Alterations in the expression of the apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE/Ref-1) and DNA damage in the caudal region of acute and chronic spinal cord injured rats treated by embryonic neural stem cells

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**Running Title:** Embryonic neural stem cell transplantation on DNA damage

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Abstract

The oxidative mechanisms of injury-induced of neurons within the spinal cord are not very well understood. We used a model of T8-T9 spinal cord injury (SCI) in the rat to induce neuronal degeneration. In this spinal cord injury model, unilateral avulsion of the spinal cord causes oxidative stress on neurons. We tested the hypothesis that apurinic/apyrimidinic endonuclease (or redox effector factor-1, APE/Ref-1) regulates this neuronal oxidation mechanism in the spinal cord region caudal to the lesion, and that DNA damage is an early upstream signal. The embryonic neural stem cell therapy significantly decreased DNA-damage levels in both of study groups, acute (followed up to 7 days after SCI), and chronic (followed up to 28 days after SCI) injured animals. Meanwhile, mRNA levels of APE/Ref-1 significantly increased after embryonic neural stem cell therapy in acute and chronic injured animals when compared to acute and chronic sham groups. Our data has demonstrated that an increase of APE/Ref-1 mRNA levels in the caudal region of spinal cord strongly correlates with DNA damage after traumatic spinal cord injury. We suggest that DNA damage can be observed both in lesional and caudal regions of the acute and chronic injury groups, however with embryonic neural stem cell therapy DNA damage is reduced.

Key words: Spinal cord injury, Stem cell transplantation, DNA damage, APE protein, Ref-1 protein

Introduction

Traumatic injury to the spinal cord triggers several secondary effects which play a major role in biochemical and pathological changes in the spinal cord tissue. The enhanced production of reactive oxygen species (ROS) during spinal cord injury (SCI) appears to play an important role in neuronal cell death and neurological dysfunction (Vaziri et al., 2004). ROS induced oxidative DNA damage is believed to be a major cause of these kinds of lesions (Azbill et al., 1997). As oxidatively modified DNA bases have mutagenic potential, their accumulation with time might be a major cause of the physiological changes associated with SCI. By spontaneous depurination of
DNA about 10,000 apurinic sites per day per cell are generated (Lindahl et al., 1972). Base modifications occur endogenously or are the result of environmental mutagens, in particular alkylating compounds or ROS. There are multiple repair pathways to excise oxidative base modifications and prevent their incorporation into DNA (Bohr et al., 1999). The DNA repair enzyme, apurinic/apyrimidinic endonuclease (or redox effector factor-1, APE/Ref-1), is involved in base excision repair of apurinic/apyrimidinic sites after oxidative DNA damage. The DNA repair enzyme apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) is a multifunctional protein involved in both the repair of oxidative and alkylating DNA damage and the regulation of gene expression. First recognized as a major APE, this enzyme catalyzes the cleavage of the phosphodiester either 5’ or 3’ to an apurinic/apyrimidinic site (Kawase et al., 1999). Apurinic sites are both mutagenic and cytotoxic (Loeb et al., 1986) and are enzymatically repaired by APE. Human APE/Ref-1 has been reported to be inducible by oxidative stress (Ramana et al., 1998) and has been shown to affect cellular susceptibility to different types of genotoxic agents including oxidative compounds. This strongly indicates that APE/Ref-1 is part of an adaptive cellular response mechanism to oxidative stress. With the use of experimental SCI models in the past few years, the effectiveness of various treatment regimens such as receptor blockers, physiologic antagonists, inhibitors of biosynthetic pathways, and membrane-stabilizing drugs have been investigated (Bambakidis et al., 2005). 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 an 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 spinal cord (Schultz, 2005).

Our aim was to show the changes in the expression levels of APE/Ref-1 in caudal region of spinal cord-injured rats during acute and chronic period. Single-cell gel electrophoresis (comet assay)
procedure was also used to measure molecular changes in caudal region of spinal cord neurons during the progression of oxidative stress. The second aim of this study was to show the changes in APE/Ref-1 expression and DNA damage in caudal region of acute and chronic spinal cord-injured rats after ENSC treatment.

Methods

Thirty adult, female Sprague-Dawley rats weighing between 250-270 g were included in the study. The animals were randomly separated into 6 groups.

**Group 1**: Sham (chronic group) (n=5),

**Group 2**: Spinal cord injury (SCI) only (chronic group) (n=5),

**Group 3**: SCI followed by ENSCs application into the lesion site 9 days after hemisection which has been followed up 28 days after the operation (chronic period) (n=5),

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

**Group 5**: SCI only (acute period) (n=5),

**Group 6**: SCI followed by ENSC application into the lesion site 9 days after hemisection which has been followed up to 7 days after the operation (acute period) (n=5). The study was approved by the Local Ethics Committee of the Ege University Hospital and was carried out in the Animal Research Laboratory of Ege University, School of Medicine, Izmir, Turkey. All procedures confirmed 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 cycle.

Spinal Cord Injury

Following anesthesia with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg), lowest ribs and the vertebrae they attach to were identified. The back musculature was excised and a laminectomy was performed at the T8/T9 level. Dura was incised above the dorsal root entry zone. Microscissor cuts were created at the rostral and caudal extents of the injury. Aspiration was used
to selectively ablate only the lateral white matter tracts and a minimal portion of the dorsal and ventral gray matter. However, the dorsal columns and central canal were untouched. Once hemostasis was achieved, NRP/GRP cells in collagen matrix were implanted into the injury cavity. The cell/matrix mixture completely filled the cavities, and in each case ~1x10⁶ cells were grafted into the injury site. The dura was closed with 9-0 suture, muscle was reapposed and the skin closed with wound clips. Body temperature was kept at 37°C during and within the first 24 hours after the operation using a heat plate. Intramuscular ampicillin at a dose of 150 mg/kg was used for prophylaxis for the next 7 days. 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. In the first 2 weeks, the bladder was emptied every 8 hours until reflex micturition was restored and twice daily afterwards. Animals were immuno-suppressed by subcutaneous administration of cyclosporine A (10 mg kg⁻¹) daily, beginning three days before grafting and continuously until sacrifice.

**Locomotor function**

Locomotor function was assessed blindly by using an open-field locomotor test and scored according to the Basso, Beattie and Bresnahan (BBB) rank scale (Basso et al., 1995). The BBB procedure uses an ordinal nonlinear 21-point scale from 0 (no observable movements) to 21 (normal locomotion) for locomotor evaluation. This test is used to assess the state of contusion injury, showing deficits and partial recovery.

**Preparation of embryonic neuronal stem cells for transplantation**

ENSCs (NRP and GRP-Neuronal and glial restricted precursors) were isolated from embryonic day 13.5 rats. The preparation of NRPs and GRPs has been described previously (Han et al., 2002; Lepore et al., 2005). Briefly, embryos were isolated in DMEM/F-12. Trunk segments were incubated in collagenase type I/dispass II/Hanks balanced salt solution (HBSS) 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, BSA, B27, basic FGF, penicillin–streptomycin, N2, and neurotrophin-3] (Sigma, St. Louis, MO, USA) on poly-L-lysine and Laminin-coated dishes. After dissection, NRP and GRP were co-cultured for 5–10 day in the complete medium to generate a mixed population for grafting. Previous studies (Lepore et al., 2005; Lepore et al., 2004) 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, 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 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.

**Comet Assay**

Caudal region of spinal cord (1 cm caudal tissue to the epicenter of injury) was collected in the injury and treatment groups. 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 analysis. Comet assay was adapted from the method of Singh et al (Singh et al., 1988). The lesioned region was minced, suspended and chilled with a homogenizing buffer (pH 7.5) containing 0.075 M NaCl and 0.024 M Na₂EDTA and then homogenized and gently using a Potter type homogenizer. Homogenates were centrifuged at 700xg for 10 min at 0°C and the precipitates were re-suspended in chilled homogenizing buffer. The cell suspension was mixed with 500 µl of
LMP agarose in PBS at 37°C, and 140 µl of this mixture was applied onto a frosted glass microscope slide pre-coated with a layer of 1% NMP agarose (150 µl). After application of a third layer of NMP agarose, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris, 1% N-sodium lauroyl sarcosinate, 1% Triton-X-100, 10% DMSO) for 1h at 4°C. The slides were then placed in an electrophoresis tank, allowing the DNA to unwind for 15 min in the alkaline solution (300 mM NaOH and 1 mM Na$_2$EDTA). Electrophoresis was then performed at 300 mA for 20 min in the same alkaline solution at room temperature. The slides were neutralized with 0.4 M tris-HCl buffer (pH 7.4) and were stained using the silver staining method described by (Nadin et al. (2001). The dried microscope slides were covered with a cover-glass prior to analyzing under an Olympus light microscope. The microscope was connected to a charge-coupled device camera and a personal computer based analysis system (CASP-Comet Analysis Software Program, 1.2.2 Version) to determine the extent of DNA damage after electrophoretic migration of DNA fragments in the agarose gel. Results were expressed as percentage of DNA in the tail (tail%) or in tail moment [Tail DNA% x Tail Length (percent of DNA in the tail) x (tail length)] or tail length (length of the comet tail measured from right border of head area to end of tail in pixels). Three samples per rat were assayed implicating 50 cells per sample. The means ± standard error means (SEM) of the three samples were calculated for each rat.

**RT-PCR**

Total RNA was extracted from the lesioned regions using TriTidy G reagent followed by phenol-chloroform extraction and isopropanol precipitation (Chomczynski, 1993). One µg of total RNA was reverse-transcribed at 42°C for 1 h in a 20 µl reaction mixture containing mouse Moloney Leukemia virus reverse transcriptase. The cDNA products were amplified for APE/Ref-1 gene expression via PCR with APE/Ref-1 specific primers. The forward primers were 5’-GCCAGAGACCAAGAAGAGTA-3’ for APE/Ref-1 and 5’-AGGTCATCCACAGAGCTGAA-3’
for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reverse primers were 5’-AAGGTCATCCAGAGCTGAA - 3’ for APE/Ref-1 and 5’-ATGTGAGCCATGAGGTCCAC-3’ for GAPDH. Amplification of 2 µl RT mixture was carried out using 5.0 µl 10XPCR buffer, 4.0 µl 25 mM MgCl2, 1.0 µl 10 mM dNTPs, 1 µl of forward and reverse primers (0.1 µg/µl) and 5 units Taq DNA polymerase in a total volume of 50 µl. Cycling parameters were: 5 min at 94°C, 45 s at 94°C followed by 35 cycles of 30 s at 60°C and 50 s at 72°C. The amplified PCR products were analyzed by electrophoresis in 1.7% agarose gels and visualized under UV illumination after ethidium bromide staining. Data are expressed as mean ± standard error means (SEM), or means with 95% confidence interval (CI), where appropriate. Differences in outcome measures between the groups were examined by Tukey test and Kruskal-Wallis. 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

The BBB open-field locomotor scoring was performed to determine the functional outcome of ENSC grafts to injured spinal cords. For all groups, preoperative baseline BBB score was 21. After injury, BBB score dropped to 0-2 and then gradually recovered. 28 days after hemisection, BBB scores reached a plateau in ENSC groups in acute and chronic groups. In the chronic group (28 days after ENSC transplantation) the animals had an average BBB score of 11.17±2.88, which was significantly higher (p<0.01, Kruskal-Wallis) when compared to the acute group (7 days after transplantation) with ENSC transplantation (4.2 ± 0.8). There were no significant differences between acute and chronic spinal cord injured rats.
Comet assay parameters (including T%, TL and TM) in caudal region of all the sham operated, acute and chronic SCI groups and ENSC therapy rats are shown in Table 1 and Fig 1. In both acute and chronic study groups, T%, TL and TM levels were significantly higher in the injury groups when compared to the sham operated groups (p<0.001). T-DNA %, TL and TM (p<0.001) levels decreased in the SCI+ENSC therapy group for both acute and chronic study groups (p<0.001).

RT-PCR and APE/Ref-1 mRNA expression results are shown in Fig 2 a and b. mRNA levels of APE/Ref-1 decreased 43.36% and increased 31.75% in acute SCI and SCI+ENSC therapy groups, respectively in acute study groups and decreased 31.97% and increased 65.13% in chronic SCI and SCI+ENSC therapy groups in chronic study groups, when compared to the sham (p<0.01).

After ENSC therapy, in both acute and chronic SCI groups APE/Ref-1 levels significantly improved (\*p<0.01 and \*p<0.01, respectively). Besides, chronic ENSC therapy was more effective than the acute (\#p<0.05) (Fig. 2b).

Discussion

The most important consequence of SCI is severe nerve injury that results from two mechanisms, primary trauma and secondary damage. The primary trauma induces mechanical compression, bleeding and electrolyte disturbances, which can lead to irreversible nerve injury. The secondary damage is due to edema, hemorrhage, inflammatory reaction, lipid peroxidative reaction, energy metabolism system disorders, ischemia and oxidative stress, which can give rise to reversible nerve injury. After SCI, the body's inflammatory cells produce highly reactive oxidizing agents including "free radicals" (Lee et al., 2004; Vaziri et al., 2004).

Reactive oxygen species (ROS) are likely to mediate apoptotic cell death through mitochondrial release of cytochrome C and activation of caspases after traumatic and ischemic
brain injury (Shimazaki, 2003; Singh et al., 1988). Several endogenous antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase can detoxify ROS. However, overproduction of ROS after injury often exceeds the capacity of these enzymes, causing oxidative stress and subsequent cell death (Singh et al., 1988).

Oxidizing agents attack molecules that are crucial for cell function by modifying their chemical structure. This process is called oxidative damage. Oxidative damage occurs in disorders ranging from slow neurodegenerative diseases like amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) and Parkinson's disease to acute events like stroke and trauma. Thus, it has been the focus of intensive research. Much of the damage that occurs in the CNS following trauma is due to secondary effects such as glutamate excitotoxicity, Ca²⁺ overload and oxidative stress, three mechanisms that in a spiraling interactive cascade lead to neuronal death (Juurlink et al., 1998; Xu et al., 2005).

Focal trauma induces oxidative damage in distinct regions of spinal cord. Several studies showed that the parameters increased during ROS-induced oxidative stress changed not only at the lesion site, but also in regions caudal and rostral to the lesion in the spinal cord (Thornwall et al., 1997). Bao et al. (2004) assessed trauma effects on the expression of APE/Ref-1 expression and DNA damage in the lesioned region of spinal cord. They have shown that DNA damage was increased after SCI while APE/Ref-1 expression was decreased. So, we were to show the changes in the expression levels of APE/Ref-1 in the caudal section of SC injured rats during acute and chronic period. Therefore, caudal section was chosen as an experimental biological material.

Transplanting genetically modified fibroblasts that secrete BDNF into the lateral funiculus lesion site can support rescue of injured neurons destined to die, regeneration of injured axons, sprouting of spared axons, and ameliorate functional deficits (Kim et al., 2001; Liu et al., 1999; Murray et al., 2002).
Our results showed a decrease on APE/Ref-1 levels and an increase in oxidative DNA damage scores (comet assay) in the nucleus of neurons after acute and chronic injury in the caudal section, although DNA damage in other cell groups including glia, infiltrating leucocytes and endothelial cells of vascular walls might also be contributing to these changes (Schwab et al., 2005). Nuclear DNA is highly susceptible to oxidative damage because of its proximity to reactive oxygen species and its poor repair mechanisms (Beal, 1995). In addition to direct DNA damage, DNA mechanism can also be impaired after SCI. Oxidized DNA normally undergoes base excision repair by APE/Ref-1 (Chopp et al., 1996) and milder forms of brain injury resulting in an increase in the expression of repair enzymes such as APE/Ref-1. APE/Ref-1 is suggested to play a central role in base excision repair by 3′-OH primer for synthesis of DNA after all types of oxidative damage. However, after the severe SCI studied APE/Ref-1 expression is reduced. Kawasse and coworkers (1999) have suggested that ROS might have an important role in the reduction of APE/Ref-1. Although the mechanism of APE/Ref-1 reduction after trauma is not clearly known, free oxygen radicals can reduce the expression of APE/Ref-1. Our results showed that comet scores were strongly correlated with APE/Ref-1 in caudal sections of acute and chronic injured spinal cords.

After ENSC therapy, comet scores (Tail-DNA %, TL and TM) clearly decreased, however APE/Ref-1 expression levels increased. The ENSC treatment reduced oxidation of DNA, presumably due to the reduction of free radical formation as a result of oxidative stress blockade. Indirectly this treatment also permitted DNA repair to be sustained in cells at the all sections of the spinal cord. We observed a similar improvement in caudal DNA damage after ENSC therapy for the acute and chronic SCI groups. This condition was expected. The significantly higher improvement in the chronic ENSC therapy group, compared to the acute ENSC group, might be due to the improvement of secondary damage events, such as edema, hemorrhage, inflammatory reaction, ischemia, oxidative stress.
In conclusion, we have shown that APE/Ref-1 decreased after acute and chronic SCI in the caudal section. After the ENSC therapy, the oxidative DNA damage improved and elevated APE/Ref-1 expression levels. Further examinations are necessary to elucidate the relationship between the potential antioxidant effects of stem cell therapy and APE/Ref-1 expression levels.

References


**Legends**

**Table 1.** Effect of ENSC therapy on caudal DNA damage comet-Tail-DNA %, TL and TM scores in sham-controls, chronic and acute SCI-rats (means ± SEM). Statistically significant differences are indicated as: *** p<0.001, ** p<0.01 versus sham group; ††† p<0.001 versus SCI group

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<th>T-DNA %</th>
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<tr>
<td>Acute sham</td>
<td>9.93 ± 0.53</td>
<td>7.33 ± 2.18</td>
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<tr>
<td>Acute SCI</td>
<td>20.12 ± 3.06**</td>
<td>14.22 ± 2.22**</td>
<td>4.98 ± 1.26**</td>
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<td>Acute SCI+ENSC</td>
<td>3.47 ± 0.40†††</td>
<td>4.75 ± 0.63†††</td>
<td>0.38 ± 0.008†††</td>
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<td>Chronic sham</td>
<td>7.575 ± 0.29</td>
<td>6.75 ± 1.18</td>
<td>1.18 ± 0.12</td>
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<tr>
<td>Chronic SCI</td>
<td>34.29 ± 3.75**</td>
<td>17.50 ± 1.44**</td>
<td>11.64 ± 2.69***</td>
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<td>Chronic SCI+ENSC</td>
<td>5.07 ± 2.56†††</td>
<td>4.67 ± 1.48†††</td>
<td>0.46 ± 0.01†††</td>
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**Fig. 1.** Typical comets from acute and chronic SCI groups with and without ENSC therapy. Silver staining. Microscope objective X20 A: Acute SCI, B: Acute SCI+ENSC, C: Acute sham group, D: Chronic SCI, E: Chronic SCI+ENSC, F: Chronic sham group

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Fig. 2. APE/Ref-1 mRNA expression in acute and chronic spinal cord injury groups with and without ENSC therapy. (a) RT-PCR products, separated and visualized by agarose gel electrophoresis, representing APE/Ref-1 mRNA levels (520 bp) in: 1. Acute Sham operated, 2. Acute SCI, 3. Acute SCI+ENSC therapy 4. Chronic Sham operated, 5. Chronic SCI, 6. Chronic SCI+ENSC therapy groups. M: 100 bp DNA Ladder molecular size marker (b) Values are presented as the ratio between the target mRNA and the corresponding GAPDH mRNA in acute and chronic groups (*,a, p<0.01 and # p<0.05).