quantitative measurement of ceria content (Figure 5). Thus, the surface treatment and surface properties of the nanoparticles play a prominent role in the catalytic activity of CeNPs and in their propensity to aggregate[58] and apparently also in their tissue distribution.

Summary

CeNPs are capable of participating in a variety of redox coupled reactions resulting in neutralization of reactive oxygen and reactive nitrogen species. The CeNPs that we used possess unique features—small size, regenerative, catalytic redox activity, and long tissue dwell time—which translate into unusually potent and durable, intracellular, redox activity in the brain. The pharmacokinetic and pharmacodynamic properties of CeNPs are still being characterized, and some, but not all, formulations seem to have good brain penetration and limited off target effects. Using a murine model of ALS, we found that twice per week intravenous administration of 20 mg/kg CeNPs prolonged survival of SOD1^{G93A} transgenic mice even when treatment was started late at the onset of muscle weakness. CeNP treatment was equally effective in male and female mice. We believe that CeNPs achieve this therapeutic effect by acting as redox active agents that reduce the concentration of reactive oxygen and reactive nitrogen species such as H_2O_2, the superoxide radical[4,59] and peroxynitrite anion.[6] The effectiveness of CeNPs in the current study suggests that CeNPs may be developed into an effective antioxidant therapy for a variety of neurodegenerative disorders.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2016.06.009.

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