POST-INJURY ADMINISTRATION OF CERIUM OXIDE NANOPARTICLES: A DOSE-RESPONSE STUDY

by

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ABSTRACT

Following traumatic brain injury (TBI), there is an excessive release of free radicals that trigger processes which contribute to cognitive and behavioral dysfunction. Cerium oxide nanoparticles (CeONPs) can scavenge free radicals, so CeONPs were examined as a potential post-injury therapeutic intervention. However, there is debate regarding the adverse side effects that can result from interfering with free radical activity. For this reason, CeONPs were administered to sham animals in order to monitor the behavioral effects. Long-Evans rats were administered a moderate TBI or were sham injured. Thirty minutes following injury, rats received an intravenous injection of CeONPs or vehicle at a dose of .05 μg/g, .50 μg/g, or 5.0 μg/g. Motor function was investigated on post-injury days 1-5 using a beam balance task, and cognitive function and anxiety levels were measured using the Morris water maze on days 11-15. Results from the beam balance assessment suggest that no dose of CeONPs significantly alters motor function for sham or injured animals. Results from water maze testing suggest a pattern of differential effects of CeONPs on cognitive functioning and anxiety levels for both injured and sham animals. Specifically, injured and sham rats treated with .05 μg/g CeONPs displayed trends towards impaired performance and increased anxiety relative to their respective vehicle comparison groups. In contrast, injured rats treated with 5.0 μg/g CeONPs displayed performance that was comparable to sham animals treated with vehicle. Sham animals treated with 5.0 μg/g CeONPs were less anxious and showed trends of performance that surpassed that of their vehicle counterparts. These results suggest that CeONPs may be useful for the treatment of TBI-induced dysfunction. However, some doses may impair behavioral outcome. Future research should seek to identify the mechanisms by which CeONPs cause these differential effects.

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Traumatic brain injury (TBI) directly affects around 2 million Americans every year (O'Connell & Littleton-Kearney, 2012; Zauner & Bullock, 1995). Medical costs and related expenses for TBI were $60 billion in the year 2000, and this number is expected to rise as more veterans return from overseas with head injuries (O'Connell & Littleton-Kearney, 2012). Brain injuries now account for 20% of cases of epilepsy, and around 40-50% of patients with severe TBI will go on to develop epilepsy that is often intractable to standard medications (Atkins et al., 2010). TBI is frequently accompanied by cognitive and motor deficits including learning and memory impairment (Hamm et al., 1996). Clearly, TBI is an expensive, often seriously debilitating, and universal problem. Thus, it is an issue that is well deserving of scientific attention. Developing new (or improving current) treatments that better the prognosis of TBI will reduce medical expenditures and improve the quality of life of those who suffer a TBI.

Damage from TBI typically occurs in two phases. *Primary injury* consists of the initial mechanical insult that results in some form of brain tissue damage. *Secondary injury* refers to the cascade of events that occur as a result of the primary injury. They can occur immediately or over a period of days, and summate with the initial injury to intensify its damage (O'Connell & Littleton-Kearney, 2012). Examples of secondary injury include inflammation, brain edema, increased intracranial pressure, ischemia, blood brain barrier (BBB) dysfunction, and excitotoxicity (O'Connell & Littleton-Kearney, 2012). These secondary processes can be incredibly detrimental by themselves. For example, cerebral inflammation triggered early on after a TBI promotes the infiltration of white blood cells and creation of pro-inflammatory agents that in turn increase vascular permeability and contribute to the formation of vasogenic edema

(Chatzipanteli, Alonso, Kraydieh, & Dietrich, 2000). Edema leads to increased intracranial pressure, which compromises blood flow to the brain (Feeser & Loria, 2011). Thus, all of these mechanisms intertwine and complicate one another to worsen secondary injury.

Free Radicals

One particular mechanism that is involved in the facilitation of these secondary processes is the production of free radicals, which are biological molecules with one or more unpaired electrons. Free radicals are a normal part of cellular functioning and their presence is essential for various physiological processes to occur. Their highly unstable state leads free radicals to react with nearby molecules, essentially stealing electrons from other molecules and rendering the other molecules dysfunctional (Hirst et al., 2011). Originating primarily from mitochondria, reactive oxygen species (ROS), a family of free radicals, are produced as byproducts from energy metabolism (Fleury, Mignotte, & Vayssiere, 2002; O'Connell & Littleton-Kearney, 2012). ROS are involved in physiological processes such as signal transduction and activating proteins (Fleury et al., 2002) and help to maintain homeostasis and protect the brain during inflammation (Hirst et al., 2011). Under normal circumstances, levels of free radicals are regulated by endogenous antioxidants such as superoxide dismutase, catalase, or glutathione (Fleury et al., 2002).

When the brain experiences some sort of insult (as in TBI), free radicals are more detrimental than beneficial because they are produced at such a rapid rate. This excessive production and subsequent accumulation of free radicals overwhelms the antioxidant system, and endogenous antioxidants are unable to regulate the free radical activity fast enough to prevent their damage. When this occurs, it is called *oxidative stress.*

Due to the involvement of ROS in the initiation phase of apoptosis (Fleury et al., 2002), oxidative stress caused by the excessive production of free radicals can lead to widespread cell death (Kannan & Jain, 2000). Apoptosis is a naturally occurring process of programmed cell death that occurs at the single cell level and is necessary for normal development and the maintenance of cellular homeostasis (Kannan & Jain, 2000). After TBI, there is a sublethal excess of ROS in the mitochondria that trigger widespread apoptotic processes. ROS can chemically destroy cell membranes through lipid peroxidation by damaging proteins and fatty acids. ROS can alter the membrane integrity of mitochondria and so they are able to decrease the energy that is available to the cell (Kannan & Jain, 2000).

In addition to interfering with mitochondrial function and initiating apoptotic processes, free radicals are also involved in the inflammatory response. As previously mentioned, ROS help to protect the brain during inflammation; however, after TBI, ROS are more detrimental than beneficial. After TBI, immune cells like neutrophils and macrophages, along with disrupted endothelial cells produce cytokines, which alter vascular permeability and worsen edema. The resultant increase in damage attracts even more inflammatory cells, which produce ROS (Chatzipanteli et al., 2000) and contribute to the overwhelming accumulation of free radicals. Normal BBB functioning is highly dependent on the ability of brain endothelial cells to defend themselves from free radicals. A state of oxidative stress weakens the defense of the endothelium, and so free radicals themselves are able to increase the permeability of the BBB (Chodobski, Zink, & Szmydynger-Chodobska, 2011).

When the membrane creating the BBB becomes more permeable after TBI, a number of complications to the initial injury arise. First, plasma water can leak through and contribute to vasogenic edema, which then increases intracranial pressure (O'Connell & Littleton-Kearney,

2012). Second, this change in the membrane allows the passage of exogenous agents into the brain that would normally be sequestered to outside this barrier. Excitatory agents are able to enter and summate with endogenous neurotransmitters to overwhelm the brain with excitation (Hayes, Jenkins, & Lyeth, 1992). In addition, the transporter mechanisms of glial cells that are normally responsible for the uptake and metabolism of the excitatory amino acid, glutamate, are not as effective when the BBB does not function properly. This results in less glutamate reuptake. Increased extracellular glutamate quickly leads to hyperexcitability, which can result in epilepsy and neuronal death (Heinemann, Kaufer, & Friedman, 2012).

Memory Deficits Following TBI

Along with the physical changes of secondary brain injury come cognitive deficits such as learning and memory impairment. Animals that receive an experimental brain injury are able to learn procedures, but their working memory is significantly impaired and they quickly forget (Hamm et al., 1996). Pathologically, this makes sense because certain areas of the brain are more susceptible to TBI than others—specifically, the hippocampus, a structure that is well-known for its involvement in learning and memory. Contusional TBI leads to neuronal death in certain areas of the hippocampus such as CA1, CA3, and the dentate gyrus. In this area, long-term potentiation (one cellular model of learning and memory) is mediated by NMDA receptors. While a normal level of excitation is required for cell functioning, the excessive level of extracellular glutamate that is characteristic of TBI renders cells and their NMDA receptors dysfunctional (Hayes et al., 1992). Thus, it makes sense that certain hippocampal-related memory deficits result from TBI.

Increased Anxiety Following TBI

In addition to impaired cognitive functioning following TBI, many individuals suffer from increased emotional distress and anxiety (Jones et al., 2008). For many who experience a mild TBI, heightened anxiety can persist for months after recovery from the initial injury, and this emotional disturbance can significantly reduce quality of life (Mooney & Speed, 2001). Mooney and Speed found that the presence of psychiatric symptoms like anxiety can impact the recovery process, and there is a significant relationship between these debilitating symptoms and poor outcome. The relationship between TBI and anxiety has also been demonstrated in rodent models. For example, injured animals engage in more freezing behavior (Rodgers et al., 2012), and animals with a severe TBI spend less time in the center of an open field; this behavior persists for up to three months following injury (Jones et al., 2008).

While there are many factors related to increased emotionality following TBI, there is evidence that implicates the immune response and subsequent inflammation. Following injury, there is increased inflammation in the amygdala, and Rodgers et al. (2012) found that by suppressing the activation of glial cells following injury (thereby reducing inflammation), symptoms of anxiety are weakened. Thus, it is likely that inflammation, one of the secondary injury processes, is related to the heightened anxiety that is evident following injury.

Treating TBI

The pathophysiology of TBI is quite complex because it involves not only the initial mechanical insult, but also the cascade of secondary processes that contribute to poor outcome. This complexity complicates treatment of TBI, and as a result, there is no standard treatment at this time. Researchers have tried to introduce therapeutic antioxidants in an attempt to assist the overwhelmed endogenous antioxidant system and reduce free radical activity. However, many

of these agents are limited in their clinical efficacy due to their inability to penetrate the BBB, short half-life, and slow uptake (Novack, Dillon, & Jackson, 1996).

Cerium Oxide Nanoparticles

Used in polishing agents and fuel additives (Horie et al., 2011), cerium oxide nanoparticles (CeONPs) mimic enzymatic antioxidants such as superoxide dismutase and catalase (Hirst et al., 2011), so they are often referred to as *free radical scavengers*. There is even evidence to suggest that the superoxide dismutase catalytic rate of CeONPs surpasses that of superoxide dismutase itself (Clark, Zhu, Sun, & Petty, 2011). The unique structure of CeONPs equips them with high "redox" potential. Redox (*red*uction-*ox*idation) potential refers to the ability to facilitate chemical reactions that change the oxidation state of atoms (Wilson & Gelb, 2002). Because endogenous antioxidants are overwhelmed after a TBI, redox potential declines (Wilson & Gelb, 2002). The ability of CeONPs to act as free radical scavengers enables them to trigger redox reactions; in other words, they can regenerate oxidation states (Das et al., 2007).

Pretreatment with CeONPs decreases the production of the free radical nitric oxide and reduces levels of lipid peroxidation after induced-liver toxicity in mice (Hirst et al., 2011). CeONPs diminish hydrogen peroxide-mediated apoptosis without damaging cell membranes (Clark et al., 2011). They are neuroprotective in spinal cord neurons and lead to higher cell survival in cells subjected to hydrogen peroxide-induced oxidative stress (Das et al., 2007). Finally, CeONPs are able to localize to sites of free radical production and appear to be regenerative; for these reasons, CeONPs are far superior to other antioxidant agents that struggle to penetrate the BBB and require repeated administrations to maintain effective concentrations (Das et al., 2007).

While it is known that excessive levels of CeONPs (such as 200 μg/g) can actually *induce* oxidative stress and significantly decrease mitochondrial activity, there are no obvious adverse effects related to moderate doses of CeONPs (such as 100 μg/g; Horie et al., 2011). With concentrations at or below this dose, CeONPs do not appear to interfere with the integrity of cell membranes or to be toxic (Clark et al., 2011). However, CeONPs do remain deposited in tissues over extended periods of time (Hirst et al., 2011), and so it is important to note that there may be cognitive side effects related to CeONPs treatment that are unknown at this time.

CeONPs and Nitric Oxide

Pretreatment with CeONPs decreases the post-injury production of the free radical, nitric oxide, by decreasing protein and mRNA levels of iNOS—an enzyme that is responsible for the synthesis of nitric oxide (Hirst et al., 2011). At normal levels, nitric oxide is involved in the establishment of long-term potentiation (LTP) in the hippocampus. This particular free radical acts as a retrograde messenger that is produced by the postsynaptic neuron; it travels back across the synapse to increase the presynaptic production of glutamate, essentially strengthening the synaptic connection between neurons (Breedlove, Watson, & Rosenzweig, 2010).

LTP is theorized to be one cellular model of learning and memory, and so if CeONPs interfere with this process by reducing the production of nitric oxide, then it is possible that CeONPs adversely affect cognitive performance. Thus, the proposed study will incorporate three doses of CeONPs to investigate their differential effects on cognition after TBI. In addition, there are currently no studies that have examined the potential behavioral side effects of nanoparticle administration in a *normally* functioning animal. Due to its superior redox capacity, CeONPs may assist or surpass endogenous antioxidants in their free radical scavenging duties. However, due to reasons already proposed, CeONPs may also interfere with the establishment of

LTP. For this reason, the proposed study will incorporate sham groups that will receive three doses of CeONPs.

As free radical scavengers, CeONPs should be able to prevent the destructive chain caused by oxidative stress. Thus, post-injury administration of CeONPs should significantly improve functional and pathophysiological outcomes following TBI. Behavioral outcomes were measured using the beam balance task (Dixon et al., 1987), a measure of gross motor ability where duration to remain balanced on a suspended beam is recorded. The Morris water maze, a hippocampal-dependent test of spatial learning that is sensitive to damage in the hippocampus (Hamm et al., 1996) was used to measure cognitive functioning. Specifically, latency to the goal platform, average proximity to the goal platform, and total distance traveled were used as variables to measure cognitive outcome. In addition, thigmotaxis (time spent in the outer zone of the water maze) was used to measure anxiety levels.

Hypotheses

There will be a main effect of day such that all animals, regardless of treatment group, will perform significantly better on the final day of motor and cognitive testing than on the first. In other words, all animals will learn both tasks and improve across trials. Animals will also become less anxious as a result of repeated exposure to the water maze.

Motor. There will be an interaction between treatment group and day of testing. Specifically, sham animals will not demonstrate impaired motor performance following sham surgery. In contrast, injured animals will experience a significant decrease in motor behavior immediately following injury, but this impairment will diminish across days of motor testing.

Cognition. There will be an interaction between treatment group and day of testing. Specifically, a significant difference between groups that is evident on the first day of testing, such that injured animals will perform worse, will diminish toward the final days of testing.

Anxiety. There will be an interaction between treatment group and day of testing. Specifically, a significant difference between groups that is evident on the first day of testing, such that injured animals will demonstrate higher levels of anxiety, will diminish toward the final days of testing.

CeONPs dose effects. The pharmacological literature suggests that there should be a differential effect of the varying doses of CeONPs. However, it is not yet clear how these effects translate into changes in behavior. Thus, there were no specific predictions regarding the differential effects of the doses of CeONPs.

CeONPs in sham animals. Because this project asks a novel research question regarding the side effects of CeONPs in normally functioning animals, there were no specific predictions about the effects of CeONPs in sham animals.

Method

Subjects

The subjects of this study were 69 male rats of Long-Evans descent that were between 3-4 months of age and weighed between 350-450 g at the time of behavioral testing. The animals were housed in group cages in the colony room and had access to standard rat chow and water *ad libitum*. The rats were maintained on a 12-hour diurnal cycle with lights on between 6:00 AM and 6:00 PM.

Animals were randomly assigned to sham + vehicle (n = 9), sham + .05 μg/g CeONPs (n = 10), sham + .50 μg/g CeONPs (n = 8), sham + 5.0 μg/g CeONPs (n = 3)[1], TBI + vehicle (n = 11), TBI + .05 μg/g CeONPs (n = 13), TBI + .50 μg/g CeONPs (n = 8), and TBI + 5.0 μg/g CeONPs (n = 7). All behavioral assessments were run blind to minimize any experimenter bias.

Table 1

Number of Animals in Each Treatment Group (N = 69)

	Vehicle	.05 μg/g	.50 μg/g	5.0 μg/g
Sham	9	10	8	3[1]
TBI	11	13	8	7

[1] *Note.* The small number of animals in this treatment group was due to the exploratory nature of this condition. The 5.0 μg/g was not included in the original research proposal, but these animals were completed for another research project that prematurely terminated. With approval from members of the thesis committee, these animals were included in data analysis. The small n obviously limits the implications of the findings related to these animals.

Attrition. Originally, this study consisted of 74 subjects. However, five animals were removed from analyses due to inadequate righting times. Following preparatory surgery and subsequent injury, three animals righted in less than 500 seconds, which suggests too mild, or complete lack, of an injury. Similarly, two animals righted in more than 1200 seconds, which suggests that their injuries were too severe to be comparable to other injured subjects.

Materials

Fluid percussion injury device. A fluid percussion injury device (VCU Biomedical Engineering, Richmond, VA) was used to injure subjects in TBI groups. This device consists of a Plexiglas cylinder that is 60 cm long and 4.5 cm in diameter. The entire device is filled with saline. At one end of the cylinder is a 2 cm metal housing unit that contains a pressure transducer. Attached to this piece is a male Leur-Loc fitting to which the rat is attached in preparation for injury. At the other end of the cylinder is a piston; to produce injury, a pendulum is released and hits the piston. This forces a small amount of saline out of the device and into the closed cranial cavity of the rat. The amount of pressure is recorded in atmospheres of pressure.

Beam balance. Gross motor function was assessed using the well-established beam balance task (Dixon et al., 1987). Subjects are placed on a narrow wooden beam (1.5 cm wide) that is suspended 90 cm above the ground. The amount of time the animal is able to balance on the beam (up to 60 seconds) is recorded.

Water maze. Originally developed by Morris (1984), the Morris water maze task has repeatedly demonstrated its ability to reliably measure spatial learning in the rat (Hamm et al., 1996). In addition, the water maze can be used to measure anxiety levels; rats are inclined to avoid large open spaces and swim near the perimeter in a novel environment (Treit & Fundytus,

1988). This behavior is called thigmotaxis and is suppressed with anxiolytic agents (Treit & Fundytus, 1988). Thus, it is an accepted measure of anxiety in the rodent.

The water maze task consisted of a plastic pool that is 170 cm in diameter and 60 cm high. The pool was painted white and filled to a depth of 32 cm with cloudy water (made so by non-toxic white paint). A platform with a 10 cm diameter was hidden 2 cm beneath the water in the southeast quadrant of the water maze; the location of this platform did not change across testing. On the walls surrounding the pool were various visual cues that did not vary throughout the experiment.

All movements in the water maze were tracked and recorded with a computer tracking system (AnyMaze, San Diego Instruments). From these records, average proximity to the goal platform, total distance traveled, and time spent in the outer zone of the water maze were recorded and analyzed. In addition, the experimenter recorded average latencies to find the goal platform with a stopwatch.

Procedure

All animal procedures were approved by and conducted in accordance with Radford University's Institutional Animal Care and Use Committee (IACUC) protocol.

General animal preparation. Twenty-four hours prior to surgery, rats were pre-trained on the beam balance task until their average scores ranged between 50 and 60 seconds. This ensured that each subject had successfully learned the task prior to injury.

Animals were fasted for 1 hour prior to surgery. In order to induce anesthesia, subjects were placed in an induction chamber into which isoflurane in a carrier of oxygen gas flowed at 4% for 4 minutes. Upon removal from the chamber, animals received subcutaneous injections of 2 mg/kg lidocaine and 7 mg/kg Marcaine and were then placed in a stereotaxic frame. Isoflurane

was maintained at 2% throughout the duration of the surgery. After a sagittal incision was made in the scalp, a 4.8-mm craniotomy was trephined into the skull above the right parietal cortex midway between bregma and lambda and 2.5 mm lateral to the midline. A modified Leur-Loc connector (plastic hub), 2.6 mm in diameter was glued in placed on the skull surrounding the exposed dura, and dental acrylic was poured around the hub to ensure its stability.

Induction of TBI. For 1 minute prior to injury, isoflurane was increased to 4% to induce deep anesthesia. Then the animal was removed from the stereotaxic frame, attached to the fluid percussion injury device, the pendulum was dropped, and a stopwatch was started. The small volume of saline injected into the closed cranial cavity caused a moderate TBI (2.0 atmospheres). After injury, the animal was removed from the fluid percussion device, and the incision was sutured. Immediately after suturing, animals were placed on their backs and the righting reflex (the amount of time required for the animal to right itself) was recorded by the stopwatch as a supplementary measure of injury (longer latencies corresponded with more severe injuries).

Sham-injured animals underwent similar surgical preparation procedures and were attached to the fluid percussion device, but the pendulum was not dropped, so they did not receive an injury. A stopwatch began recording once the animal was removed from anesthesia.

CeONPs administration. In order to prevent agglomeration of the nanoparticles, CeONPs and vehicle were sonicated for 10 minutes prior to injection. Thirty minutes post-injury, animals were returned to the induction chamber and anesthetized with isoflurane at 4% for 4 minutes upon which time isoflurane was reduced to 2%. While under anesthesia, a single intravenous injection of CeONPs was administered to animals receiving .05 μg/g, .5 μg/g, or 5.0 μg/g CeONPs. A single injection of vehicle was administered to animals receiving vehicle.

CeONPs or vehicle administration required 2 minutes to complete in order to prevent rupture of the blood vessel.

Assessment of behavior, cognitive functioning, and anxiety. On days 1-5 post-injury, gross motor function was assessed with the beam balance task. Length of time the animal remained balanced on the beam (duration) was recorded and averaged across 3 trials for each of the 5 days following injury.

On days 11-15 post-injury, spatial learning and anxiety levels were assessed with the water maze task. At the beginning of testing on each day, start locations (either north, south, east, or west) were randomly selected for each of the 4 trials that day. The first start location was quasi-random because it was never the same as the previous day.

Each animal was placed into the water with his nose facing the wall of the water maze at the previously selected start location and the experimenter started a stopwatch. Animals were allowed 2 full minutes to find the goal platform; the location of this platform never changed. If the rat found the platform, he was permitted to remain on the platform for 15 seconds to allow for observation of extramaze visual cues. If the rat did not find the platform within 2 minutes, the experimenter guided the rat to the platform where he remained for 15 seconds. The computer tracking software recorded total distance traveled, time spent in the outer zone, and proximity to goal platform. The experimenter recorded latency to find the goal platform. Each measure was averaged across all 4 trials for each of the 5 days of water maze testing.

Perfusion and harvesting of brain. At the conclusion of behavioral testing (post-injury day 16), all rats were euthanized with an intraperitoneal injection of 150mg/kg sodium pentobarbital. Animals were then transcardially perfused with 50 mL of saline followed by 300 mL of formalin. Their brains were removed and stored in formalin.

Results

The purpose of this study was twofold. One goal was to investigate the efficacy of CeONPs as a post-injury therapeutic intervention. To test whether any dose of CeONPs would improve motor and cognitive function following TBI, a 5 (treatment groups) X 5 (days) mixed model ANOVA[2] was run for each dependent variable. *Treatment groups* refers to subjects categorized by both injury status and dose received (TBI + vehicle, TBI + .05 μg/g CeONPs, TBI + .50 μg/g CeONPs, TBI + 5.0 μg/g CeONPs, sham + vehicle, sham + .05 μg/g CeONPs, sham + .50 μg/g CeONPs, and sham + 5.0 μg/g CeONPs). Thus, there are eight treatment groups. However, in order to discuss the findings of this study in a manner consistent with TBI literature, the first primary analysis of each dependent variable examined only five of the eight treatment groups. These five groups were TBI + vehicle, TBI + .05 μg/g CeONPs, TBI + .50 μg/g CeONPs, TBI + 5.0 μg/g CeONPs, and sham + vehicle.

The second goal of this study was to investigate the side effects of CeONPs in normally functioning animals. To test this novel research question, a 4 (sham treatment groups) X 5 (days) mixed model ANOVA was run for each dependent variable. These four groups were sham + vehicle, sham + .05 μg/g CeONPs, sham + .50 μg/g CeONPs, and sham + 5.0 μg/g CeONPs. Analyses of sham animals will be addressed later on in this section.

Note that the majority of statistical analyses violated equal assumptions of variance; thus, all repeated measure main effects and interactions are reported as Greenhouse Geiser. This particular statistic uses altered degrees of freedom, which is why many include decimals.

The average injury magnitude of injured animals was 2.00 atmospheres (*SD* = 0.17) and ranged from 1.57 to 2.28 atm. The righting times (average amount of time it took the animal to

[2] *Note.* This analysis was favored over a 2 X 4 X 5 mixed model ANOVA in order to preserve power and test a priori hypotheses. The inclusion of a sham + vehicle comparison group alongside TBI treatment groups is consistent with the TBI literature.

roll from its back onto its paws) of all injured animals were compared to those of all sham animals using an independent samples *t*-test. This analysis revealed a significant difference between groups, $t(66) = -8.98$, $p < .001$. Injured animals took significantly longer to right themselves ($M = 825.97$, $SD = 186.47$) than sham animals ($M = 403.53$, $SD = 200.02$). This is evidence of the injured animals' brain damage.

The Effects of CeONPs in Injured Animals

Beam balance duration. Figure 1 displays animals' gross motor performance during pre-training (PRE) and following injury or sham surgery. As can be seen in the figure, animals performed at similar levels during pre-training. This was confirmed by a one-way ANOVA that revealed no significant difference between treatment groups for pre-training beam balance means, $F(4, 43) = .44$, $p = .78$, $\eta^2 = .04$.

To test whether practice would improve performance in injured animals on the beam balance task, a 5 (TBI + vehicle, TBI + .05 μg/g, TBI + .50 μg/g, TBI + 5.0 μg/g, and sham + vehicle) X 5 (days) mixed model ANOVA was run. As can be seen in Figure 1, all animals, even injured animals that initially lost the ability to adequately perform the task, improved their performance throughout the five days of motor testing. This was confirmed by the analysis that revealed a significant main effect of day on beam balance performance, $F(2.82, 121.28) = 14.21$, $p < .001$, $\eta^2 = .248$.

Figure 1 does not suggest a main effect of treatment group on beam balance performance. This was confirmed by the analysis, $F(4, 43) = 1.47$, $p = .227$, $\eta^2 = .12$. In contrast, Figure 1 suggests an interaction between treatment group and day of testing. Specifically, sham animals and TBI + 5.0 μg/g CeONPs animals did not exhibit significantly impaired motor ability on PID1, while injured animals receiving vehicle, .05 μg/g CeONPs, and .50 μg/g CeONPs initially

suffered but were then able to recover. This interaction was not supported by the analysis between treatment group and day of testing, $F(11.28, 121.28) = .81$, $p = .63$, $\eta^2 = .07$. This does not support the hypothesis that predicted an interaction between treatment group and day of testing for motor performance.

While Figure 1 seems to suggest that motor performance immediately following surgery (PID1) was significantly different between groups, a one-way ANOVA failed to support this, $F(4, 43) = 1.73$, $p = .162$, $\eta^2 = .138$. However, it is interesting to note that the effect size was large, and so a lack of significance could be attributed to an insufficient number of animals in each treatment group. Paired samples *t*-tests comparing motor means on PRE to PID1 revealed a significant decrease in balance duration for injured animals that received vehicle, .05 μg/g, and .50 μg/g CeONPs (all $p < .003$), but no significant difference between days for injured animals that received 5.0 μg/g CeONPs and sham animals (both $p > .301$). Thus, even though the one-way ANOVA did not reveal a significant difference between groups on PID1, the results of these paired samples *t*-tests suggest that injured animals treated with vehicle, .05 μg/g, and .50 μg/g CeONPs were significantly impaired on PID1 compared to their pre-surgery performance.

Paired samples *t*-tests comparing motor means on PRE to PID2 revealed a significant difference for injured animals that received vehicle, .05 μg/g, and .50 μg/g CeONPs (all $p < .052$), but no significant difference between days for injured animals that received 5.0 μg/g CeONPs or sham animals ($p > .707$). Paired samples *t*-tests comparing motor means on PRE to PID3, PID4, and PID5 revealed no significant differences between days for any treatment group (all $p > .066$). Thus, all animals eventually recovered enough gross motor functioning to prevent discrimination between groups on PID5. This was supported by a one-way ANOVA, which failed to demonstrate a significant difference between treatment group beam balance means on

post-injury day 5, $F(4, 43) = .16$, $p = .959$, $\eta^2 = .01$. This suggests that all animals began water maze testing with similar gross motor ability.

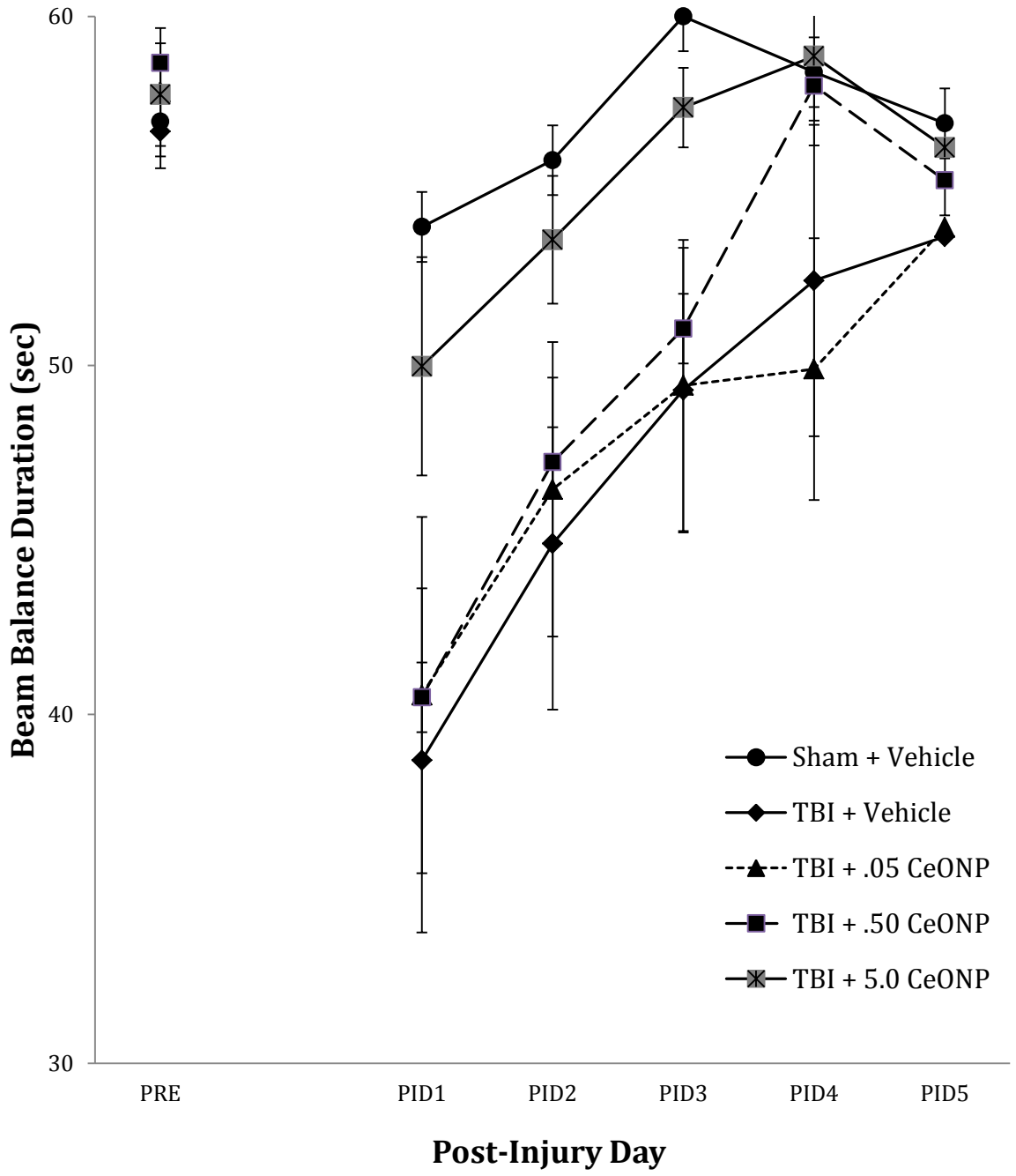

Figure 1. Beam balance duration means (sec) of treatment groups. Note: PRE = pre-training on the beam balance (prior to injury or sham surgery), PID = post-injury day. Data presented as mean ± standard error.

Latency to the hidden platform. Cognitive functioning was assessed with the water maze task that animals completed on PIDs 11-15. Animals were required to learn the location of a goal platform hidden beneath the opaque surface of the water using extramaze visual cues. To test whether practice would decrease latency to find the hidden platform in all treatment groups, a 5 (TBI + vehicle, TBI + .05 μg/g, TBI + .50 μg/g, TBI + 5.0 μg/g, and sham + vehicle) X 5 (days) mixed model ANOVA was run.

Figure 2 displays average latency to the hidden goal platform of the four injured treatment groups and the sham animals that received vehicle. As is evident in Figure 2, all animals learned to find the hidden platform in fewer seconds across days of testing. This was confirmed by the analysis, which revealed a significant main effect of day on latency, $F(2.87, 120.54) = 82.71$, $p < .001$, $\eta^2 = .663$. The average latency to the water maze platform decreased across days of testing.

Figure 2 suggests a differential effect of the varying doses of CeONPs such that injured animals that received .05 μg/g CeONPs performed worse that injured animals that received vehicle. In contrast, injured animals that received 5.0 μg/g CeONPs seemed to perform at a level similar to that seen in sham animals. This was supported by the analysis, which revealed a significant main effect of treatment group on water maze latency means, $F(4, 42) = 3.21$, $p = .022$, $\eta^2 = .234$.

Fisher LSD post-hoc analyses suggest that injured animals that received .05 μg/g CeONPs performed significantly worse ($M = 66.13$, $SD = 29.50$) than sham animals treated with vehicle ($M = 35.99$, $SD = 18.54$, $p = .008$), and injured animals treated with 5.0 μg/g CeONPs ($M = 29.24$, $SD = 12.82$, $p = .003$). However, there was no significant difference in performance between sham animals and injured animals treated with 5.0 μg/g CeONPs, ($p = .587$).

Figure 2 does not suggest the presence of an interaction between treatment group and day of testing. Even though all treatment groups improved across days of testing, animals that began water maze testing with inferior behavioral performance compared to that of other treatment groups concluded testing with inferior performance. This was confirmed by the analysis, which failed to reveal a significant interaction between treatment group and day of testing on latency to the platform, $F(11.48, 120.54) = .83$, $p = .616$, $\eta^2 = .073$.

This finding does not support the hypothesis, which predicted that there would be an interaction between treatment group and day. However, these results provide evidence of the potential for at least one dose of CeONPs (.05 μg/g) to be detrimental to cognitive functioning while another dose (5.0 μg/g CeONPs) may actually be beneficial following TBI.

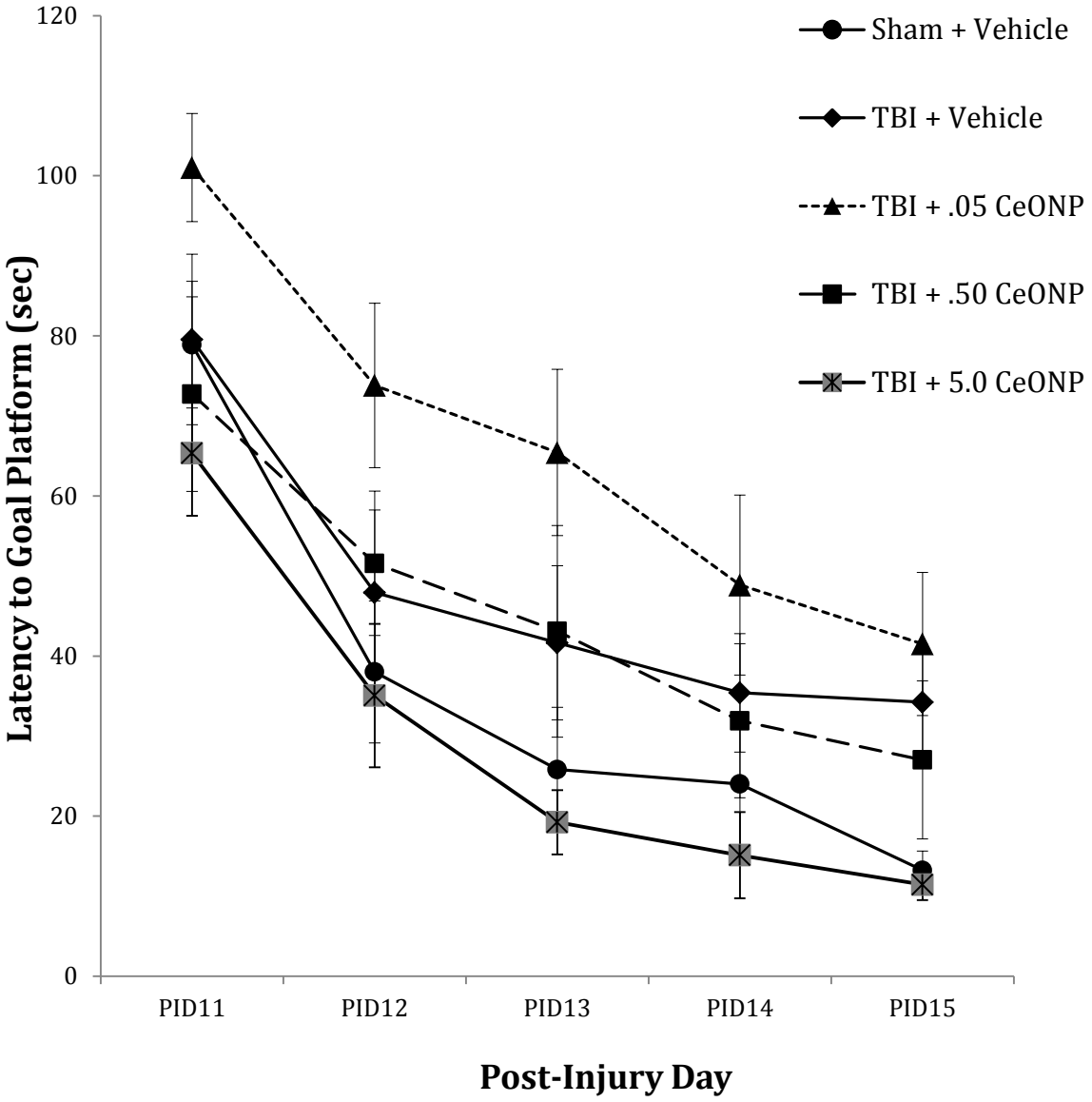

Figure 2. Latency (sec) to locate the hidden goal platform in the water maze of treatment groups across days. Note: PID = post-injury day. Data presented as mean ± standard error.

Average proximity to the goal platform. To test whether practice across days would decrease the average proximity to the hidden platform in all treatment groups, a 5 (TBI + vehicle, TBI + .05 μg/g, TBI + .50 μg/g, TBI + 5.0 μg/g, and sham + vehicle) X 5 (days) mixed model ANOVA was run.

Figure 3 displays the average proximity to the goal platform of the four injured treatment groups and the sham animals that received vehicle. Figure 3 suggests that all treatment groups were able to decrease their average proximity to the goal platform across days of testing. All animals exhibited shorter proximities to the goal platform on the final days of testing compared to the first few days. This was confirmed by the analysis, which revealed a significant main effect of day, $F(3.51, 147.20) = 91.36$, $p < .001$, $\eta^2 = .685$. The average proximity to the water maze platform decreased across days of testing in all animals, regardless of treatment group.

It is clear in Figure 3 that no treatment group demonstrated exceptionally inferior or superior proximity behavior compared to all other groups. This was confirmed by the analysis, which revealed no significant main effect of treatment group on proximity means, $F(4, 42) = 1.49$, $p = .221$, $\eta^2 = .125$. No group exhibited significantly closer proximities to the platform than another group.

While Figure 3 demonstrates that all treatment groups began water maze testing with similar goal proximities, their proximities on PID15 differ. For example, injured animals that received .05 μg/g CeONPs and 5.0 μg/g CeONPs began water maze testing with similar proximity means. However, their means on PID15 significantly differ, which was supported by the analysis that revealed a significant interaction between treatment group and day of testing, $F(14.02, 147.20) = 1.86$, $p = .035$, $\eta^2 = .15$.

Post-hoc analyses revealed no simple effect of treatment group on PID11, $F(4, 42) = 1.16$, $p = .343$, PID12, $F(4, 42) = 1.85$, $p = .138$, PID13, $F(4, 42) = 1.63$, $p = .184$, or PID14, $F(4, 42) = .81$, $p = .527$. However, on PID15, there was an effect of treatment group that approached statistical significance, $F(4, 42) = 2.52$, $p = .055$. Simple comparisons of proximity means on PID15 show that injured animals that received .05 μg/g CeONPs performed significantly worse ($M = 52.00$, $SD = 9.98$) than sham animals receiving vehicle ($M = 44.02$, $SD = 9.96$, $p = .012$), and injured animals that received 5.0 μg/g CeONPs ($M = 41.97$, $SD = 8.42$, $p = .013$). However, injured animals that received 5.0 μg/g CeONPs did not perform any worse than sham animals treated with vehicle, ($p = .864$).

These findings support the hypothesis, which predicted that there would be an interaction between treatment group and day of testing. In addition, these results provide evidence of the potential for one CeONPs dose (.05 μg/g) to be detrimental to cognitive functioning while another dose (5.0 μg/g) may be beneficial.

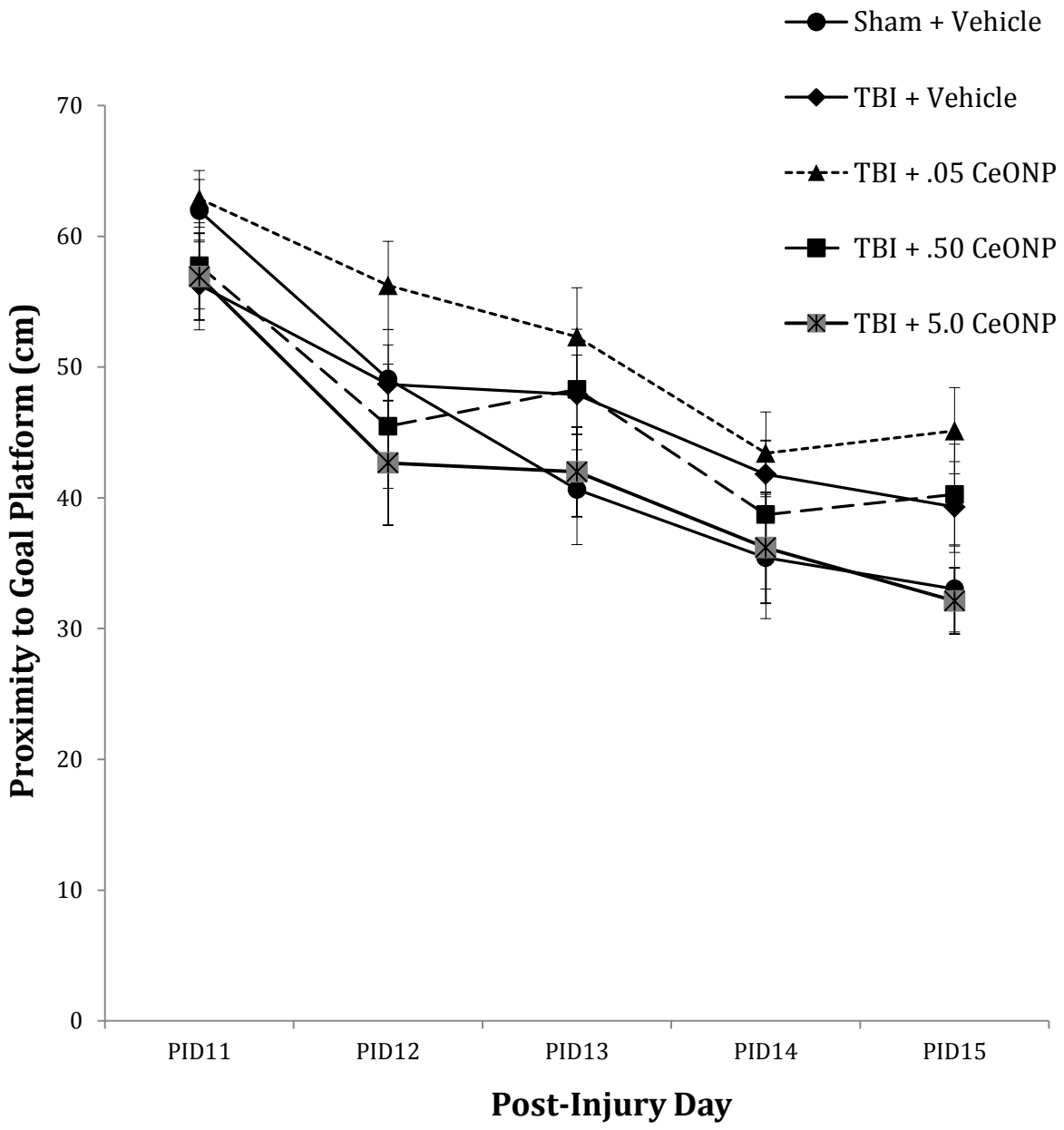

Figure 3. Average proximity (cm) to the hidden goal platform in the water maze of treatment groups across days of testing. Note: PID = post-injury day. Data presented as mean ± standard error.

Total distance traveled. To test whether animals learned to travel shorter distances in the water maze following practice, a 5 (TBI + vehicle, TBI + .05 μg/g, TBI + .50 μg/g, TBI + 5.0 μg/g, and sham + vehicle) X 5 (days) mixed model ANOVA was run.

Figure 4 displays the total distance traveled in the water maze of the four injured treatment groups and sham animals that received vehicle. Data presented in Figure 4 suggest that all animals were able to improve their performance following practice. This was confirmed by the analysis, which revealed a significant main effect of day on total distance traveled, $F(2.94, 123.28) = 82.33$, $p < .001$, $\eta^2 = .662$. Animals traveled shorter distances during the final days of testing compared to the first few days of testing, suggesting that they learned to take a more direct route to the platform.

Figure 4 suggests that injured animals that received .05 μg/g CeONPs traveled greater distances overall compared to all other treatment groups. This implies that their cognitive performance was negatively affected by the dose of CeONPs; however, the other two doses of CeONPs did not produce this impairment. This differential effect of CeONPs was supported by the analysis, which demonstrated a significant main effect of treatment group on total distanced traveled, $F(4, 42) = 2.83$, $p = .037$, $\eta^2 = .212$.

LSD post-hoc analyses suggest that injured animals treated with .05 μg/g CeONPs traveled significantly greater distances in the water maze ($M = 1544.01$, $SD = 618.94$) than injured animals treated with vehicle ($M = 1081.54$, $SD = 554.97$, $p = .045$), .50 μg/g CeONPs ($M = 1050.24$, $SD = 543$, $p = .05$), and 5.0 μg/g CeONPs ($M = 789.44$, $SD = 363.05$, $p = .005$), or sham treated with vehicle ($M = 944.23$, $SD = 493.16$, $p = .015$). It is interesting to note, however, that injured animals treated with 5.0 μg/g CeONPs performed no differently than sham animals treated with vehicle, ($p = .57$).

All treatment groups improved their performance at a similar rate, and so Figure 4 suggests no interaction between treatment group and day of testing. This was supported by the analysis, $F(11.74, 123.28) = .97$, $p = .48$, $\eta^2 = .085$.

These findings do not support the hypothesis, which predicted that there would be an interaction between treatment group and day of testing. However, because injured animals treated with .05 μg/g CeONPs performed significantly worse than all other treatment groups—including injured animals treated with vehicle—this suggests that the .05 μg/g CeONPs dose is detrimental to cognitive functioning. In contrast, injured animals treated with 5.0 μg/g CeONPs performed at levels similar to those seen in sham animals treated with vehicle. This suggests that the high dose of CeONPs may prevent impairment to or promote superior search strategies.

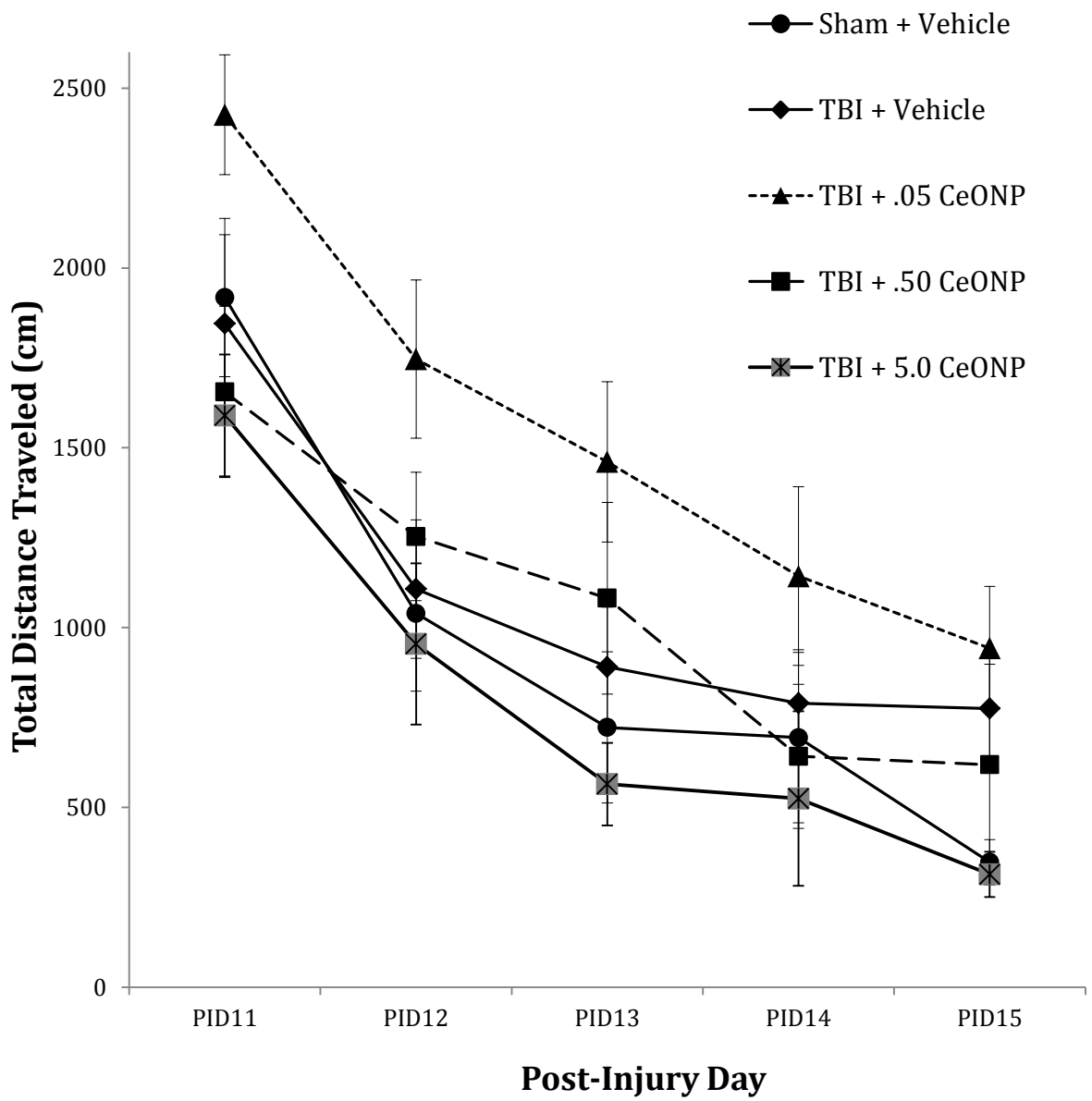

Figure 4. Total distance traveled (cm) in water maze of treatment groups across days of testing.

Note: PID = post-injury day. Data presented as mean ± standard error.

Thigmotaxis. To test whether repeated exposure would decrease anxiety levels, a 5 (TBI + vehicle, TBI + .05 μg/g, TBI + .50 μg/g, TBI + 5.0 μg/g, and sham + vehicle) X 5 (days) mixed model ANOVA was run.

Figure 5 displays the average amount of time each treatment group spent in the outer zone of the water maze on each day (also known as *thigmotaxis*). Evident in this graph, all animals spent considerably less time in the outer zone in the final day of testing compared to the start of testing. This was confirmed by the analysis, which revealed a significant main effect of day on thigmotaxis, $F(2.65, 111.40) = 95.08$, $p < .001$, $\eta^2 = .694$. This indicates that all animals, regardless of treatment group, became less anxious across days of water maze testing—the average amount of time spent in the outer zone of the water maze decreased.

Figure 5 seems to suggest that injured animals that received .05 μg/g CeONPs were more anxious than all other treatment groups (especially compared to injured animals that received 5.0 μg/g CeONPs). However, this was not confirmed by the analysis, which demonstrated a main effect of treatment group on thigmotaxis that just failed to reach conventional levels of statistical significance, $F(4, 42) = 2.16$, $p = .09$, $\eta^2 = .171$. This suggests that no dose of CeONPs significantly altered the amount of time animals spent in the outer zone of the water maze.

Figure 5 suggests that all treatment groups became less anxious in the water maze at similar rates. This does not suggest an interaction between treatment group and day of testing, which was confirmed by the analysis, $F(10.61, 111.40) = .61$, $p = .815$, $\eta^2 = .055$. These findings do not support the hypothesis, which predicted that there would be an interaction between treatment group and day of testing. These results suggest that CeONPs do not significantly decrease anxiety levels of injured animals.

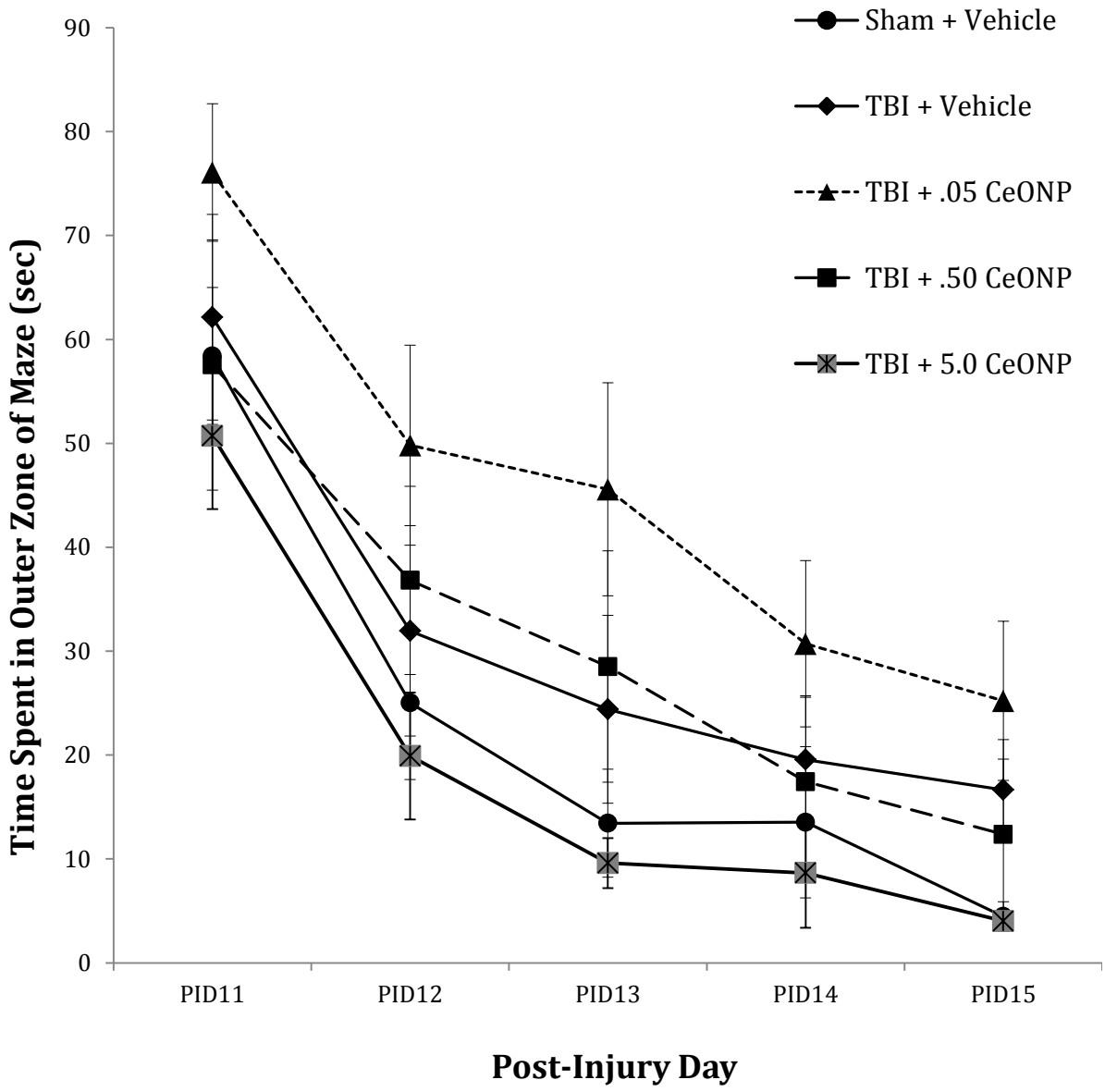

Figure 5. Thigmotaxis of treatment groups across days of testing. Note: PID = post-injury day. Data presented as mean ± standard error.

The Effects of CeONPs in Normally Functioning Animals

In addition to investigating the efficacy of CeONPs as a therapeutic intervention following traumatic brain injury, this study explored the potential beneficial or detrimental side effects of CeONPs in normally functioning animals. In order to examine these effects on motor behavior, a 4 (sham + vehicle, sham + .05 μg/g, sham + .50 μg/g, and sham + 5.0 μg/g CeONPs) X 5 (days) mixed model ANOVA was conducted—for *sham* animals.

Beam balance duration. Figure 6 displays average duration to remain on the beam balance of the four sham animal treatment groups. As shown in Figure 6, it appears that sham animals did not experience significant motor impairment following sham surgery, and so their motor performance remained at relatively constant levels. This was supported by the analysis, which failed to reveal a significant main effect of day on beam balance means for sham animals, $F(3.68, 95.74) = 1.71$ $p = .159$, $\eta^2 = .062$. This suggests that their gross motor functioning was not significantly altered by the sham surgery or CeONPs administration.

Figure 6 suggests that the varying doses of CeONPs differentially affected the motor performance of sham animals. However, the analysis failed to support this main effect of treatment group, $F(3, 26) = 1.22$, $p = .321$, $\eta^2 = .124$.

Data presented in Figure 6 seem to suggest that some doses of CeONPs may have detrimental effects on motor functioning as evidenced by initial drops in performance following sham surgery. These effects eventually disappear toward final days of motor testing, which suggests an interaction between treatment group and day of testing. However, the analysis revealed no significant interaction between day and treatment group, $F(11.05, 95.74) = 1.55$, $p = .128$, $\eta^2 = .151$. This evidence demonstrates no beneficial or detrimental effects of CeONPs on the motor ability of normally functioning animals.

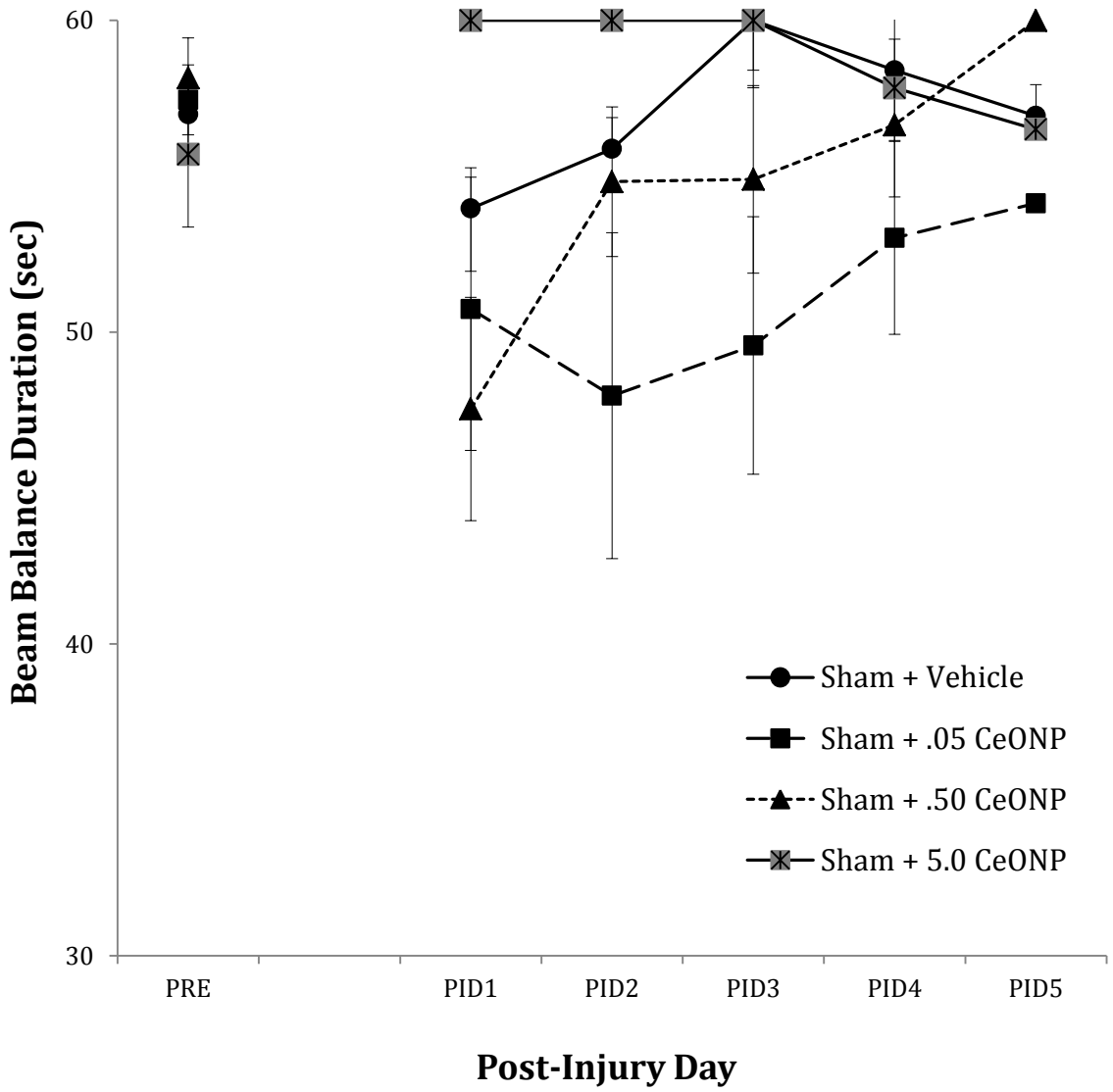

Figure 6. Beam balance duration means (sec) of treatment groups across days of testing. Note: PRE = pre-training on the beam balance (prior to injury or sham surgery), PID = post-injury day. In addition, the 4 X 5 mixed model ANOVA did NOT include pre-training day means. They have been included in the graph for purposes of visual demonstration. Data presented as mean ± standard error.

Latency to the hidden platform. In order to examine the potential cognitive side effects of CeONPs in normally functioning animals, a 4 (sham + vehicle, sham + .05 μg/g, sham + .50 μg/g, and sham + 5.0 μg/g CeONPs) X 5 (days) mixed model ANOVA was conducted to look at mean latencies to the hidden platform of sham animals.

Figure 7 displays average latencies to the hidden goal platform of all four sham animal treatment groups. Figure 5 shows that all sham animals improved their water maze performance through practice. For all groups, latency to the hidden platform decreased across days of testing. This was supported by the analysis, which revealed a significant main effect of day on latency means for sham animals, $F(3.03, 78.88) = 68.82$, $p < .001$, $\eta^2 = .726$. Latencies to the hidden goal platform decreased across days of testing.

Evident in Figure 7, animals that received .05 μg/g CeONPs seemed to perform worse than the other treatment groups, although this impairment eventually disappeared in final days of testing. This differential effect of the varying doses of CeONPs was not supported by the analysis, which failed to demonstrate a significant main effect of treatment group on latency means, $F(3, 26) = 1.99$, $p = .141$, $\eta^2 = .186$.

Likewise, there was no significant interaction between treatment group and day of testing in sham animals, $F(9.1, 78.88) = 1.72$, $p = .097$, $\eta^2 = .166$. Thus, the initial disadvantage the .05 μg/g CeONPs gave to the sham animals was not significant. However, it is important to note that this interaction approached conventional levels of statistical significance.

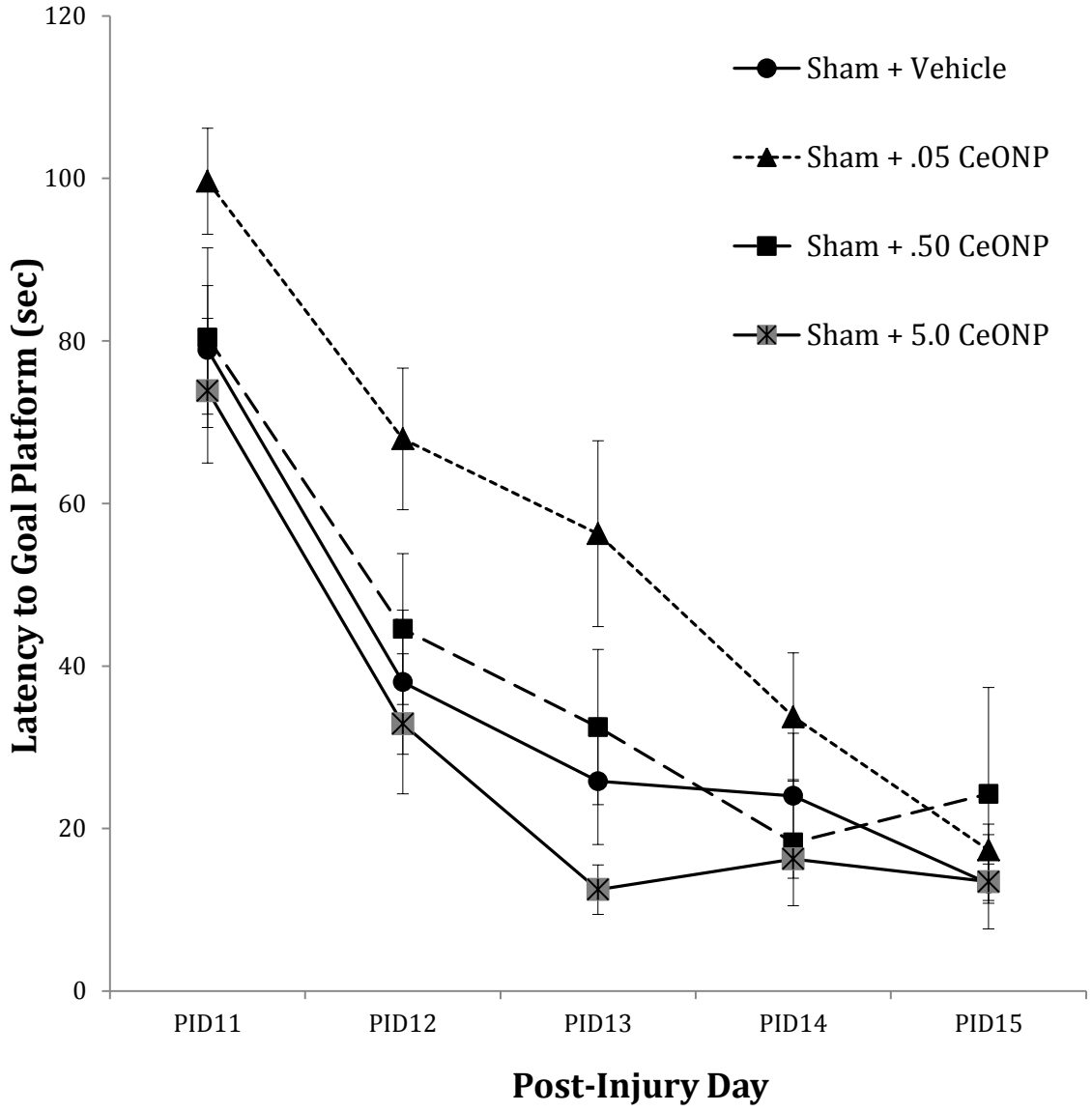

Figure 7. Latency (sec) to the hidden goal platform in the water maze of treatment groups across days of testing. Note: PID = post-injury day. Data presented as mean ± standard error.

Average proximity to the goal platform. In order to examine the cognitive effects of CeONPs in normally functioning animals, a 4 (sham + vehicle, sham + .05 μg/g, sham + .50 μg/g, and sham + 5.0 μg/g CeONPs) X 5 (days) mixed model ANOVA was conducted to look at goal proximity means of sham animals.

Figure 8 displays the average proximity to the goal platform of all 4 sham animals' groups. Clearly displayed in Figure 8, sham animals were able to learn the location of the water maze platform, thus exhibiting shorter proximities to the goal platform at later days of testing. This was confirmed by the analysis, which revealed a significant main effect of day on proximity means for sham animals, $F(3.19, 83.04) = 85.0$, $p < .001$, $\eta^2 = .766$. This suggests that no dose of CeONPs interfered with the sham animals' ability to learn the location of the water maze platform.

Similar to the effects seen in injured animals, the varying doses of CeONPs did not seem to differentially affect goal proximity behavior, which was supported by the analysis, which failed to demonstrate a significant main effect of treatment group on proximity means, $F(3, 26) = .61$, $p = .613$, $\eta^2 = .066$.

Likewise, Figure 8 suggests that all treatment groups learned to find the goal platform at a similar rate, and so there does not appear to be an interaction between treatment groups and days of testing. This was confirmed by the analysis, which revealed no significant interaction between treatment group and day of testing, $F(9.58, 83.04) = .75$, $p = .667$, $\eta^2 = .08$.

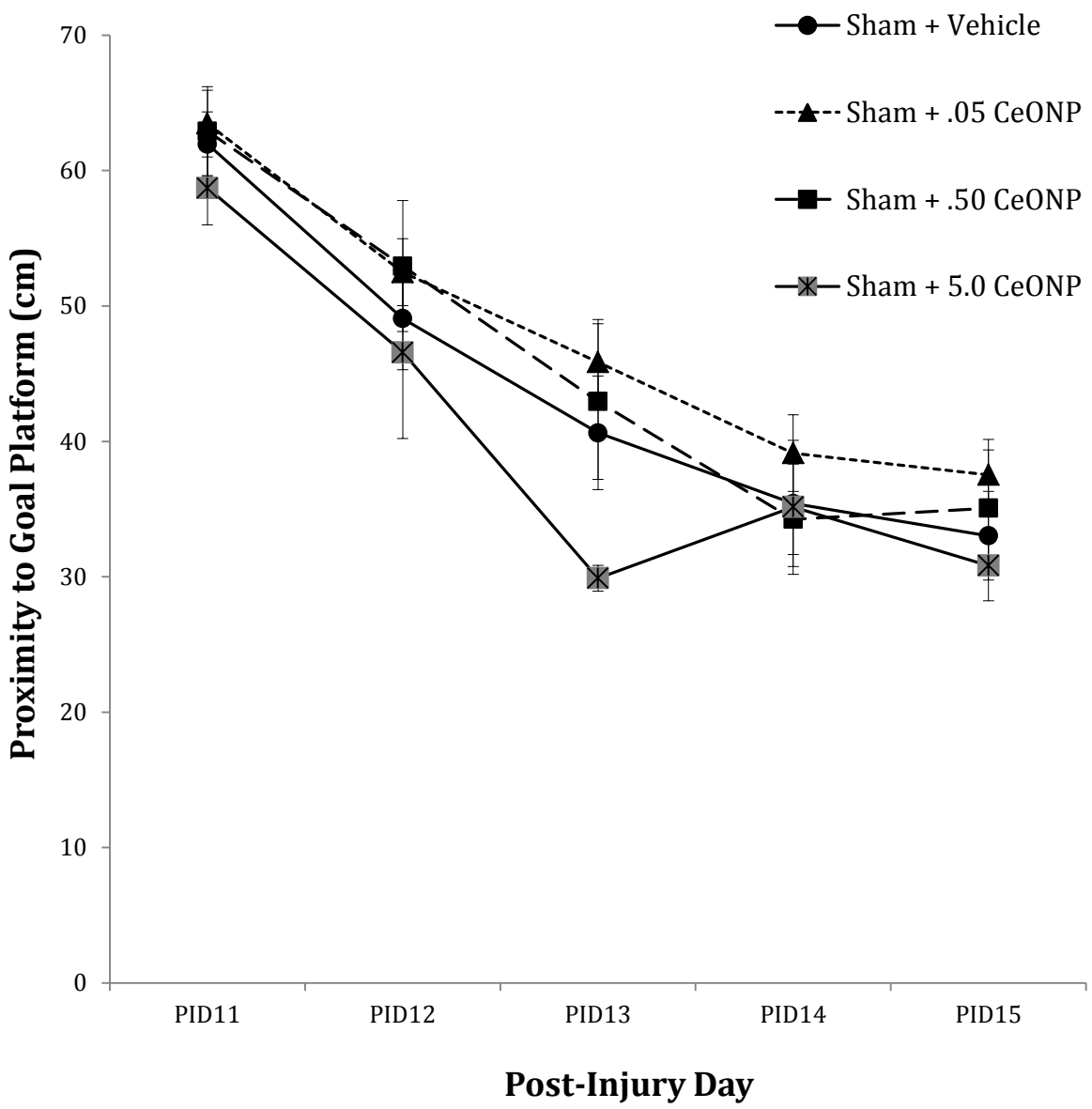

Figure 8. Average proximity (cm) to the hidden goal platform in the water maze of treatment groups across days of testing. Note: PID = post-injury day. Data presented as mean ± standard error.

Total distance traveled. In order to examine cognitive effects of CeONPs in normally functioning animals as evidenced by their total distance traveled in the water maze, a 4 (sham + vehicle, sham + .05 μg/g, sham + .50 μg/g, and sham + 5.0 μg/g CeONPs) X 5 (days) mixed model ANOVA was conducted.

Figure 9 displays the total distanced traveled in the water maze of the four sham treatment groups. Figure 9 suggests that all sham animals improved their cognitive performance following practice. This was confirmed by the analysis, which revealed a significant main effect of day of testing on total distance traveled, $F(3.17, 82.29) = 72.60$, $p < .001$, $\eta^2 = .736$. All sham animals traveled shorter distances during the final days of testing compared to the first few days of testing, suggesting that they learned to take a more direct route to the hidden platform.

Data presented in Figure 9 do not seem to indicate a differential effect of the varying doses of CeONPs on distance traveled. While sham animals that received .05 μg/g CeONPs traveled greater distances throughout testing compared to other treatment groups, this difference does not appear to be substantial. This was confirmed by the analysis, which failed to demonstrate a significant main effect of treatment group on total distance traveled, $F(3, 26) = 1.17$, $p = .341$, $\eta^2 = .119$.

Figure 9 suggests that the rate of learning of all sham treatment groups was relatively similar; the total distance traveled of each treatment group decreased at the same rate. This does not indicate an interaction between treatment group and day of testing, which was confirmed by the analysis, $F(9.50, 82.29) = 1.12$, $p = .358$, $\eta^2 = .114$.

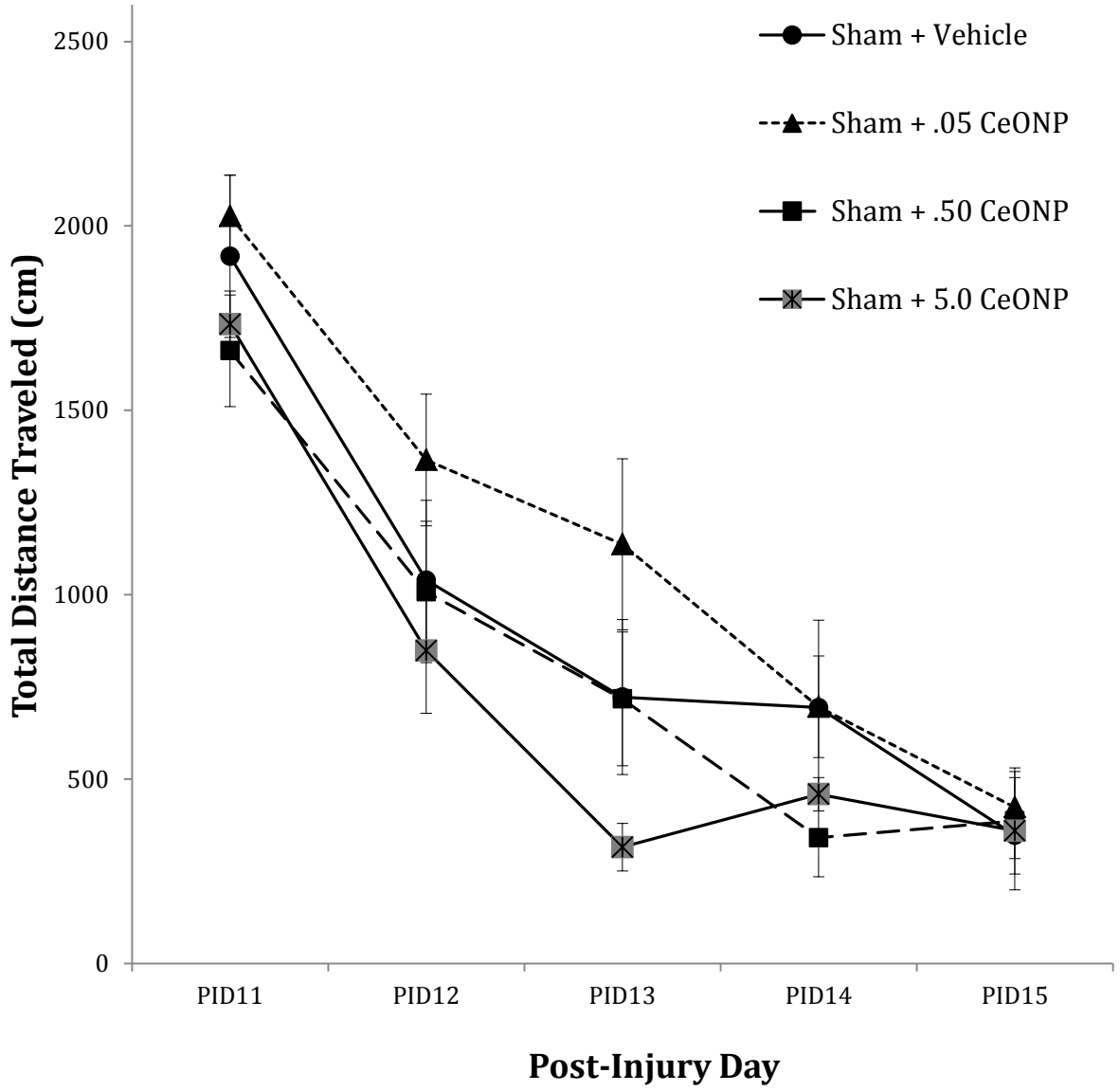

Figure 9. Total distance traveled (cm) in water maze of treatment groups across days of testing.

Note: PID = post-injury day. Data presented as mean ± standard error.

Thigmotaxis. In order to examine the effects of CeONPs on anxiety levels of normally functioning animals, a 4 (sham + vehicle, sham + .05 μg/g, sham + .50 μg/g, and sham + 5.0 μg/g CeONPs) X 5 (days) mixed model ANOVA was conducted for thigmotaxis.

Figure 10 displays the average amount of time the four sham animal treatment groups spent in the outer zone of the water maze on each day. The Figure clearly demonstrates that all animals became less anxious across days of testing. This was confirmed by the analysis, which revealed a significant main effect of day on thigmotaxis, $F(3.04, 78.97) = 61.99$, $p < .001$, $\eta^2 = .705$. All sham animals spent considerably less time in the outer zone towards the final days of testing compared to commencement.

As is evident in Figure 10, the .05 μg/g CeONPs dose appeared to increase anxiety levels of sham animals in comparison to the other doses. However, this differential effect of CeONPs was not quite confirmed by the analysis, which demonstrated a main effect of treatment group on thigmotaxis that just failed to meet conventional levels of statistical significance, $F(3, 26) = 2.83$, $p = .058$, $\eta^2 = .246$.

Data in Figure 10 suggest an interaction between treatment group and day of testing. Sham animals that received .05 μg/g CeONPs began water maze testing with more anxiety than the other sham treatment groups. However, this heighted anxiety diminished toward the final days of testing. The analysis revealed an interaction that just failed to reach conventional levels of statistical significance, $F(9.11, 78.97) = 1.82$, $p = .076$, $\eta^2 = .174$.

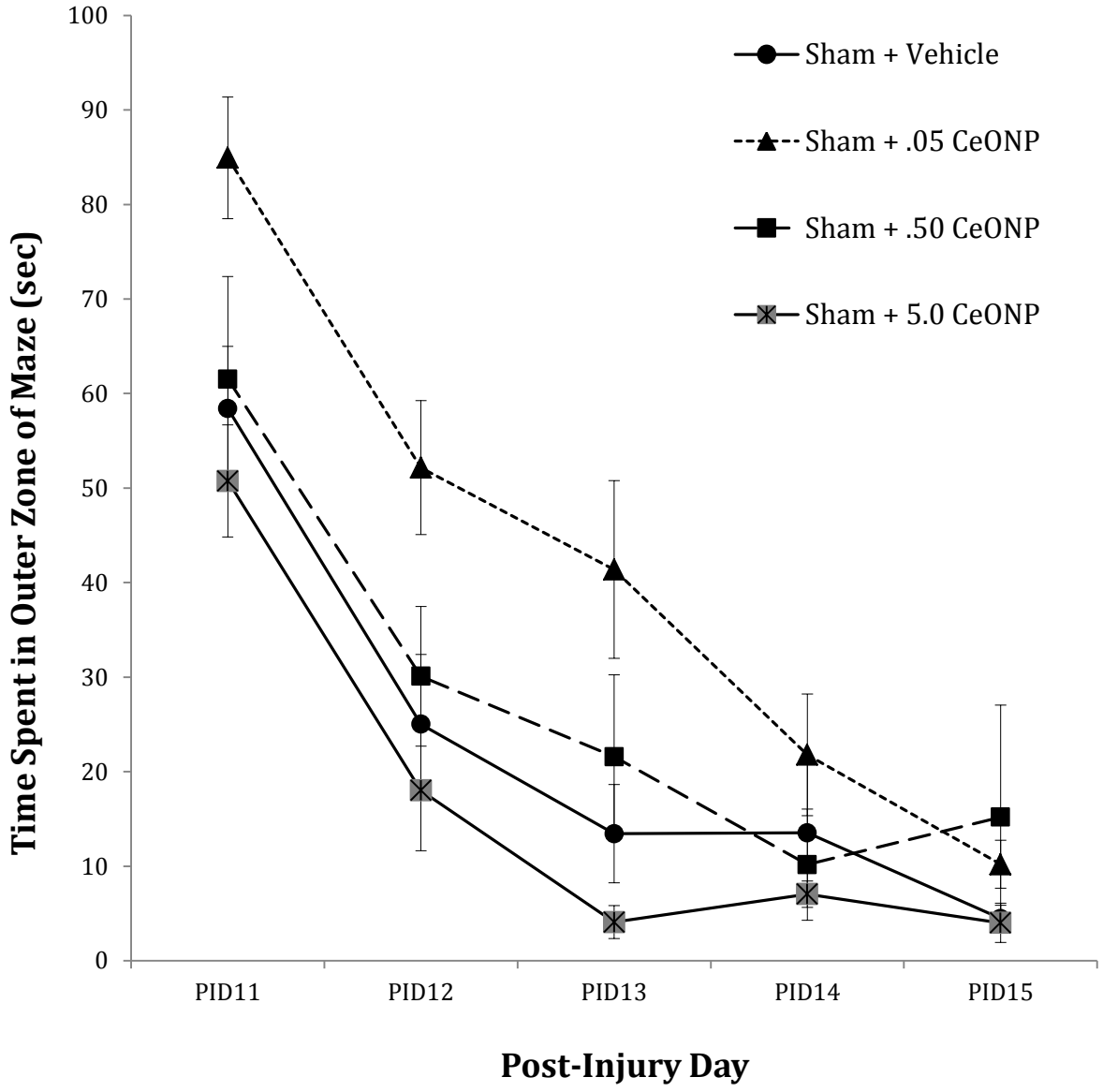

Figure 10. Thigmotaxis of treatment groups across days of testing. Note. PID = post-injury day. Data presented as mean ± standard error.

Discussion

The purpose of the current study was to investigate the ability of CeONPs to promote behavioral and cognitive recovery and to reduce anxiety levels following TBI. In addition, the current study aimed to explore the side effects of CeONPs in normally functioning animals. To do this, various doses of CeONPs were administered 30 minutes post-injury or sham surgery; then, all animals were tested on the beam balance task to assess gross motor functioning and on the Morris water maze to assess spatial learning, hippocampal function, and anxiety levels.

The findings clearly show that all animals, regardless of injury status or CeONPs dose received, improved their motor and cognitive performance with practice. This is consistent with past literature that demonstrated the ability of injured animals to learn new procedures (Hamm et al., 1996). They also became less anxious in the water maze. Thus, the primary foci of this research were the effects of CeONPs on the rate of learning and degree of impairment between groups.

The Effects of CeONPs in Injured Animals

Motor. Injured animals were tested on the beam balance task in order to evaluate their gross motor functioning. Immediately following injury, injured animals that received 5.0 μg/g CeONPs did not display impaired behavior that was evident in all other injury groups. This suggests that the 5.0 μg/g dose has the ability to promote motor recovery following injury. Despite the initial decline in motor functioning seen in injured animals treated with vehicle, .05 μg/g, and .50 μg/g CeONPs, all treatment groups recovered motor functioning and began water maze testing with similar motor ability.

Cognition. Analyses of cognitive tasks demonstrate that the major effect of CeONPs was to alter the average latency to the hidden goal platform and the total distance traveled by

injured animals in the water maze task. The findings suggest that CeONPs have the potential to be neuroprotective; however, the beneficial effects of CeONPs seem to depend on the particular dose that was administered.

Injured animals that received 5.0 μg/g repeatedly performed at levels like those seen in sham animals that did not receive a brain injury. For example, injured animals that received this dose did not significantly differ from sham animals in their average latency to the water maze platform, average proximity from the platform, or total distance traveled in the water maze. Even when the performance of injured animals treated with 5.0 μg/g CeONPs was not significantly better than that of injured animals treated with vehicle, the same pattern of results (where injured animals treated with 5.0 μg/g CeONPs performed at levels similar to sham animals) was evident for all cognitive measures. This suggests that the CeONPs dose of 5.0 μg/g may be beneficial to cognitive functioning following TBI.

In contrast, the moderate dose of CeONPs (.50 μg/g) appeared to exert no more effect on cognitive functioning than vehicle. Injured animals treated with this dose performed no better or worse than injured animals treated with vehicle.

Surprisingly, the lowest dose of CeONPs that was administered (.05 μg/g) was consistently shown to be detrimental to cognitive functioning. Animals that received .05 μg/g CeONPs took significantly longer to find the hidden platform and traveled greater distances in the water maze than other groups. In addition, animals that received .05 μg/g CeONPs performed worse than injured animals that received vehicle—this clearly indicates that this particular dose of CeONPs has the potential to be detrimental to cognitive functioning.

Anxiety. No dose of CeONPs significantly altered the anxiety levels of injured animals. However, it is interesting to note the same pattern of differential dose effects that is evident in

the cognitive variables. Specifically, injured animals treated with 5.0 μg/g CeONPs exhibited anxiety levels similar to those of sham animals. This suggests that the high dose of CeONPs has the potential to prevent the development of one psychiatric symptom that is common following brain injury. Injured animals treated with .50 μg/g CeONPs were no more or less anxious than injured animals treated with vehicle. Finally, injured animals treated with .05 μg/g CeONP exhibited the highest levels of anxiety compared to any other treatment group. This suggests that the low dose of CeONPs may actually induce or worsen anxiety following brain injury.

To my knowledge, the current study is the first to investigate the ability of CeONPs to promote recovery in a mammalian *in vivo* model of TBI, so there are no analogous behavioral studies with which to compare the findings of this study. However, the results that indicate a beneficial effect of the 5.0 μg/g CeONPs dose are consistent with CeONPs literature, which suggests that CeONPs *should* be beneficial to behavioral and cognitive outcome. For example, (Hirst et al., 2011) demonstrated the ability of CeONPs to reduce lipid peroxidation, which is a process that can alter cell membranes and increase permeability (Gutowski & Kowalczyk, 2013). Clark et al. (2011) found that CeONPs can diminish apoptotic processes that are triggered by hydrogen peroxide-mediated oxidative stress, and Das et al. (2007) provided evidence of CeONPs's ability to be neuroprotective in spinal cord neurons. All of these findings demonstrate CeONPs's ability to be beneficial to cellular and molecular functioning.

The findings of the current study suggest that this beneficial effect can be translated into improvements in behavioral and cognitive functioning. However, it is important to note that this effect is dose-dependent. While injured animals treated with 5.0 μg/g performed similar to sham animals, injured animals treated with .05 μg/g performed significantly worse than injured animals treated with vehicle. Thus, the beneficial effects of CeONPs do not always translate into

improvements in behavioral and cognitive functioning. Obviously, this warrants further investigation into the mechanisms by which the varying doses of CeONPs exert their effects.

While considering the findings of the current study, it is important to note a statistical outcome other than that of significance. Specifically, the effect sizes of each analysis suggest that the findings can be interpreted differently. All dependent variables (motor, cognitive, and anxiety) exhibit medium to large effect sizes, regardless of the presence or absence of a statistically significant effect. In addition, many effects just failed to reach conventional levels of statistical significance. The effect sizes and alpha levels suggest that, even when the analyses did not reveal a significant effect of treatment group, this outcome could be attributed to a small sample size instead of a true absence of effect.

The Effects of CeONPs in Normally Functioning Animals

Analyses of motor, cognitive, and anxiety tasks show that no dose of CeONPs significantly alters the behavior of normally functioning animals. However, the pattern of the differential effects of CeONPs dose seen in injured animals was similar to the pattern seen in sham animals. Even though non-significant, sham animals treated with .05 μg/g performed worse and exhibited higher levels of anxiety than sham animals treated with vehicle. In contrast, sham animals treated with 5.0 μg/g performed either similarly to or better than sham animals receiving vehicle. Sham animals treated with 5.0 μg/g CeONPs also exhibited either similar or lower levels of anxiety compared to sham animals treated with vehicle. The medium to large effect sizes associated with each analysis suggest that the lack of any significant effect of CeONPs could be attributed to a small sample size and ultimate lack of power.

The Effects of CeONP Doses (Independent of Injury Status)

Due to the relative lack of power in the analyses, additional analyses were conducted where animals were categorized according to the dose of CeONPs they received and not by their injury status. Thus, injured animals and sham animals that received .05 μg/g CeONPs were analyzed as one group, etc., and each analysis consisted of a 4 (vehicle, .05 μg/g, .50 μg/g, 5.0 μg/g) X 5 (days) mixed model ANOVA. From these analyses with increased sample sizes, it was possible to detect a significant effect of dose on latency to the hidden platform, $F(3, 64) = 5.01$, $p = .004$, $\eta^2 = .19$, total distance traveled, $F(3, 64) = 4.08$, $p = .01$, $\eta^2 = .16$, and thigmotaxis, $F(3, 64) = 4.59$, $p = .006$, $\eta^2 = .177$. All dependent variables exhibited medium to large effect sizes ($\eta^2 > .093$; with the exception of motor). LSD post-hoc analyses showed that animals that received .05 μg/g CeONPs required longer latencies to find the hidden platform ($M = 61.07$, $SD = 7.37$, $p < .019$), traveled greater distances ($M = 1355.67$, $SD = 105.57$, $p < .032$), and exhibited more thigmotaxis ($M = 43.94$, $SD = 4.32$, $p < .029$), than any other group. In addition, animals treated with 5.0 μg/g CeONPs performed no different than animals treated with vehicle (all $p > .153$).

Trending effects of the various doses of CeONPs that were evident in both injured and sham animals were also made clear when animals were categorized by CeONPs dose received. Even though these effects were not always significant, it is interesting to note that they emerge in a similar manner for every dependent variable and in all animals. Specifically, animals that received .50 μg/g CeONPs behaved no differently than animals that received vehicle whereas animals that received .05 μg/g CeONPs performed worse or were more anxious than vehicle (these effects were evident in injured and sham animals alike). Injured animals that received 5.0 μg/g performed similarly to shams and shams that received 5.0 μg/g seemed to perform better

than their vehicle counterparts. This suggests that there is a clear pattern of differential effects of CeONPs, even though these effects were not significant. See Figure 11 for a visual display of the differential effects of CeONPs in injured and sham animals and when categorized by dose.

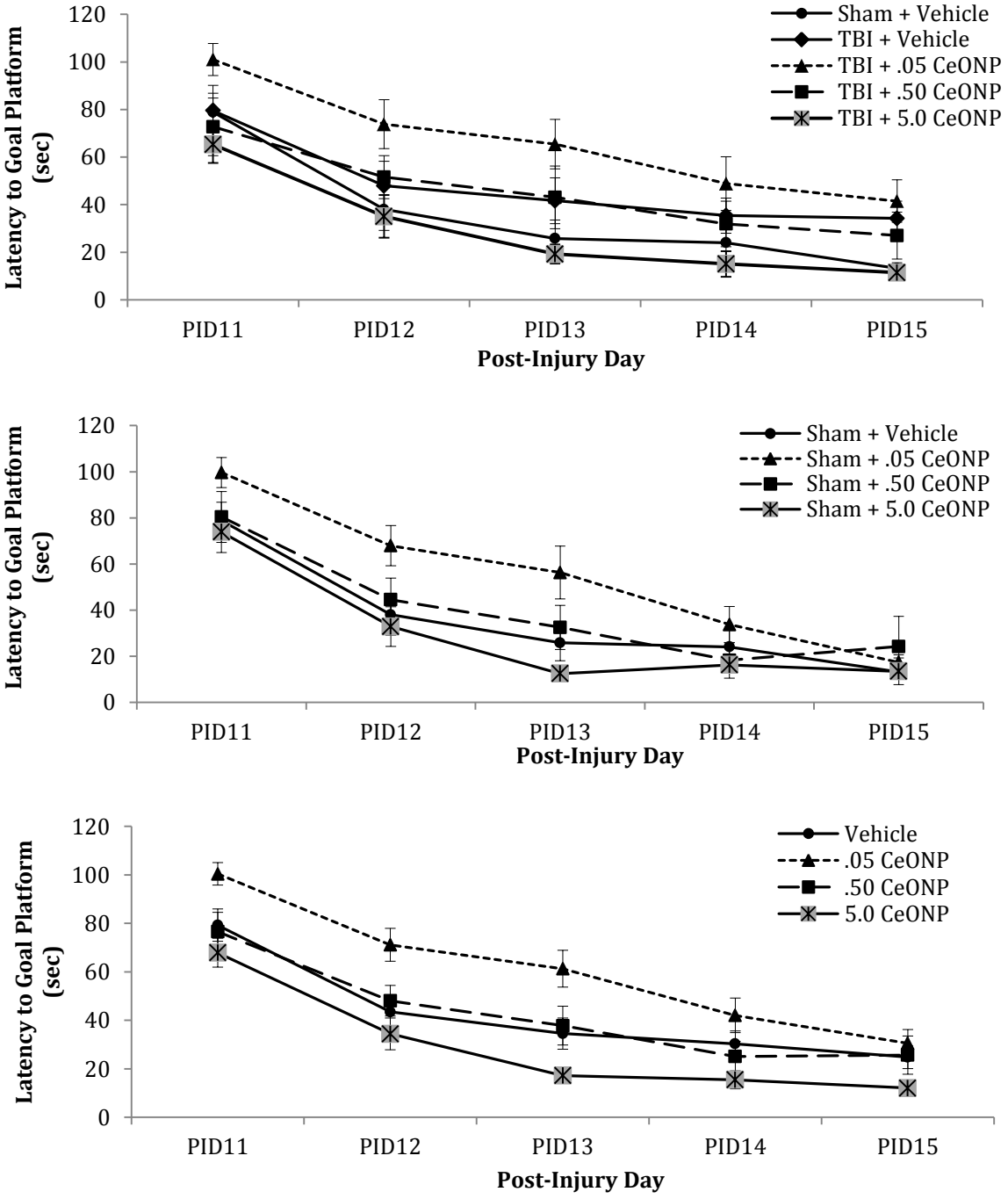

Figure 11. Three graphs displaying the average latency to find the hidden platform. The first graph displays data from injured animals and sham + vehicle. The second graph displays data from the four sham treatment groups. The third graph displays data from animals when categorized by CeONPs dose received (ignoring injury status). Note that the pattern of effects of each CeONPs dose is similar in all three graphs.

Possible Explanations for the Differential Effects of CeONPs

It is no surprise that one dose of CeONPs should alter cognitive behavior while another does not. Throughout the pharmacological literature it is evident that myriad drugs exhibit qualitatively different effects at varying doses due to the differential activation of distinctive mechanisms. Specifically, this pattern of physiological responses to a drug can be seen when discussing agents that act as partial agonists. Drugs that are partial agonists show affinity for particular receptors, but show less efficacy than a true agonist (Julien, Advokat, & Comtay, 2011). For example, the antipsychotic aripiprazole is a partial agonist at D_2 and 5-HT_{1A} receptors while simultaneously acting as an antagonist at 5-HT_2 receptors. This drug binds to dopamine receptors, but due to its antagonism at 5-HT_2, it is less effective than a true dopamine agonist. When dopamine levels are high, aripiprazole binds to dopamine receptors, but its effects are not as strong as the endogenous dopamine neurotransmitter. However, when natural dopamine levels are low, aripiprazole increases the action of dopamine (Julien et al., 2011).

Unlike aripiprazole, CeONPs do not exert their effects by activating neuronal receptors. It is possible, however, that CeONPss' differential manipulation of free radical activity activates distinctive mechanisms that in turn produce dissimilar cognitive effects. This particular discussion will focus on CeONPss' manipulation of the free radical, nitric oxide (NO).

Nitric oxide. Nitric oxide is a free radical that can easily diffuse through cellular membranes (Gutowski & Kowalczyk, 2013). It is released from endothelial cells (Charriaut-Marlangue et al., 2012) and promotes the relaxation of vascular smooth muscle in the walls of blood vessels, which lowers blood pressure (Gutowski & Kowalczyk, 2013). Nitric oxide reduces platelet aggregation and can increase arterial blood flow through vasodilation (Charriaut-Marlangue et al., 2012). Nitric oxide helps to regulate the immune response and is involved in

neurotransmission and synaptic plasticity in the central nervous system (Gutowski & Kowalczyk, 2013). Under the right condition, this particular free radical can even act as an antioxidant and stop the chain reactions caused by free radicals that cultivate oxidative stress (Gutowski & Kowalczyk, 2013). Thus, nitric oxide can be an incredibly beneficial free radical.

At other times, nitric oxide is more detrimental than beneficial. When produced in excess (such as after a TBI), nitric oxide may be an influential mediator of inflammation (Hirst, Karkoti, Tyler, Sriranganathan, Seal, & Reilly, 2009). Nitric oxide reacts with superoxide radicals to form peroxynitrite, a very toxic free radical (Estevez et al., 2011). Nitric oxide and superoxide radicals are not strong oxidants by themselves (Dowding, Dosani, Kumar, Seal, & Self, 2012), but peroxynitrite can provoke inflammation and cell death either by initiating necrotic or apoptotic processes (Estevez et al., 2011). In addition, peroxynitrite damages the brain's capillary endothelial cells, which can increase edema (Gutowski & Kowalczyk, 2013).

CeONPs are able to scavenge nitric oxide (Dowding et al., 2012). However, it is possible that the differential effects of the varying doses of CeONPs are due to the varying mechanisms of action of this particular antioxidant. Perhaps the .05 μg/g dose of CeONPs works in such a way as to promote the detrimental effects of nitric oxide and inhibit the beneficial effects of nitric oxide. This contrasting effect may not be as prominent in the higher doses of CeONPs whose scavenging abilities are able to capitalize on the beneficial effects of nitric oxide and promote its optimum level.

Promotion of amyloid beta fibrillation. Nanoparticles like CeONPs exert a wide array of physiological effects in addition to scavenging free radicals. For example, the nanoparticle titanium dioxide (TiO_2) can significantly enhance the rate of amyloid beta fibrillation (Wu et al., 2008). Amyloid beta fibrillation is thought to be a major feature in the pathogenesis of

Alzheimer's disease, which is why therapeutic interventions focus on inhibiting the formation of fibrillation (Wu et al., 2008).

Similar to other nanoparticles like TiO_2, CeONPs have also been shown to increase the rate of fibrillation (Linse et al., 2007). Thus, one effect of CeONPs may be to promote pathophysiology comparable to that seen in individuals with Alzheimer's disease. It is possible that one dose of CeONPs capitalizes on this process, which is why it is detrimental to cognitive functioning. Another dose of CeONPs may not have as strong an effect on fibrillation, and so the beneficial effects of CeONPs are promoted, and the result is cognitive recovery.

Neural mechanisms of thigmotaxis. It is interesting to note that when animals were categorized according to the CeONPs dose they received, a significant effect of dose on thigmotaxis became evident. Animals treated with .05 μg/g CeONPs spent considerably more time in the outer zone of the water maze compared to all other groups, which suggests that they were the most anxious. Because these animals spent the majority of their time in the perimeter of the water maze attempting to escape, it is no surprise that they should also exhibit longer latencies to the hidden platform and greater distances traveled.

Perhaps the differential effects of CeONPs are a result of the differential activation of systems that mediate thigmotaxis and anxiety. If the .05 μg/g dose caused animals to become highly anxious and preoccupied with escape, then a corresponding deficit in place learning would be expected.

In addition, the differential effects of CeONPs on anxiety could be related to their potential anti-inflammatory properties. Inflammation resulting from the immune response is associated with increased anxiety in the rodent, and suppression of immune cell activation results in reduced anxiety (Rodgers et al., 2012). Because CeONPs are able to inhibit the production of

inflammatory immune cells by reducing the production of ROS free radicals (Hirst et al., 2009), it is possible that CeONPs reduce anxiety. Thus, the 5.0 μg/g CeONPs dose may promote the anti-inflammatory properties of CeONPs while the .05 μg/g dose prevents them.

When considering the dose-dependent effects of CeONPs it is important to keep in mind that nanoparticles such as CeONPs do not act like traditional pharmacological drugs. They are much smaller, and are categorized by molecular size instead of mass number (Singh, Cohen, & Rzigalinski, 2007). Most nanoparticles are small enough to easily bypass the BBB and access the brain (Wu et al., 2008). CeONPs in particular are small in size, but they have a large surface area that allows these nanoparticles to react with nearby free radicals to reduce oxidation. Due to their reactive surface area, the dosing parameters of CeONPs are not comparable to regular drugs, and so a 10-fold difference in CeONPs doses requires a different interpretation than the same difference in a regular drug. Likewise, CeONPs may actually exert their effects *within* a cell as opposed to at its receptor (Singh et al., 2007).

Limitations

As is repeatedly demonstrated throughout the discussion of this study's findings, the statistical analyses suffered from a lack of power. The medium to large effects sizes suggest that an increased sample size may have provided enough power to detect a significant effect of treatment group. Smith, Levine, Lachlan, and Fediuk (2002) argue that effect size and sample size are the two most important issues that can affect the probability of making a Type II error. Specifically, smaller sample sizes and smaller effect sizes increase the risk of making this sort of statistical error. While it is a common practice among psychological scientists to set alpha at .05 to avoid making a Type I error, it is important to consider the consequences of this choice. It is possible that the underpowered nature of this research study (due to too few subjects in each

treatment condition) resulted in the inability to detect a significant effect of treatment. Thus, there may be effects of some CeONPs doses on motor or cognitive functioning that were not detected, which is by definition a Type II error.

Future Directions

The findings of this research project clearly indicate that the investigation into the efficacy of CeONPs as a post-injury intervention requires further examination. Future studies of CeONPs must increase the number of subjects in each treatment group in order to increase power and the ability to detect significant effects. In addition, future studies would significantly benefit from the addition of both histological and biochemical analyses.

Histological analyses of the brain tissue of subjects (such as Nissl staining that allows for neuronal cell counts) would allow researchers to quantify the amount of brain damage in animals. Thus, it would be possible to relate changes in behavioral functioning to quantifiable changes in brain structure. The water maze task has repeatedly shown itself to be highly dependent on intact hippocampal functioning (Hamm et al., 1996); animals with damage to their hippocampus perform worse on this task than normally functioning animals. However, analysis of the brains of subjects would provide tangible evidence of brain damage.

Biochemical assays such as those that look for biomarkers of oxidative stress, antioxidant activity, and lipid peroxidation would provide the opportunity to more clearly interpret the differential effects of CeONPs. With solely behavioral data, it is only possible to theorize about how the varying doses of CeONPs differentially activate mechanisms in order to exert their detrimental or beneficial effects on cognitive functioning. Future studies should incorporate these additional analyses in order to have physiological correlates to the behavioral data.

If the 5.0 μg/g CeONPs dose is able to significantly improve behavioral, cognitive, and emotional outcome following TBI, future studies should attempt to lengthen the duration of time between injury and CeONPs administration. This alteration in methodology would more closely mimic real-world instances of brain trauma where there is often a significant delay before medical staff has the opportunity to introduce therapeutic intervention.

Conclusion

As one of the first studies that investigated the efficacy of CeONPs as a post-injury intervention in an *in vivo* model of TBI, the findings of this study should serve as the foundation for future behavioral analyses of CeONPs. Although these findings suggest that one dose of CeONPs may be detrimental to cognitive functioning and may increase anxiety, there is the potential for a larger dose to be beneficial. Due to the large effect sizes and underpowered nature of the statistical analyses, it would be unwise to conclude that CeONPs should be abandoned as a potential therapy for TBI. In addition, it is important to continue investigation into the effects of CeONPs in normally functioning animals. CeONPs are present in substances that many individuals encounter regularly. Thus, if there is potential for CeONPs to be detrimental to cognitive functioning and to increase anxiety, this should be addressed. Likewise, if CeONPs actually improve cognitive performance and reduce anxiety in the normally functioning individual, this suggests the potential for it to serve as a performance-enhancing agent or anxiolytic. Clearly, further analyses of this free radical scavenger are warranted.

References

Atkins, C. M., Truettner, J. S., Lotocki, G., Sanchez-Molano, J., Kang, Y., Alonso, O. F., Sick, T. J., Dietrich, W. D., & Bramlett, H. M. (2010). Post-traumatic seizure susceptibility is attenuated by hypothermia treatment. *European Journal of Neuroscience, 32*, 1912-1920. doi:10.1111/j.1460-9568.2010.07467.x

Breedlove, S. M., Watson, N. V., & Rosenzweig, M. R. (2010). *Biological Psychology: An Introduction to Behavioral, Cognitive, and Clinical Neuroscience.* Sinauer Associates, Inc.

Charriaut-Marlangue, C., Bonnin, P., Gharib, A., Leger, P-L., Villapol, S., Pocard, M…Baud, O. (2012). Inhaled nitric oxide reduces brain damage by collateral recruitment in a neonatal stroke model. *Stroke, 43*, 3078-3084. doi:10.1161/STROKEAHA.112.664243

Chatzipanteli, K., Alonso, O. F., Kraydieh, S., & Dietrich, W. D. (2000). Importance of posttraumatic hypothermia and hyperthermia on the inflammatory response after fluid percussion brain injury: Biochemical and immunocytochemical studies. *Journal of Cerebral Blood Flow and Metabolism, 20*, 531-542.

Chodobski, A., Zink, B. J., & Szmydynger-Chodobska, J. (2011). Blood-brain barrier pathophysiology in traumatic brain injury. *Translational Stroke Research, 2*, 492-516.

Clark, A., Zhu, A., Sun, K., & Petty, H. R. (2011). Cerium oxide and platinum nanoparticles protect cells from oxidant-mediated apoptosis. *Journal of Nanoparticles Research, 13*, 5547-5555. doi:10.1007/s11051-011-0544-3

Das, M., Patil, S., Bhargava, N., Kang, J., Riedel, L. M., Seal, S., & Hickman, J. J. (2007). Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Biomaterials, 28*, 1918-1925.

Dixon, C. E., Lyeth, B. G., Povlishock, J. T., Findling, R. L., Hamm, R. J., Marmarou, A., Young, H. R., & Hayes, R. L. (1987). A fluid percussion model of experimental brain injury in the rat. *Journal of Neurosurgery, 67*, 110-119.

Dowding, J. M., Dosani, T., Kumar, A., Seal, S., & Self, W. T. (2012). Cerium oxide nanoparticles scavenge nitric oxide radical. *Chemical Communications, 48*, 4896-4898.

Estevez, A. Y., Pritchard, S., Harper, K., Aston, J. W., Lynch, A., Lucky, J. J…Erlichman, J. S. (2011). Neuroprotective mechanisms of cerium oxide nanoparticles in a mouse hippocampal brain slice of ischemia. *Free Radical Biology and Medicine, 51*, 1155-1163. doi:10.1016/j.freeradbiomed.2011.06.006

Feeser, V. R., & Loria, R. M. (2011). Modulation of traumatic brain injury using progesterone and the role of glial cells on its neuroprotective actions. *Journal of Neuroimmunology, 237*, 4-12. doi:10.1016/j.neuroim.2011.06.007

Fleury, C., Mignotte, B., & Vayssiere, J. (2002). Mitochondrial reactive oxygen species in cell death signaling. *Biochimie, 84*, 131-141.

Hamm, R. J., Temple, M. D., Pike, B. R., O'Dell, D. M., Buck, D. L., & Lyeth, B. G. (1996). Working memory deficits following traumatic brain injury in the rat. *Journal of Neurotrauma, 13*, 317-323.

Hayes, R. L., Jenkins, L. W., & Lyeth, B. G. (1992). Neurotransmitter-mediated mechanisms of traumatic brain injury: Acetylcholine and excitatory amino acids. *Journal of Neurotrauma, 9*, S173-S187.

Heinemann, U., Kauffer, D., & Friedman, A. (2012). Blood-brain barrier dysfunction, TGFβ signaling, and astrocyte dysfunction in epilepsy. *Glia, 00*, 1-7. doi:10.1002/glia.22311

Hirst, S. M., Karakoti, A., Singh, S., Self, W., Tyler, R., Seal, S., & Reilly, C. M. (2011). Bio-distribution and in vivo antioxidant effects of cerium oxide nanoparticles in mice. *Environmental Toxicology, 00*, 1-12. doi:10.1002/tox.20704

Hirst, S. M., Karakoti, A. S., Tyler, R. D., Sriranganathan, N., Seal, S., & Reilly, C. M. (2009). Anti-inflammatory properties of cerium oxide nanoparticles. *Small, 5*, 2848-2856. doi:10.1002/smll.200901048

Horie, M., Nishio, K., Kato, H., Fujita, K., Endoh, S., Nakamura, A., Miyauchi, A., Kinugasa, S., Yamamoto, K., Niki, E., Yoshida, Y., Hagihara, Y., & Iwahashi, H. (2011). Cellular responses induced by cerium oxide nanoparticles: Induction of intracellular calcium level and oxidative stress on culture cells. *Journal of Biochemistry, 150*, 461-471. doi:10.1093/jb/mvr081

Jones, N. C., Cardamone, L., Williams, J. P., Salzberg, M. R., Myers, D., & O'Brien, T. J. (2008). Experimental traumatic brain injury induces a pervasive hyperanxious phenotype in rats. *Journal of Neurotrauma, 25*, 1367-1374. doi:10:1089/neu.2008.0641

Julien, R. M., Advokat, C. D., & Comaty, J. E. (2011). *A primer of drug action* (12th ed.). New York, NY: Worth Publishers.

Kannan, K., & Jain, S. K. (2000). Oxidative stress and apoptosis. *Pathophysiology, 7*, 153-163.

Linse, S., Cabaleiro-Lago, C., Xue, W-F., Lynch, I., Lindman, S., Thulin, E…Dawson, K. A. (2007). Nucleation of protein fibrillation by nanoparticles. *Proceedings of the National Academy of Sciences of the United States of America, 104*, 8691-8696. doi:10.1073/pnas.0701250104

Mooney, G., & Speed, J. (2001). The association between mild traumatic brain injury and psychiatric conditions. *Brain Injury, 15*, 865-877.

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *Journal of Neuroscience Methods, 11*, 47-60.

Novack, T. A., Dillon, M. C., & Jackson, W. T. (1996). Neurochemical mechanisms in brain injury and treatment: A review. *Journal of Clinical and Experimental Neuropsychology, 18*, 685-706.

O'Connell, K. M. & Littleton-Kearnery, M. T. (2012). The role of free radicals in traumatic brain injury. *Biological Research for Nursing*, 1-11. doi:10.1177/1099800411431823

Rodgers, K. M., Bercum, F. M., McCallum, D. L., Rudy, J. W., Frey, L. C., Johnson, K. W., Watkins, L. R., & Barth, D. S. (2012). Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury. *Journal of Neurotrama, 29*, 1886-1897. Doi:10.1089/neu.2011.2273

Singh, N., Cohen, C. A., & Rzigalinski, B. A. (2007). Treatment of neurodegenerative disorders with radical nanomedicine. *Annals of the New York Academy of Sciences, 1122*, 219-230. doi:10.1196/annals.1403.015

Smith, R. A., Levine, T. R., Lachlan, K. A., & Fediuk, T. A. (2002). The high cost of complexity in experimental design and data analysis: Type I and Type II error rates in multiway ANOVA. *Human Communication Research, 28*, 515-530.

Treit, D., & Fundytus, M. (1988). Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacology, Biochemistry, and Behavior, 31*, 959-962. doi:10.1016/0091-3057(88)90413-3

Wilson, J. X. & Gelb, A. W. (2002). Free radicals, antioxidants, and neurologic injury: Possible relationship to cerebral protection by anesthetics. *Journal of Neurosurgical Anesthesiology, 14*, 66-79.

Wu, W-h., Sun, Y-p. Y., Hu, J., Zhao, L., Liu, Q., Zhao, Y-f., & Li, Y-m. (2008). TiO_2 nanoparticles promote β-amyloid fibrillation *in vitro*. *Biochemical and Biophysical Research Communications, 373*, 315-318.

Zauner, A. & Bullock, R. (1995). The role of excitatory amino acids in severe brain trauma: Opportunities for therapy: A review. *Journal of Neurotrauma*, *12*, 547-554.