Injection of Cerium Oxide Nanoparticles To Treat Spinal Cord Injury In Rats

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Research Article

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Abstract

Introduction: The present study, investigated the local injection of cerium oxide nanoparticles (CeONPs) into Spinal cord injury (SCI) lesions in rats, and the effect on motor performance and neuropathic pain, together with biochemical markers.

Methods: 36 adult male Wistar rats were divided into 4 groups: control group (healthy animals); sham group (laminectomy); SCI group (laminectomy+SCI induction); treatment group (laminectomy + SCI induction + intrathecal injection of 10 μ L of CeONPs (1000 μ g/mL) immediately after injury). SCI was induced by application of an aneurysm clip at the T12-T13 vertebral region. Immediately after SCI, CeONPs were injected into the treatment group with a Hamilton syringe and micropipet. H&E staining and measurement of the size of the cavity were performed after 6 weeks, and the BBB motor performance test and pain threshold test were performed weekly. GCSF expression, P44/42 MAPK (ERK1/ERK2), P-P44/42 MAPK (ERK1/ERK2), total Tau, total MAG, β -actin were evaluated after 6 weeks.

Results: The BBB score and pain threshold improved in animals receiving CeONPs compared with SCI animals. The size of the cavity decreased in the treatment group. GCSF protein expression levels were similar in animals receiving CeONPs compared with the SCI group, but the expression of ERK1/ERK2 and phospho-ERK was lower compared with the SCI group. The expression levels of Tau and MAG were significantly increased in treated animals compared to the SCI group.

Conclusion: The use of CeONPs in SCI could improve motor functional recovery, reduce pain and increase nerve cell regeneration.

Introduction

Spinal cord injury (SCI) is one of the most common and complex clinical problems worldwide, and leads to many debilitating symptoms such as impaired motor performance, sensory disorders, and neuropathic pain[1]. In SCI, the initial stage begins immediately after the injury, which causes the death of some nerve cells due to direct compression or reduced blood flow[2]. However, major injuries continue to occur in the secondary injury phase, including apoptosis, inflammation, microglial activation, demyelination, and axonal degeneration [3, 4]. Axonal regeneration is an essential step in the healing process of SCI. However, axons in the adult central nervous system (CNS) cannot be easily regenerated, which has so far prevented adequate restorative treatment of SCI. Several efforts have been made to promote regeneration of the spinal cord and some progress has been obtained [5, 6].

In addition, neuropathic pain (pain caused by primary damage or dysfunction of the central or peripheral nervous system) due to SCI also causes many problems for patients. Neuropathic pain is one of the most important complications of SCI, and occurs in more than 53% of patients after injury [7]. Neuropathic pain is often confusing and frustrating for both patients and physicians, because there often does not appear to be a reason for the poor response to standard treatment, and the pain can worsen over time leading to

So far, the therapeutic effects and overall outcomes in SCI patients have been disappointing. This necessitates the discovery of new and effective methods for the treatment of SCI, which may allow the restoration of functionally active neuronal cells, the repair of SCI, and the subsequent reduction of neuropathic pain. Reactive oxygen species (ROS) are dramatically increased during the inflammatory process following SCI, and are thought to be a major cause of secondary injury and damage to nerve cells, axonal degeneration, neuropathic pain, and motor dysfunction [9]. Therefore, reducing the levels of ROS is expected to improve the biological functions of acutely damaged nerve cells. Previous studies have shown that ROS-reducing approaches can reduce nerve cell apoptosis, and also improve the motor function.

Cerium oxide is a rare earth metal that can exist in both 3^+ and 4^+ oxidation states. Cerium oxide exists as both CeO_2 and Ce_2O_3 in the bulk state. At the nanoscale, however, cerium oxide is a mixture of cerium III and cerium IV on the nanoparticle surface, giving the ability to undergo redox reactions[10, 11]. With a decrease in nanoparticle diameter, the number of Ce(III) sites on the surface increases and some oxygen atoms are lost (oxygen vacancies), which increases its catalytic activity. Cerium oxide nanoparticles (CeONPs) are a non-toxic nanomaterial that can act as a catalyst for redox reactions and ROS destruction [12, 13]. In addition, CeONPs are thought to be able to mimic the properties of some enzymes such as, superoxide dismutase, catalase, peroxidase, oxidase, as well having the ability to quench hydroxyl and nitric oxide radicals [14, 15]. Due to the antioxidant properties of CeONPs, they are expected to help nerve regeneration after SCI. The effect of CeONPs has been examined for the recovery of nerve tissue in-vitro [16], in sciatic nerve crush injury in vivo[17], and in functional recovery after SCI [18]. Despite the fact that CeONPs can act as powerful antioxidants, and these are known to benefit SCI, so far there is no report on whether CeONP administration could help neuron regeneration and relieve neuropathic pain after SCI. In this study we investigated the effects of 10 μL of CeONPs (1000 $\mu\text{g}/\text{ml}$) injected into the lesion area, on the repair, nerve regeneration, and pain reduction after SCI in rats.

Material And Methods

Study design

Adult male Wistar SD rats (weighing 150–185 g, $n = 36$) were kept under controlled conditions (12 hours of light and 12 hours of darkness, 6 am to 6 pm) and temperature ($22 \pm 2^\circ\text{C}$) in the animal laboratory of Iran University of Medical Sciences. Four rats were placed in each cage and the animals in all groups had free access to water and pelleted chow. The experimental animal research protocol was approved by the ethics committee of Iran University of Medical Sciences (98-4-99-16788). In this study, the animals were randomly divided into 4 groups ($n = 9$).

1- Control group (Healthy animals)

2- Sham group (Laminectomy)

3- SCI group (Laminectomy + SCI induction)

4- Treatment group (Laminectomy + SCI + Intraspinal injection of CeONPs immediately after injury).

SCI model and nanoparticle injection

The method of inducing SCI and injection of nanoparticles were similar to that of Behroozi et al [19]. Animals were anesthetized by injection of a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg ip). After identifying areas T12 to T13, the skin at the site of surgery was shaved, cleaned, and disinfected. A midline incision was performed, the skin and superficial muscles were separated, and the spinal cord was exposed by a laminectomy. The last rib was an important guide for confirming the injury site at the T13 vertebra [20]. Then we applied an aneurysm clip (force = 20 g/cm², FST company) onto the spinal cord for 90 seconds. The muscles and skin were closed by separate sutures (0.3 suture thread). The BBB test was performed to confirm the presence of moderate spinal cord injury [20, 21]. The average BBB score of all animals was 5.5 ± 1.5 . Post-surgical care included tetracycline spray on the injury site to prevent infection and 70% alcohol spray in the cage daily for one week. In addition, the bladder of animals in the third and fourth groups was emptied manually twice a day until the spontaneous reflex returned (between 3 to 7 days after SCI).

In group 4, after induction of SCI, 10 μ L of sonicated CeONPs (1000 μ g/mL) (purchased from Sigma Aldrich #796077) were injected immediately using a Hamilton syringe. This dose was selected based on a previous study that examined the effect of several doses on the motor functional recovery of animals with SCI[18]. The needle was inserted into a glass micropipette. Then the Hamilton syringe was fixed in a stereotaxic device and the CeONPs were injected into 4 locations (2 locations at the right and 2 locations on the left side of the lesion, the depth of injection was 1 mm from the surface of the dura). 2.25 μ L of nanoparticles were injected in each site. The injection was done manually at the rate of 1 μ L/min. To prevent nanoparticle leakage, the micropipette was held in place for two minutes after injection and was slowly removed from the spinal cord. Finally, the muscle and skin were sutured and animals received post-operative care after the surgery.

Histological H&E staining was used to confirm the SCI the model, and to measure the size of the cavity due to SCI. The Basso, Beattie, Bresnahan (BBB) behavioral test was performed to evaluate the motor performance. The BBB is a semi-quantitative scale based on the locomotor response of rats that can range from zero to 21.

Thermal hyperalgesia was performed with the plantar test to measure the pain threshold. All behavioral tests, including BBB and thermal hyperalgesia was performed weekly by a blinded assessor for 6 weeks after injury.

After 6 weeks, the rats were deeply anesthetized and the spinal tissue in the T12 to T13 region was prepared by perfusion and removed to be frozen at -80°C for histology and Western blotting.

Transcardiac perfusion and spinal fixation for tissue assessment

At the sixth week, after the last behavioral assessment, the animals underwent transcardial perfusion in order to stabilize the spinal cord for tissue removal. For this purpose, the animal was first deeply anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine and then fixed on a surgical board. The needle of the perfusion system was inserted into the left ventricle from the apex of the heart and the fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2–7.4) circulated in the arteries for 35–40 minutes. After these steps, the spinal cord was removed from the spine and placed in 4% paraformaldehyde to ensure tissue stabilization, until dissection. The spinal cord at the T12-T13 vertebral region was dissected and post fixed in 4% paraformaldehyde for 24 hours. The tissue sample was embedded in paraffin. and 5 μ m thickness sections cut for tissue staining.

Western blotting

About 0.5 cm of spinal cord tissue ($n = 3$ in each group) was rapidly excised, and stored for protein extraction and Western blotting at -80°C . For every 100 mg of tissue, 300 μL of RIPA buffer was added and the mixture was left on ice for 30 minutes and then homogenized. Tissues were centrifuged (13,000 g, 30 minutes, 4°C). The supernatants were isolated and the protein concentration was estimated using a Nanodrop spectrophotometer (Thermo Science, USA). In the next step, supernatants were used for Western blotting. The lysates (containing 50 μg of protein) were placed on SDS gel and the proteins were separated at 15V and transferred to a polyvinylidene-fluoride (PVDF) membrane (time: 25 minutes). After blocking with a fresh blocking buffer (5% skim milk and 0.1% Tween-20 in Tris-buffered saline, pH 7.4) at room temperature for 2 hr, the membranes were incubated with the primary antibodies including GCSF antibody (orb308858, MW: 90 kDa.), P44/42 MAPK (ERK1/ERK2) antibody (1:1000, 9102S, MW: 42–44 kDa.), phospho-P44/42 MAPK (ERK1/ERK2) (4376S), total Tau antibody (1/1000, orb158145, MW: 52/79 kDa), total MAG (1/1000, orb536682, MW: 63 kDa), β -actin antibody (1:500, sc-47778, MW:45KD) overnight. The membranes were then washed with TBST (10, 3, 3 minutes) and incubated with goat horseradish peroxidase conjugated IgG (1/1000, sc-516102). Protein bands were detected by enhanced luminescence chemistry (ECL) and captured by the ChemiDoc imaging system. β -Actin was the internal standard for the Western blotting. The results were quantified by Image J software.

Results

Six weeks after intraspinal CeONPs injection, one rat died due to surgical complications, and was replaced in the study. The data from 36 animals were included in the final analysis.

Effectiveness of cerium oxide nanoparticle on locomotor

The results showed that the induction of SCI led to a reduction in the BBB score over the 6-week experiment, compared to baseline measurement ($DF = 18,199$; $F = 77.46$; $P < 0.0001$). No significant differences were observed between Sham and Control group from the first week after SCI to the sixth week. A significant reduction in the motor function in SCI animals began in the first week and continued until the end of the study compared with the control group ($p < 0.0001$). The BBB score in animals, which received CeONPs improved at one week after injury in comparison with SCI animals ($p < 0.0001$) and this improvement continued up to the sixth week. However, the locomotor performance of CeONPs treatment group did not reach the control level by the sixth week ($p = 0.0001$) (Fig. 1A).

Effectiveness of cerium oxide nanoparticle on heat hyperalgesia

The results showed that the induction of SCI led to a reduction in the paw withdrawal threshold after heat stimulation during the 6-week experiment ($DF = 18, 192$; $F = 6.149$; $P < 0.0001$). A significant reduction in the paw withdrawal threshold in SCI animals began in the second week and continued until the end of the study versus the control group ($p < 0.0001$). The paw withdrawal threshold in animals which received CeONPs treatment improved in comparison with SCI animals ($p < 0.0001$), and returned to the control level at weeks 4 and 5, but it did not reach the control level at the sixth week, however, it still showed a significant increase compared to the SCI group ($p = 0.0005$) (Fig. 1B).

Cavity formation after SCI

Cavity formation after SCI was visualized by staining with H&E (Fig. 2). The total cavity % area was markedly reduced in the group that received CeONPs injection. Only very small cavities were identified within the SCI lesions in the treatment group.

Figure 2 shows the difference in the % area of cavities at 28 days after SCI in the groups, Control, Sham, CeONPs, and SCI. The % cavity size was significantly different between CeONPs treatment and SCI groups ($P < 0.001$).

Effects of CeONPs on the expression of granulocyte colony-stimulating factor (G-CSF)

The results showed a significant difference in the expression of granulocyte colony-stimulating factor (G-CSF) between groups in the sixth week of the experiment ($DF = 3$; $F = 7.227$; $p = 0.011$). The G-CSF expression was significantly lower in the SCI group (0.0577 ± 0.016) compared to the control group (0.7177 ± 0.028 , $p = 0.038$). The expression level of G-CSF protein in rats which received CeONPs was increased, but its increase was not statistically significant (0.19 ± 0.015) compared to SCI group (Fig. 3).

Effects of CeONPs on the expression of P44/42 MAPK (ERK1/ERK2)

The results showed a significant difference in the expression of P44/42 MAPK (ERK1/ERK2) between groups at the sixth week ($DF = 3$; $F = 23.149$; $p < 0.0001$). The P44/42 MAPK expression was significantly higher in the SCI group (0.515 ± 0.432) compared to the control (0.1543 ± 0.066 , $p = 0.002$) and sham (0.078 ± 0.35 , $p < 0.0001$) groups. The expression level of P44/42 MAPK in animals, which received CeONPs was significantly lower (0.086 ± 0.002) compared to SCI group ($p < 0.0001$). There was no significant difference in P44/42 MAPK expression between the treatment and control group (Fig. 3B).

Effects of CeONPs on the expression of phospho-P44/42 MAPK (ERK1/ERK2)

The result showed significant difference in phospho-ERK1/ERK2 expression between groups in the sixth week of the experiment ($DF = 3$; $F = 5.211$; $p = 0.028$). The phospho-ERK1/ERK2 expression was significantly increased in the SCI group (1.492 ± 0.367) compared to the control group (0.5513 ± 0.566 , $p = 0.046$). The expression level of phospho-ERK1/ERK2 protein in animals which received CeONPs was significantly lower (0.545 ± 0.51) compared to the SCI group ($p = 0.045$). In addition, there was no significant difference in phospho-ERK1/ERK2 expression between the treatment and control group (Fig. 3C).

Effects of CeONPs on the expression of Tau protein

The result showed a significant difference in the expression of Tau between groups at the sixth week of the experiment ($DF = 3$; $F = 8.651$; $p = 0.007$). Tau expression was significantly lower in the SCI group (0.286 ± 0.04) compared to the control group (0.717 ± 0.022 , $p = 0.017$). The expression level of Tau protein in rats which received CeONPs (0.7 ± 0.96) was significantly higher compared to SCI rats ($p = 0.02$). There was no significant difference in Tau expression between treatment and control groups (Fig. 4A).

Effects of CeONPs on the expression of myelin-associated glycoprotein (MAG)

The result showed a significant difference in the expression of MAG between groups at the end study ($df = 3$, $F = 6.245$; $p = 0.017$). MAG expression was significantly lower in the SCI group (0.127 ± 0.097) compared to the control group (1.29 ± 0.385 , $p = 0.026$). The expression level of MAG protein in rats, which received CeONPs (1.2 ± 0.054) was significantly higher compared to the SCI group ($p = 0.037$). There was no significant difference in Tau expression between treatment and control group (Fig. 4B).

Discussion

In this study, the induction of SCI caused locomotor impairment, hyperalgesia, and glial scar formation. Treatment with CeONPs improved the locomotor ability, decreased glial scar size, and had an important effect on reducing pain. In the third and fourth weeks of the study, the thermal hyperalgesia was completely improved, but at the sixth week, the pain returned again in both groups compared to the control. However, the pain was still significantly lower than the SCI group at the 6th week. These findings indicate the effectiveness of CeONPs as an anti-oxidant treatment, which can improve some important symptoms of SCI, such as locomotor impairment and neuropathic pain.

After SCI, a lack of growth factors, and the suppression of axonal outgrowth caused by the secretion of inhibitory molecules, means that SCI almost always has devastating consequences such as permanent pain and disability.

ERK1/ERK2 are factors that are increased after SCI, where they contribute to pain and inflammation by regulating the phosphorylation of Cdk5 at serine159. However, after treatment with CeONPs their expression returned to the normal level. When SCI is in the acute phase, increased production of nitric oxide not only increases the phosphorylation level of ERK1/2, but also increases the level of activated protein kinases in microglia/macrophages within the injured area. In addition, these proteins play an important role in neuronal degeneration following acute SCI[22]. Therefore, motor dysfunction and pain will occur and a cavity is created in the lesion zone. Previous studies have reported that antioxidants were not able to control inflammation and reduce ROS generation by decreasing ERK1/ERK2[23, 24], but we observed that CeONPs prevented an increase in ERK1/ERK2. The nanoproperties of the CeONPs may allow them to remain in place for a longer time within the injured region of the spinal cord, to reduce the formation of cavities and lessen the pain and inflammation.

MAG is a protein present on the surface of oligodendrocytes, which participates in the formation of a glial scar and promotes cavity formation. MAG is thought to inhibit axon growth via binding to Nogo receptors (chondroitin sulfate proteoglycan receptors)[25, 26]. There are some controversies about the role of MAG in axon growth, with suggestions it plays a dual role, either promoting or inhibiting neuronal outgrowth depending on the age of the neuron. MAG inhibits most mature neurons, but has a regenerative role for the growth of new axons in damaged white matter[25, 27]. Studies from the Filbin group have explicitly shown the inhibitory role of MAG, and suggested that MAG could be partly responsible for the lack of CNS neuron regeneration in vivo [28]. Studies have shown that MAG knockout mice failed to show axon regeneration in the optic nerve, or the CST, suggesting that MAG may be required for sprouting of corticospinal axons[29]. On the other hand, MAG is a transmembrane glycoprotein expressed in the axonal membrane of oligodendrocytes between axons and the inner myelin sheath, and has a function in the maintenance of myelinated axons in the adult nervous system[5, 27]. Therefore, a reduction in MAG could indicate damage and detachment of myelinated axons in the SCI model [27, 30].

In the present study, induction of SCI reduced MAG expression. Therefore it is possible that the lower Loading [MathJax]/jax/output/CommonHTML/jax.js al scar formation in the chronic phase of SCI. The injection of

antioxidant CeONPs led to increased MAG expression, which may stimulate the growth of the axons after injury[31]. On the other hand, the increased MAG expression may have been caused by inhibiting progressive cell damage and preserving the environment in the spinal cord.

Tau is another marker that can play a dual role in the nervous system. Tau is a major microtubule associated protein that contributes to a number of cellular processes, including axonal trafficking, myelination, synaptic plasticity, and is also involved in pain perception[32, 33]. Tau is a cytoplasmic neuronal marker that maintains the stability of axons, and its presence in CSF or serum is considered to be an indicator of axonal degeneration or damage in conditions such as Alzheimer's disease or SCI[34–36]. According to reports, in the first hours and days after SCI, Tau expression remains high, indicating that the process of neuronal death and axonal injury continues[36, 37]. In the chronic phase of SCI, we observed a decrease in Tau expression compared to the control group.

In healthy nervous tissue, Tau facilitates microtubule stabilization within cells and is particularly abundant in neurons[38]. In agreement with this, we observed high levels of Tau protein in the control group. Administration of CeONPs may have prevented the reduction of Tau protein in neurons due to the inhibition of neuronal damage.

There are many different studies that have confirmed the antioxidant properties of CeONPs in different tissues and in serum [39–41]. In a study by Kim et al. in 2017, they showed under conditions similar to our experiment, that injection of the same amount of CeONPs could reduce the ROS levels at the lesion site after SCI. Oxidative stress and ROS production is often associated with sustained activation of the ERK1/2 pathway. ROS-induced ERK1/2 activation (increase of P-ERK1/2) has been shown in a wide variety of cells including neurons, and may promote neuronal death [42]. ROS is a cellular stress that can modulate Tau phosphorylation, and several antioxidants have been tested in different models of tauopathy, with some interesting therapeutic effects. On the other hand, the accumulation of hyperphosphorylated Tau has been shown to cause oxidative stress[43]. Therefore, the effect of CeONPs, on the neural markers ERK1/2 and Tau could possibly be explained by its ROS scavenging properties.

The expression of GCSF, which inhibits neuropathic pain[44–46], was higher in the treatment group compared to the SCI group, but this increase was not statistically significant in our study.

In conclusion, we found that CeONPs injected into the injured spinal cord improved the functional recovery, reduced pain, and cavity formation. The mechanisms are proposed to be the inhibition of oxidative stress, leading to the restoration of neurodegenerative factors such as MAG, Tau and ERK1/2 back to normal levels. Further study is warranted before clinical trials can be designed.

Declarations

* Ethics approval and consent to participate

Not applicable

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* Consent for publication

Not applicable

* Availability of data and materials

Data are available from corresponding authors (FR and AJ) by reasonable request

* Competing interests

The authors declare no conflict of interest.

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* Authors' contributions

FR: conceptual, methodology, writing

AJ: methodology, surgical of animals, writing

LB; surgical of animals, behavioral tests, western blotting, Statistical analysis

BR: surgical of animals, behavioral tests

FN: reading, material providing,

MH; writing, reviewing and editing

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Figures

Figure 1

The effect of CeONP injection immediately after the induction of spinal cord injury (SCI) on locomotor function recovery (BBB score) (A), heat hyperalgesia (B). Data are mean $\pm$ SEM (n=9 in each group). *p<0.05, **p<0.01, ***p<0.001 versus SCI groups. #p <0.05, ##p<0.01, ###p<0.001 versus control group. \$p<0.05,

$$p < 0.01,$$

\$p<0.001, versus group which received CeONPs.

Figure 2

Histological staining (H&E) of longitudinal sections of the spinal cord (5μm) that showed the % cavity size in control, sham, SCI, and treatment group. As shown in the graph, at week sixth of SCI induction, the cavity size in the SCI group was significantly larger than the CeONP group. Data are expressed as mean $\pm$ SEM. (**p < 0.01).

Figure 3

Expression of Granulocyte colony-stimulating factor (GCSF), P44/42 MAPK (ERK1/ERK2) and phospho-P44/42 MAPK (ERK1/ERK2) proteins by western blot. The test was performed thrice on proteins and integrated optical density of each band was normalized to corresponding β -actin level. GCSF protein (A); phospho-P44/42 MAPK (ERK1/ERK2) protein (B); phospho-ERK1/ERK2 protein (C); western bolt bands of proteins (D). Data are shown as the mean $\pm$ SEM. ## $p < 0.01$, # $p < 0.05$ compared to control group and

$\$p < 0.001$,

$p < 0.01$, \$ $p < 0.05$ compared to treatment group.

A **B** **C** **Figure 4**

Western blot analysis of expression of Tau and MAG proteins. The test was performed three times and integrated optical density of each band was normalized to corresponding β -actin level. Total tau (A); Total MAG (B) Western blotting images (C). Data are shown as the mean $\pm$ SEM. [#] $p < 0.05$ compared to control group and ^{\$} $p < 0.05$ compared to CeONPs group