Effects of Embryonic Neural Stem Cell Therapy on Oxidative and Nitrosative Stress Biomarkers in Acute and Chronic Spinal Cord Injured Rats

Akut ve Kronik Omurilik Hasarlı Sıçanlarda Oksidatif ve Nitrosatif Streste Embriyonik Nöral Kök Hücre Tedavisinin Etkisi

Sibel KONYALIOĞLU, MD,[a]
Mert ÖZGÖNÜL, MD,[b]
Taner DAĞCI, MD[c]

[a]Department of Biochemistry, Ege University, Faculty of Pharmacy,
[b]Department of Biochemistry, Ege University, Faculty of Medicine,
[c]Department of Physiology, Ege University, Faculty of Medicine, Center for Brain Research, İzmir

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Yazışma Adresi/*Correspondence:*
Sibel KONYALIOĞLU, MD
Ege University, Faculty of Pharmacy, Department of Biochemistry, İzmir, TÜRKİYE/TURKEY
sibel.konyalioglu@ege.edu.tr

ABSTRACT Objective: Primary trauma to the spinal cord triggers a cascade of cellular and molecular events that promote tissue damage and expansion of the lesion for extended periods following the initial injury. Oxidative and nitrosative stresses play an important role in the progression of spinal cord injury (SCI). The experimental embryonic stem cell (ENSC) therapy is being applied to a wide range of SCIs; however, the effects of the ENSC therapy on oxidative and nitrosative stresses are not clear yet. Therefore, we aimed to investigate the effects of ENSC transplantation on antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX)], nitric oxide synthase (NOS) and nitric oxide (NO) levels. **Material and Methods:** Antioxidant enzymes, NOS activities and NO levels were measured by using spectrophotometric methods in spinal cord (SC) tissues of rats 7 (acute group) and 28 (chronic group) days after surgical resections of a 10 mm segment of the cord below T8-T9 and sham-operation. **Results:** SOD levels of acute or chronic control, damage and therapy groups did not show any considerable differences. NOS activities in acute and chronic injury groups increased as compared to their control groups; similarly NO levels significantly elevated after acute and chronic injury and stem cell therapies. Acute and chronic injured groups had a significantly higher CAT activity than their control groups. GPX activity in the acutely induced injury increased as compared to the acute control groups. The stem cell therapy diminished GPX activity in this group; however, it had a significantly higher level of GPX activity than acute controls. **Conclusion:** Stem cell therapy is suggested to cause further increases in oxidative and nitrosative stresses. Application of antioxidants coupled with stem cell therapy can be used for treatment of SCI.

Key Words: Embryonic stem cells; spinal cord injuries; catalase; nitric oxide

ÖZET Amaç: Omuriliğe uygulanan birincil travma, bu hasarın başlangıcından itibaren ilerleyen zaman dilimlerinde lezyonun yayılması ve doku hasarının ilerlemesini sağlayan hücresel ve moleküler olayların başlama basamaklarını tetikler. Oksidatif ve nitrosatif stres omurilik hasarının ilerlemesinde önemli rol oynar. Günümüzde deneysel embriyonik nöral kök hücre tedavisi omurilik hasarlarında yaygın olarak uygulanmaktadır, buna rağmen bu tedavinin oksidatif ve nitrosatif stres üzerine olan etkileri henüz tam olarak bilinmemektedir. Bu nedenle ENSC tedavisinin antioksidan enzimler [süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GPX)], nitrik oksit sentaz (NOS) ve nitrik oksit (NO) düzeyleri üzerindeki etkilerini araştırmayı hedefledik. **Gereç ve Yöntemler:** Bu amaçla, embriyonik nöral kök hücre uygulamasının etkilerini antioksidan enzimler ile NO sentaz aktiviteleri ve NO düzeylerini 7 (akut grup) ve 28 (kronik grup) günlük travmalı ve kontrol sıçanlarının T8-T9 omurilik bölgesinin cerrahi rezeksiyon sonucu elde edilen, 10 mm'lik bir segment dokusunda spektrofotometrik yöntemler kullanarak ölçtük. **Bulgular:** SOD düzeyleri akut ve kronik kontrollere kıyasla ne hasarlı bölgede, ne de terapi görmüş grupta değişmemiştir. NOS aktivitesi hem akut hem de kronik kontrollere göre akut, kronik ve tedavi görmüş gruplarda artmıştır, bununla birlikte NO düzeyleri de sayılan gruplarda yükselmiştir. Kendi kontrol gruplarına göre tüm hasarlı gruplarda CAT aktivitesi artmıştır. GPX aktivitesi akut hasarlı grupta yükselmiş, terapi GPX aktivitesini azaltmasına rağmen yine de sonuçlar akut kontrol gruptan yüksek bulunmuştur. **Sonuç:** Kök hücre tedavisi oksidatif/nitrosatif stres göstergelerini arttırmıştır. Kök hücre uygulamalarının tedavisi için, antioksidan maddeler ile birlikte uygulanması yoluna gidilebilir.

Anahtar Kelimeler: Embriyonik kök hücreler; omurilik hasarı; katalaz; nitrik oksit

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Traumatic injury of the central nervous system (CNS) results in the delayed dysfunction and death of neuronal population near and distant to the site of injury.[1] Traumatic spinal cord injury (SCI) causes tissue damage through both primary and secondary mechanisms. The primary mechanical injury results in damage to neuronal and vascular tissue. Most of the damage that follows the primary mechanical injury is exacerbated by secondary mechanisms such as glutamate excitotoxicity, ischemia, edema, Ca^{2+} overload, compromised energy metabolism, and oxidative stress.[1-3] Neurological dysfunction often results more from secondary changes than from primary neuronal damage. After SCI, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated through a series of cellular pathways by inflammatory cells, including NADPH oxidase, nitric oxide synthase (NOS), phospholipase A_2-cyclooxygenase, and the Fenton and Haber-Weiss reactions, leading to the formation of ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hydroxyl radical (OH).[4-6]

NO is known to be closely involved in the development of post-traumatic spinal cord cavitation as well as playing an important role in the development of the secondary pathological process in vivo.[4,7,8] This is occasioned by antioxidant depletion and/or excess production of oxygen free radicals and NO at the zone of injury. Oxygen free radicals avidly react with NO to produce highly reactive and cytotoxic products, namely, $ONOO^-$ and peroxynitrous acid (ONOOH).

Oxygen free radicals and ONOO can cause cytotoxicity by damaging lipids, proteins and nucleic acids.[3,9] Furthermore, NO-induced pathology has been linked with a large upregulation of NOS[3]. As noted earlier, oxidative and nitrosative stress play a major part in continuous expansion of spinal cord lesion after the initial injury.[1-3]

Embryonic neural stem cells (ENSCs), which exist in various regions of the CNS throughout the mammalian lifespan, can be expanded and induced to differentiate into neurons and glia in vitro and in vivo. Because of these properties, there has been increasing interest in the identification and characterization of ENSCs and neural progenitor cells both for basic developmental biology studies and for therapeutic applications to the damaged SC.[10,11] Transplantation of neural stem cells or their derivatives into a host brain and the proliferation and differentiation of endogenous stem cells by pharmacological manipulations are potential treatments for many neurodegenerative diseases and brain injuries, such as Parkinson's disease, brain ischemia and spinal cord injury.[11,12] The ENSC therapy is applied experimentally to a wide range of SCIs; however, effects of the ENSC therapy on oxidative stress that is an important secondary mechanism in SCI, is not well known yet.[12] In the present study, effects of the ENSC therapy on NOS activity and NO level, as well as the main antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities were investigated in the spinal cord tissue of rats obtained 7 and 28 days after spinal cord transection. The results were compared with the corresponding sham-operated groups.

MATERIAL AND METHODS

SUBJECTS

This study was approved by the Local Ethics Committee of the University Hospital and was carried out in the Animal Research Laboratory of Ege University, Faculty of Medicine, Izmir, Turkey. All procedures conformed to the recommendations of the Declaration of Helsinki on care and use of animals. All animals were kept in well ventilated, humidified and temperature controlled rooms with 12/12-h light/dark cycles. Thirty adult female Sprague-Dawley rats weighing between 250 to 270 grams were included in the study. The animals were randomly allocated into 6 groups.

Group 1: Sham (chronic period) (n= 5),

Group 2: Spinal cord transection only (chronic period) (n= 5),

Group 3: Spinal cord transection followed by ENSCs application into the lesion site 9 days after transection and followed up for 28 days after the operation (chronic period) (n= 5),

Group 4: Sham (acute period) (n= 5),

Group 5: Spinal cord transection only (acute period) (n= 5),

Group 6: Spinal cord transection followed by ENSCs application into the lesion site 9 days after transection and followed up for 7 days after the operation (acute period) (n= 5).

PREPARATION OF EMBRYONIC NEURAL STEM CELLS FOR TRANSPLANTATION

ENSCs (NRP and GRP-neuronal and glial restricted precursors cells) were isolated from rats on embryonic day 13.5. The preparation of NRPs and GRPs was described previously.[13,14] Briefly, embryos were isolated in DMEM/F-12. Trunk segments were incubated in collagenase type I/dispase II/HBSS (Hank's buffered salt solution), for 8 min at room temperature to remove meninges from the cords. Cords were dissociated using a 0.05% trypsin/EDTA (invitrogen) solution for 20 min at 37°C. Cells were then plated in complete medium [(DMEM/F-12, bovine serum albumine (BSA), B27, fibroblast growth factor (basic FGF), penicillin-streptomycin, N2, and neurotrophin-3] on poly-L-lysine and Laminin-coated dishes. After dissection, NRP and GRP were co-cultured for 5-10 days in the complete medium to generate a mixed population for grafting. Previous studies[14,15] verified that these cultures contained only precursors and were devoid of multipotent stem cells and mature cell types. NRPs and GRPs were dissociated from culture flasks using 0.05% trypsin/EDTA, were washed, and resuspended at a concentration of 100.000 cells/μL (in basal media) for transplantation.

Cells were placed on ice throughout the grafting session and were grafted in NRP/GRP (1:3). After the completion of the grafting session, cell viability was assessed using the trypan blue assay. Viability was always >90%. The composition of the NRP/GRP cultures, with respect to the absence of mature cells, was verified before grafting by staining for the mature neurons (neural-specific nuclear protein), astrocytes (glial fibrillary acidic protein), and oligodendrocytes (receptor interacting protein). The purity of the culture with respect to lineage-restricted precursors was verified by staining for the immature neural marker nestin, and the ratio of NRP to GRP was determined by their corresponding markers, embryonic neural cell adhesion molecule and A2B5.

PROCEDURE OF SPINAL CORD INJURY

Following anesthesia with intramuscular ketamine (50 mg/kg) + xylazine (5 mg/kg), lowest ribs and the vertebrae were attached to be identified. A 2 cm dorsal midline incision was performed and vertebral body was exposed. The paraspinal muscles were dissected from the spinal process and were retracted laterally. Following laminectomy of the T8-T9 vertebras, the spinal cord was completely sectioned with an iris scissors. To complete section of the spinal cord a 5 mm gap was made between the proximal and distal ends and collagen matrix was placed in-between. The paraspinal muscles were joined in the midline and the skin incision was closed with 4-0 catgut suture. Body temperature was kept at 37°C during and within the first 24 hours after the operation by using a hot plate. Intramuscular ampicillin (150 mg/kg) was used for prophylaxis for the next 7 days.

EMBRYONIC NEURAL STEM CELL TRANSPLANTATION

1 x 10^6 ENSCs were transplanted by collagen matrix into lesion site nine days after transection between early and late period.

POST-OPERATIVE CARE OF THE ANIMALS

Each rat was housed individually in a cage with a dry floor and material that prevents formation of decubitus ulcers. The positions of the rats were changed twice a day in the first week. After the operation the bladder was emptied every 8 hours until reflex micturition was restored for two weeks and twice daily afterwards.

IMMUNOSUPPRESSION WITH CYCLOSPORIN A

All animals received subcutaneous administration of cyclosporin (CsA) injection solution (Sandimmune, Novartis) 1 mg/100 g per 24 h, starting 3 days before the transplantation and continuing for 2 weeks after transplantation. After this, oral CsA solution (50 μg/mL) was administered through the drinking water until the animals were sacrificed.

CHEMICAL MATERIALS

DMEM-F-12, collagenase Type I, dispase II, HBSS, BSA, B27, basic FGF, penicillin-streptomycin, neurotrophin-2, and neurotrophin-3 were purchased from Sigma Chemical Co., (St. Louis, MO, USA). GPX activity was determined by a commercial enzyme kit (Ransel, RANDOX/RS-504 (Randox Laboratories, Crumlin, UK) and SOD activity was determined by a commercial enzyme kit Ransod, RANDOX/SD-125 (Randox Laboratories). NOS activity was measured by a commercial enzyme kit (Bioxytech Nitrosative Test Kits, Oxis Internationale Inc., Portland, USA) and NO level was determined by using a commercial kit (Bioxytech Nitrosative Test Kits, Oxis Laboratories).

SPINAL CORD TISSUE COLLECTION

At the end of the day 7 and day 28 observation period, lesioned region of the spinal cord (10 mm long segments) or ENSCs transplanted region were collected in the injury and treatment groups under anesthesia with intramuscular ketamine (50 mg/kg) + xylazine (5 mg/kg). In the sham operated control group, a corresponding 2 cm long segment of the spinal cord was collected. These spinal cord tissue samples were used for the analyses.

PREPARATION OF SPINAL CORD EXTRACTS

Homogenates (1:10, w/v) of spinal cord segments were prepared in cold phosphate buffer (PBS, 0.01 M; pH: 7.4) at 4°C using a polytron homogenizer. Homogenates were centrifuged at 15.000 g for 15 min at 4 °C. Concentration of soluble protein in pooled samples was determined by the method described by Lowry et al.,[16] using bovine serum albumin (BSA) as a standard.

METHODS

Catalase Activity

The CAT activity was determined spectrophotometrically according to Aebi et al.[17] The reaction mixture consisted of 1 mL PBS (50 mM, pH: 7.00) and 2 mL of diluted tissue homogenate. The mixture was incubated at 25°C for 3 min and the reaction was started by the addition of 1 mL of 30 mM H_2O_2. The decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm at 25°C measured at temperature-controlled Shimadzu UV-1601 spectrophotometer. The results were expressed as Aebi Unit (AU) /mg protein.

Superoxide Dismutase Activity

This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium-chloride to form the red formazan dye. The SOD activity was measured by the degree of inhibition of this reaction and was expressed as U/mg protein.

Glutathione Peroxidase Activity

In this method, GPX catalyses the oxidation of reduced glutathione (GSH) by H_2O_2. In the presence of glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbances at 340 nm was measured spectrophotometrically and was expressed as U/mg protein.

Nitric Oxide Synthase Activity

The principle of this assay is based on the measurement of NO_2 produced in the sample during a timed reaction compared with a heat-inactivated control sample. Nitrate reductase was utilized for the enzymatic reduction of nitrate to nitrite. Spectrophotometric quantitation of nitrite using Griess Reagents is straightforward and sensitive. In acidic solution, nitrite converts to HNO_2, which diazotises sulfanilamide. This sulfonilamide-diazonium salt then reacts with N-(1-Naphthyl)-ethylenediamine to produce a chromophore which was measured at 540 nm and was expressed as nmol/mg protein/min.

Measurement of No Levels

This kit employs granular cadmium metal for chemical reduction of nitrate to nitrite prior to quantitation of nitrite using Greiss reagent. In acid solution, nitrite converts to HNO_2 which diazotizes sulfanilamide. This sulfonilamide-diazonium salt was then reacts with N-(1-Naphthyl)-ethylenediamine to produce a chromophore, which was mea-

sured at 540 nm and was expressed as mmol/mg protein.

Light Microscope and Nissl Staining

Spinal cord was removed from the animal, followed by cryoprotection in 30% sucrose (Fisher)/0.1 M phosphate buffer at 4^0C for 3 days. The tissue was embedded in OCT (Fisher), fast frozen with dry ice, and stored at -80^0C until processed. Spinal cord tissue blocks were cut in the transverse plane at 20 μm thicknesses. Sections were collected on gelatin coated glass slides and were stored at -80^0C. Nissl staining was used to demonstrate Nissl substance in tissue sections. Nissl substance is lost after cell injury and if the axon degenerates, the myelin covering also breaks down .

Statistical Analysis

Results are expressed as mean ± standard error of means (SEM), or means with 95% confidence interval (CI), where appropriate. Differences in outcome measures between the groups were examined by the Student's t-test, one way and multivariate analysis of variances (ANOVA) and post hoc Bonferroni tests. A p value < 0.05 was considered statistically significant. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Version 13.0 for Windows (SPSS Inc, USA).

RESULTS

Comparisons between SOD levels of acute or chronic control, damage and therapy groups did not show any considerable differences (p> 0.05), which presents a compromise with the results of the following studies (Figure 1). NOS activities in acute and chronic injury groups increased as compared to their control groups (p< 0.01) and continued to increase following the stem cell therapy (p< 0.01) (Figure 2). In the same way, NO levels significantly elevated after acute and chronic injury and stem cell therapies (p< 0.01) (Figure 3). Acute and chronic injured groups have a significantly higher CAT activity than their control groups (p< 0.01) (Figure 4). The stem cell therapy applied both to acute and chronic groups further increased CAT activity (p< 0.01) than their control groups (p< 0.01). GPX activity in the acutely induced injury increased as compared to the acute control groups (p< 0.01), (Figure 6). The stem cell therapy diminished GPX activity in this group; however, it had a significantly higher level of GPX activity than acute controls (p< 0.01). GPX activities of chronically injured groups were significantly higher than the activities of the control group (p< 0.05 ve p< 0.01). All the results of this study are summarized in Table 1.

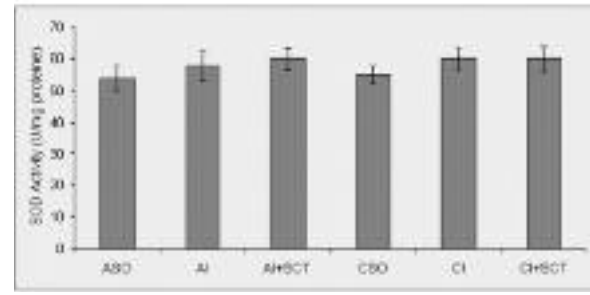

FIGURE 1: Effects of ENSC therapy on SOD activities of acute and chronic lesion region in SC tissue (ASO: Acute sham operated, AI: Acute injury group, AI + SCT: Acute injury +stem cell therapy group, CSO: Chronic sham operated, CI: Chronic injury group, CI + SCT: Chronic injury + stem cell therapy group). The data represent the mean ± S.E.M. SOD activities did not show significant differences between study groups (p> 0.05).

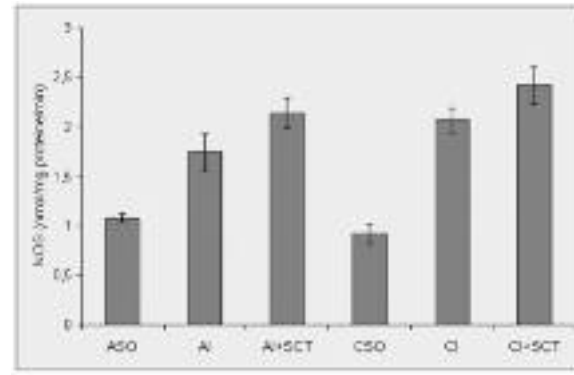

FIGURE 2: Effects of ENSC therapy on NOS activities of acute and chronic lesion region in SC tissue. The data represent the mean ± S.E.M. (ASO: Acute sham operated, AI: Acute injury group, AI + SCT: Acute injury + stem cell therapy group, CSO: Chronic sham operated, CI: Chronic injury group, CI + SCT: Chronic injury + stem cell therapy group). NOS activities in acute and chronic injury groups increased as compared to their control groups (p< 0.01) and continued to increase following the stem cell therapy (p< 0.01).

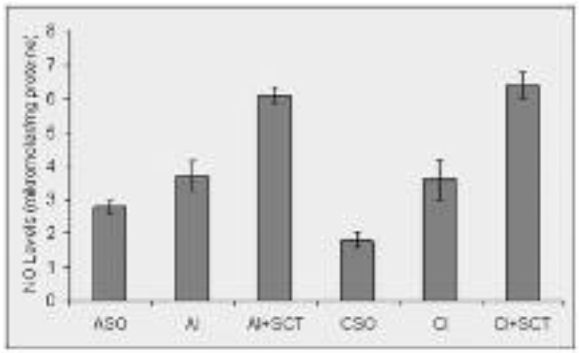

FIGURE 3: Effects of embryonic neural stem cell therapy on NO levels of acute and chronic lesion region in SC tissue. The data represent the mean ± S.E.M. (ASO: Acute sham operated, AI: Acute injury group, AI + SCT: Acute injury + stem cell therapy group, CSO: Chronic sham operated, CI: Chronic injury group, CI + SCT: Chronic injury + stem cell therapy group) NO levels significantly elevated after acute and chronic injury and stem cell therapies ($p < 0.01$).

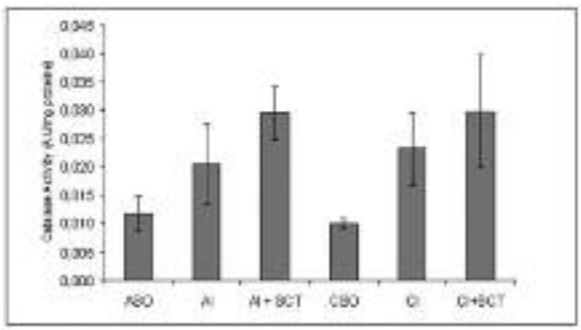

FIGURE 4: Effects of embryonic neural stem cell therapy on CAT activities of acute and chronic lesion region in SC tissue. The data represent the mean ± S.E.M. (ASO: Acute sham operated, AI: Acute injury group, AI + SCT: Acute injury + stem cell therapy group, CSO: Chronic sham operated, CI: Chronic injury group, CI + SCT: Chronic injury + stem cell therapy group). Catalase activities significantly elevated after acute and chronic injury and stem cell therapies ($p < 0.01$).

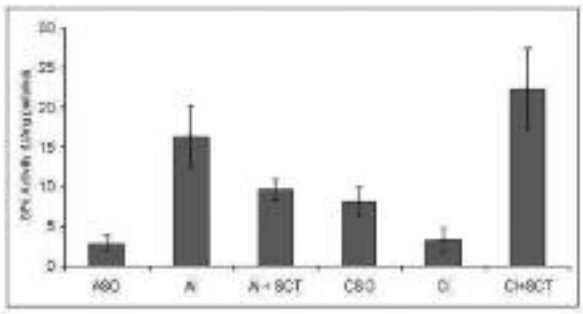

FIGURE 5: Effects of embryonic neural stem cell therapy on glutathione peroxidase activities of acute and chronic lesion region in SC tissue. The data represent the mean ± S.E.M. (ASO: Acute sham operated, AI: Acute injury group, AI + SCT: Acute injury + stem cell therapy group, CSO: Chronic sham operated, CI: Chronic injury group, CI + SCT: Chronic injury + stem cell therapy group). Stem cell therapy diminished GPX activity in the AI + SCT group (p 0.01), but GPX activities of chronically injured groups were significantly higher than those of the sham operated group ($p < 0.01$).

DISCUSSION

Considering previous investigations, there has been no study on the effects of the ENSC therapy on antioxidant enzymes, NOS activity and NO levels of the acute and chronic SCI rats. This study is the first to examine the effects of ENSC therapy on CAT, GPX, SOD, NOS activities and NO levels.

A complete transection of the spinal cord results in an immediate loss of all voluntary somatic and autonomic motor control as well as loss of all sensory input from below the lesion. Although several treatment strategies are being explored,[3,18] at present no effective therapy is available for SCI. However, use of experiment models of SCI and advances in understanding of the secondary mechanisms of injury may lead to greater progress in this area.[2,19] ENSCs are a potential therapeutic source for cellular transplantation because of their stability in vitro through multiple passages without loss of their multipotent nature. Furthermore, it is possible to induce them to differentiate into enriched populations of glial or neural progenitors.[20-23] ENSC therapy used autologous hematopoietic progenitor stem cells in order to avoid the problems associated with immunologic rejection and graftversus-host (GVH) reactions, which are frequently caused by allograft therapy.[24] Thus, it may be possible to design stem cell transplants customized to repair quite specific focal lesions in the injured CNS.[19,22]

Nissl staining results are presented in Figure 6. The pictures showing neuronal cellular development expected from both groups were obtained by Olympus B x 50 microscope in 10-fold magnification.

According to the results of this study, in groups with damage and stem cell transplantations, NOS, CAT and GPX activities and NO levels increased. SOD activity did not vary in both chronic and acute damage and therapy groups; this indicates that antioxidant enzyme activities increase in the site of damage to compensate for reactive oxygen species released due to both chronic and acute spinal cord injuries in a general picture.

TABLE 1: Results of SOD, NOS, CAT, GPx activities and NO levels of six study groups in SC tissue (± SD, n= 5). *p< 0.01 as compared with sham-controls and *p< 0.01 as compared with injury groups by ANOVA followed by post hoc Bonferroni test.

Study Groups	SOD Activity (U/mg proteine)	NOS Activity (nmol/mg)proteine/min)	NO Levels µmol/mg proteine)	CAT Activity (U/mg proteine)	GPX Activitiy (U/mg proteine)
Acute injury (AI)	56.163 ± 5.120	1.375 ± 0.038	3.680 ± 0.461*	0.023 ± 0.004*	16.837 ± 0.386
Acute injury + stem cell therapy (AI + SCT)	59.480 ± 4.681 (NS)	2.142 ± 0.194***	6.100 ± 0.242***	0.030 ± 0.008***	10.487 ± 0.474***
Acute sham operated groups (ASO)	51.144 ± 3.790 (NS)	1.545 ± 0.100	2.769 ± 0.196	0.012 ± 0.004	2.766 ± 0.386
Choronic injury (CI)	60.530 ± 4.778 (NS)	2.067 ± 0.197*	3.591 ± 0.643*	0.024 ± 0.007*	4.256 ± 0.919*
Choronic injury + stem cell therapy (CI + SCT)	60.284 ± 2.948 (NS)	2.419 ± 0.157***	6.400 ± 0.300***	0.034 ± 0.007***	23.352 ± 1.200***
Choronic sham operated (CSO)	52.039 ± 3.085 (NS)	0.917 ± 0.054	1.805 ± 0.216	0.09 ± 0.002	9.598 ± 0.533

SOD: Superoixde dismutase, NOS: Nitric oxide synthase, CAR: Catalase, GPX: Glutathione peroxidase.
(N.s.: Non-significant)

Taskiran et al.[25] reported that the SOD levels in the lesion did not change two days after SCI in rats (2000). Lee and associates[2] found that SOD activity in the site of spinal cord injury in rats did not vary during the follow-up from the first day through the fourth month. Kaynar et al.[5] measured SOD, GPX and CAT activities at different times (1 hours, 4 hours and 24 hours) following standardized and reversible experimental spinal injury in the rat. They found that the enzyme activities in the rostral, lesioned and caudal segments were indifferent whereas CAT activity displayed a prominent increase in general. Their findings indicated that H_2O_2 or its radical species were not the causative factors in tissue breakdown in experimental spinal trauma. Azbill et al.[1] showed evidence of oxidative stress, mitochondrial dysfunction, increased CAT activity but unchanged SOD activity in the rat spinal cord tissue obtained 24 hours after traumatic SCI.

Diaz-Ruiz et al[26] reported that NOS activity increased at 4 and 8 hours after trauma-induced SCI rats as compared with controls. Another study observed that NOS activity increased following chronic six-week injury in T8-10 site in rates.[3] Hamada et al demonstrated that NO levels measured immediately after the injury induced in spinal cord of rats demonstrated a greater increase than in the control group.[27] Liu[28] reported that NO levels increased in the tissue following SCI induced by weight-drop method.

Low NO levels help regulate neuroprotective neurotransmission in the nervous system; however, neurodegenerative disorders such as Parkinson and Alzheimer diseases increase NOS activity,

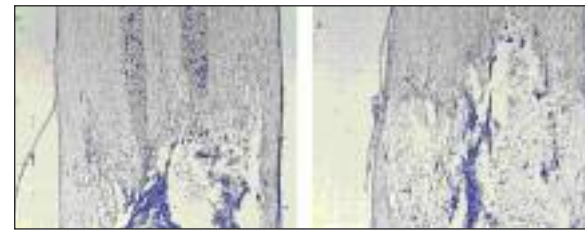

FIGURE 6: Nissl staining was used to demonstrate Nissl substance in acute (a) and chronic (b) spinal cord tissue sections. These sections were Nissl-stained and were examined under an Olympus B, x 50 microscope at 10-fold magnification.

with high levels of NO being synthesized. Therefore, many nitrogen-derived free radicals contribute to increased neurodegeneration as well. Elevated NO levels and NOS activities have neurodegenerative impacts on tissue, which in combination with free radicals also induce DNA damage, cytotoxicity, necrosis and apoptosis in cellular structure.[2] Since SOD enzyme activities did not increase sufficiently in both acutely and chronically injured rats and stem cell transplanted therapy groups, this study showed that superoxide anion in the medium could not be efficiently eliminated and a significant amount of NO possibly turned into peroxynitrite leading to increased neurotoxic effect in the lesion site. Therefore, we may suggest that adding NOS inhibitors as well as antioxidant substances into the medium can be useful to increase the chance of success in the stem cell therapy.

In a study in rats, which were traumatically injured at T-10 of the spinal cord, CAT activities at 1, 4 and 24 hours after the injury were induced. Accordingly, CAT activity remained the same as that of the control at the first hour but increased later.[1]

Our study showed that both acutely and chronically induced injuries presented high CAT activity. Lee observed that CAT activities in control and spinal cord injured groups of rats increased at 4 months after the induction of injuries in the injured and treated groups, respectively.[2]

Following chronically induced injury, GPX activity tends to decrease significantly as compared to acutely induced injury. However, stem cell transplantation increases GPX activity in the chronic group more significantly than in the acute therapy group (Figure 6).

Lee observed that GPX activity increased in the injured during the chronic period and continued to increase in the neuron-transplanted group.[2] Vaziri et al reported that GPX expression did not change on the first day after the partial T8 transsection of the spinal cord in rats, but increased considerably more than that in the control group after14 days.[3]

Our results showed a significant difference in the SC tissue antioxidant enzymes, NOS activities and NO levels in ENSC therapy group compared to the injury groups. Our data indicated the presence of local oxidative/nitrosative stress, which can contribute to the extension of the spinal cord injury. In general, stem cell therapy increased NOS activity and NO levels as well as antioxidant enzyme activity levels. Application of antioxidants in combination with stem cell therapies may be used to increase the chance of success of therapy. Finally, further studies are needed to explore the possible effects of ENSC transplantation with inhibitors of NOS or scavengers of their byproducts on progression of SCI.

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REFERENCES

1. Azbill RD, Mu X, Bruce-Keller AJ, Mattson MP, Springer JE. Impaired mitochondrial function, oxidative stress and altered antioxidant enzyme activities following traumatic spinal cord injury. Brain Res 1997;765(2):283-90.
2. Lee YS, Sindhu RK, Lin CY, Ehdaie A, Lin VW, Vaziri ND. Effects of nerve graft on nitric oxide synthase, NAD(P)H oxidase, and antioxidant enzymes in chronic spinal cord injury. Free Radic Biol Med 2004;36(3):330-9.
3. Vaziri ND, Lee YS, Lin CY, Lin VW, Sindhu RK. NAD(P)H oxidase, superoxide dismutase, catalase, glutathione peroxidase and nitric oxide synthase expression in subacute spinal cord injury. Brain Res 2004;995(1):76-83.
4. Hall ED, Braughler JM. Central nervous system trauma and stroke. II. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation. Free Radic Biol Med 1989;6(3):303-13.
5. Kaynar MY, Hanci M, Kuday C, Belce A, Gumustas K, Kokoglu E. Changes in the activity of antioxidant enzymes (SOD, GPX, CAT) after experimental spinal cord injury. Tokushima J Exp Med 1994;41(3-4):133-6.
6. Liu JB, Tang TS, Yang HL, Xiao DS. Antioxidation of melatonin against spinal cord injury in rats. Chin Med J (Engl) 2004;117(4):571-5.
7. Matsuyama Y, Sato K, Kamiya M, Yano J, Iwata H, Isobe K. Nitric oxide: a possible etiologic factor in spinal cord cavitation. J Spinal Disord 1998;11(3):248-52.
8. Kwak EK, Kim JW, Kang KS, Lee YH. The role of inducible nitric oxide synthase following spinal cord injury in rat. J Korean Med Sci 2005;20(4):663-9.
9. Lawler JM, Song W. Specificity of antioxidant enzyme inhibition in skeletal muscle to reactive nitrogen species donors. Biochem Biophys Res Commun 2002;294(5):1093-100.
10. Silani V, Corbo M. Cell-replacement therapy with stem cells in neurodegenerative diseases. Curr Neurovasc Res 2004;1(3):283-9.
11. Kimura H, Yoshikawa M, Matsuda R, Toriumi H, Nishimura F, Hirabayashi H, et al. Transplantation of embryonic stem cell-derived neural stem cells for spinal cord injury in adult mice. Neurol Res 2005;27(8):812-9.
12. Shimazaki T. Biology and clinical application of neural stem cells. Horm Res 2003;60 (Suppl 3):1-9.
13. Han SS, Kang DY, Mujtaba T, Rao MS, Fischer I. Grafted lineage-restricted precursors differentiate exclusively into neurons in the adult spinal cord. Exp Neurol 2002;177(2):360-75.
14. Lepore AC, Fischer I. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. Exp Neurol 2005;194(1): 230-42.
15. Lepore AC, Han SS, Tyler-Polsz CJ, Cai J, Rao MS, Fischer I. Differential fate of multipotent and lineage-restricted neural precursors following transplantation into the adult CNS. Neuron Glia Biol 2004;1(2):113-26.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.
17. Aebi H. Catalase in vitro. Methods Enzymol 1984;105:121-6.
18. Fawcett J. Repair of spinal cord injuries: where are we, where are we going? Spinal Cord 2002;40(12):615-23.
19. Cao Q, Benton RL, Whittemore SR. Stem cell repair of central nervous system injury. J Neurosci Res 2002;68(5):501-10.
20. Mayer-Proschel M, Kalyani AJ, Mujtaba T, Rao MS. Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. Neuron 1997;19(4):773-85.
21. Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, et al. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci U S A 2002;99(5):3024-9.
22. Faulkner J, Keirstead HS. Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl Immunol 2005;15(2):131-42.

23. Eaton MJ. Cell and molecular approaches to the attenuation of pain after spinal cord injury. J Neurotrauma 2006;23(3-4):549-59.

24. Deda H, Inci MC, Kürekçi AE, Kayihan K, Ozgün E, Ustünsoy GE, et al. Treatment of chronic spinal cord injured patients with autologous bone marrow-derived hematopoietic stem cell transplantation: 1-year follow-up. Cytotherapy 2008;10(6):565-74.

25. Díaz-Ruiz A, Ibarra A, Pérez-Severiano F, Guízar-Sahagún G, Grijalva I, Ríos C. Constitutive and inducible nitric oxide synthase activities after spinal cord contusion in rats. Neurosci Lett 2002;319(3):129-32.

26. Taskiran D, Tanyalcin T, Sozmen EY, Peker GO, Gulmen V, Cagli S, et al. The effects of melatonin on the antioxidant systems in experimental spinal injury. Int J Neurosci 2000; 104(1-4):63-73.

27. Hamada Y, Ikata T, Katoh S, Tsuchiya K, Niwa M, Tsutsumishita Y, et al. Roles of nitric oxide in compression injury of rat spinal cord. Free Radic Biol Med 1996;20(1):1-9.

28. Liu S, Qu Y, Stewart TJ, Howard MJ, Chakrabortty S, Holekamp TF, et al. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci U S A 2000;97(11):6126-31.