Effects of Embryonic Neural Stem Cell Therapy on DNA Damage Products in Urine and Tissue After Spinal Cord Injury in Rats

Embriyonik Nöral Kök Hücre Tedavisinin Farelerde Omurilik Yaralanmasından Sonra İdrar ve Dokudaki DNA Hasar Ürünleri Üzerindeki Etkileri

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ABSTRACT Objective: We tested the hypothesis that embryonic neural stem cell (ENSC) therapy reduces neuronal DNA damage in the lesional spinal cord (SC) region and assessed the significance of some DNA breakdown products in urine and tissue samples as biomarkers of efficiency of the ENSC therapy. **Material and Methods:** DNA damage was measured by single cell gel electrophoresis (SCGE/Comet Assay) in spinal cord tissue 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 operations. As biomarkers of DNA damage, urinary 5-(hydroxymethyl) uracil (5HMU) and 2'-deoxyuridine (2dU) were analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection (ED). **Results:** Comet scores of the lesioned tissues were significantly higher in the acute and chronic spinal cord injury (SCI) compared to the sham operated groups (p=0.009 and p=0.009, respectively). After the ENSC transplantation, tail %, tail length and tail moment (T%, TL and TM, respectively) decreased in acute (p=0.009, p=0.009 and p=0.009, respectively) and chronically (p=0.009, p=0.009, p=0.009 respectively) injured rats. 5HMU and 2dU were significantly higher in the acute and the chronic groups than in the sham operated groups (p=0.009 and p=0.009, respectively). 5HMU concentration in the acute SCI group had a stronger correlation strongly with tail moment (r=0.9039, p=0.02). In the chronic injury group, 5HMU and 2dU concentrations correlated significantly with tail length (r=0.9990, p=0.001 and r=0.9272, p=0.016, respectively). While 5HMU seems to be a better biomarker for acute injury, both HMU and 2dU excretions increase in chronic damage. Besides, neurological functions of all the study animals were measured with the Basso-Beattie-Bresnahan (BBB) score. **Conclusion:** Our results demonstrated that ENSC therapy of the lesioned tissue significantly decreased the levels of DNA breakdown products in urine and tissue samples of rats with acute and chronic SCI. Assessment of neurological recovery with the BBB test confirmed these findings.

Key Words: Spinal cord injuries; comet assay; chromatography, high pressure liquid; 2'-deoxyuridylic acid; 5 hydroxymethyluracil

ÖZET Amaç: Embriyonik nöral kök hücre (ENKH) tedavisinin bozulan omurilik bölgesinde sinirsel DNA hasarını azalttığı hipotezini test ettik ve ENKH tedavisinin etkinliğini yansıtmak üzere idrar ve doku örneklerinde bazı DNA yıkım ürünlerinin önemini değerlendirdik. **Gereç ve Yöntemler:** DNA hasar düzeyleri, 7 (akut grup) ve 28 (kronik grup) günlük travmalı sıçanların ve kontrol sıçanlarının T8-T9 omurilik bölgesinin cerrahi rezeksiyonu ile elde edilen 10 mm'lik bir segment dokusunda tek hücre jel elektroforez tekniği (THJE/Comet Testi) kullanılmak suretiyle ölçüldü. İdrarda DNA hasarının biyolojik göstergeleri olarak, 5-hidroksimetil-urasil (5HMU) ve 2'-deoksiüridin (2dU), elektrokimyasal saptama (ES) ile yüksek performanslı sıvı kromatografisi (YPSK) ile incelendi. **Bulgular:** Kontrol grupları ile karşılaştırıldığında, bozulan dokuların Comet skorları akut ve kronik omurilik yaralanmasında anlamlı düzeyde daha yüksekti (sırasıyla, p=0,009 ve p=0,009). Akut (sırasıyla, p=0,009, p=0,009 ve p=0,009) ve kronik (sırasıyla, p=0,009, p=0,009, p=0,009) hasarlı farelerde ENKH transplantasyonundan sonra kuyruk yüzdesi, kuyruk uzunluğu ve kuyruk momenti azaldı. 5HMU ve 2dU düzeyleri, kontrol grupları ile kıyaslandığında akut ve kronik gruplarda anlamlı ölçüde daha yüksekti (sırasıyla p=0,009 ve p=0,009). Akut omurilik hasarı olan gruplarda, 5HMU düzeyi ile kuyruk momenti arasında daha güçlü bir ilgileşim olduğu saptandı (r=0,9039, p=0,02). Kronik hasarlı grupta ise 5HMU ve 2dU düzeylerinin ise kuyruk uzunluğu ile ilgileşim gösterdiği belirlendi (sırasıyla, r=0,9990, p=0,001 and r=0,9272, p=0,016). 5HMU akut hasarda daha iyi bir biyolojik gösterge gibi görünürken, kronik hasarlarda hem 5HMU hem de 2dU'nun idrarla atılımının arttığı belirlendi. Bunun yanı sıra, çalışmadaki tüm hayvanların nörolojik işlevleri Basso-Beattie-Bresnahan (BBB) skoru ile değerlendirildi. **Sonuç:** Bulgularımız, bozulan dokuda ENKH tedavisinin, akut ve kronik omurilik yaralanması olan farelerin idrar ve doku örneklerinde DNA yıkım ürünlerinin düzeylerini belirgin olarak azalttığını ortaya koymuştur. Nörolojik iyileşmenin BBB testi ile değerlendirilmesi bu bulguları doğrulamıştır.

Anahtar Kelimeler: Omurilik yaralanmaları; comet assay; kromatografi, yüksek basınçlı sıvı; 2'-deoksiüridilik asit; 5-hydroxymethyluracil

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Traumatic spinal cord injuries (SCI) cause tissue damage through both primary and secondary mechanisms.[1] The primary mechanical injury results in damage to neuronal and vascular tissues. Most of the damage that follows the primary mechanical injury are exacerbated by secondary mechanisms such as glutamate excitotoxicity, ischemia, edema, Ca^{2+} overload, compromised energy metabolism, and oxidative stress.[1-4] Neurological dysfunction often results in secondary changes rather 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 reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthase (NOS), phospholipase A2-cyclooxygenase, and the Fenton and Haber-Weiss reactions, leading to the formation of ROS such as the superoxide anion (O_2), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hydroxyl radical ($^{\cdot}OH$).[2,3,5]

Free radical-induced DNA damage is believed to be a major cause of various processes including aging and cancer and the rate of formation of DNA oxidation products reflects the extent of genetic damage caused by ROS.[5] As oxidatively modified DNA bases have mutagenic potential, their accumulation with time might be a major cause of the physiological changes associated with SCI. There are multiple repair pathways to excise oxidative base modifications and prevent their incorporation into DNA.[6,7] After excision from DNA, the oxidatively induced lesions are released into the blood stream and consequently into the urine, where their measurement has been acknowledged to be reflective of overall oxidative stress.[7,8]

2'-deoxyuridine (2dU) is an oxypyrimidine, which is very polar and weakly acidic in nature. 2dU levels in plasma and urine have been quantitated in several experimental and human studies of oxidative DNA damage.[9,10] 5-(hydroxymethyl) uracil (5HMU) is an OH-mediated uracyl oxidation product and its urinary excretion should also represent oxidative DNA damage.[11,12]

In terms of oxidative DNA damage, much interest has been focused on modifications of DNA bases, with over 20 products identified, although only a few have been investigated in great detail.[12,13] Oxidative DNA damage products in urine are generally measured by high-power liquid chromatography (HPLC) methods.[14,15]

Otherwise, the single-cell gel assay (also termed comet assay) is used to measure DNA damage levels of tissue. This is a sensitive method for detecting DNA strand breaks (including those created by alkali-labile sites) at the level of individual cell.[5,16] It is simpler and faster than other conventional genotoxicity techniques. The parameter often used to measure DNA damage is the "tail moment", which is defined as the product of the tail length and the fraction of total DNA content that is present in the tail.[16,17] The second aim of this study was to investigate the relationship between tissue comet parameters and urinary HPLC analyses and to determine the urinary oxidative DNA damage products that correlate most with tissue comet results.

Our review of the medical literature revealed no reports on the effects of oxidatively modified DNA products in the pathogenesis of the neurological damage in acute and chronic SCI. Thus, the first aim of the present study was to investigate the effects of SCI on the levels of DNA damage products in urine and lesioned tissue samples in rats.

MATERIAL AND METHODS

We analyzed urinary excretion of all the substances described above in acute and chronic SCI rats and after embryonic neural stem cell (ENSC) transplantation. We used a HPLC procedure with electrochemical detection for the measurements of urinary 5HMU and 2dU. In addition, tissue DNA damage of all study groups were measured with the Comet assay.

CHEMICALS

Neurotrophin-3-human (NT-3), fibroblast growth factor (FGF), low-melting-point (LMP) agarose, normal-melting-point (NMP) agarose, RPMI 1640, N-sodiumlauroyl sarcosinate, 5HMU standarts were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2dU was obtained from Acros

Organics (Geel, Belgium). Ultra-pure o-phosphoric acid was purchased from Riedel-de Haen (Hannover, Germany).

EXPERIMENTAL PROCEDURE

Animals

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, İzmir, 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 250-270 g 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 ENSC application into the lesion site 9 days after transection, which has been followed up until 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 ENSC application into the lesion site 9 days after transection, which has been followed up until 7 days after the operation (acute period) (n=5).

Preparation of ENSCs

ENSCs [neural restricted precursors NRP and glial restricted precursors (GRP)] were isolated from embryonic day 13.5 rats. The preparation of NRPs and GRPs has been described previously.[18,19] Briefly, embryos were isolated in Dulbecco's Modified Eagle Medium Nutrient Mixture F (DMEM/F)-12. Trunk segments were incubated in collagenase type I/dispase II/Hanks balanced salt solution (HBSS) for 8 minutes at room temperature to remove meninges from the cords. Cords were dissociated using a 0.05% trypsin/ethylene diamine tetraacetic acid (EDTA) (Invitrogen) solution for 20 minutes 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, NRPs and GRPs were co-cultured for 5–10 days in the complete medium to generate a mixed population for grafting. Previous studies verified that these cultures contained only precursors and were devoid of multipotent stem cells and mature cell types.[19,20] 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 found to be >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 SCI

Following anesthesia with intramuscular ketamine (50 mg/kg)+xylazine (5 mg/kg), 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 formed 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.

Neurological Assessment

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

ENSC transplantation

At day 9 after after transection between acute and chronic period, $1x10^6$ ENSCs were transplanted by collagen matrix into lesion site.

Post-operative Care of the Animals

Each rat was housed individually in a cage with a dry floor and material that prevents the 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.

Sample Collection

Urine: Spot urine samples of acute and chronic study groups were collected after the injury, therapy and laminectomy on days 7 and 28. The urine samples were transferred to sterile plastic tubes and were stored at -80°C until the HPLC analysis.

Spinal cord tissue: At the end of day 7 and day 28, animals were anesthetized by intramuscular ketamine (50 mg/kg)+xylazine (5 mg/kg). Lesioned region (10 mm long segments) or the ENSC transplanted region of the spinal cord were collected in the injury and treatment groups. In the sham operated control group, a corresponding 10 mm long segment of the spinal cord was collected. These spinal cord tissue samples were used for the analyses.

BIOCHEMICAL PROCEDURES

Preparation of Spinal Cord Extracts for 5HMU and 2dU

Homogenates (1:10, w/v) of spinal cord segments were prepared in cold phosphate-buffered saline (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., using BSA as a standard.[22]

5-HMU and 2dU Measurements

The clean up of urine samples included a two-step solid phase extraction (SPE). The analyses of 5HMU and 2dU were performed by HPLC-EC as previously described.[23] Briefly, urine fractions were thawed in a 37°C water bath. Urine samples of control group were spiked with biomarkers of interest (5 ppm for 5HMU and 2dU, n=10 for each). Before application of the spiked samples the SPE cartidges had to be activated as stated by the producer. During the activation process cartidges were washed with 10 mL of methanol, 5 mL of ultra pure bi-distilled water and 10 mL of 25 mM KH2PO4 solution, respectively at a flow rate of 0.5mL/minute. Subsequently, the sample was evaporated by pure argon (Ar) gas until dryness to remove methanol. The remnant was brought to a final volume of 0.5 mL with the mobile phase. Prior to application, the solutions were filtred through 0.45 mm mesh filters (16 555 Minisart® Sartorius AG). An Agilent 1100 series (USA) isocratic pump system (G1310A) equipped with a pulse damper and a manual injector block (G1328A Rheodyne 7725 I) with 20 mL loop volume was connected to a programmable electrochemical detector (HP 1049). The electrochemical cell was equipped with a glassy carbon-working electrode, operated at +1000 mV versus an Ag/AgCl reference electrode. The system was operated at 0.5 mA full range deflection. Data acquisition was performed with HP ChemStation for LC Rev. A. 06. 03 [509] software. The separation of 5-HMU and 2-dU were carried out on an analytical C18 column (Hichrom 5 C18, 7.75x300 mm, 5 mm particle size). The mobile phase was 10 mM $MeOH/H_3PO_4$ (20/80,-v/v). Each injection was

done after a defined pretreatment procedure for the analytical column and the EC-electrode. The retention times of 5-HMU and 2-dU were 21.22 and 24.80 minutes, respectively. Recovery was checked by a spiked urine pool (pooled urine fractions from ten individuals). All results were corrected for the recovery rate. Standards were used after every fifth sample to monitor the accuracy.

Tıssue DNA Damage Determlnatıon by Comet Assay

Comet assay was adapted from the method of Singh et al 1988. The lesioned region was minced, was suspended with chilled homogenizing buffer (pH 7.5) containing 0.075 M NaCl and 0.024 M Na_2EDTA and then was homogenized gently using a Potter type homogenizer. Homogenates were centrifuged at 700xg for 10 minutes at 0°C and the precipitates were resuspended in chilled homogenizing buffer. Cell concentration and the percentage of viable cells were determined with trypan blue staining using a haemocytometer. Only samples with a viability of >90% were used for the subsequent comet assay.

The cell suspen sion was mixed with 500 µl of low melting point (LMP) agarose in PBS at 37°C, and 140 µl of this mixture was applied on to afrosted glass microscope slide pre-coated with a layer of 1% normal melting point (NMP) aga ro se (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_2EDTA, 10 mM Tris, 1% N-sodium lauroyl sarcosinate, 1% Triton-X-100, 10% DMSO) for 1 hour at 4°C. The slides were then placed in an electrophoresis tank, allowing the DNA to unwind for 15 minutes in the alkaline solution (300 mM NaOH and 1 mM Na_2EDTA). Electrophoresis was performed at 300 mA for 20 minutes in the same alkaline solution at room temperature. The slides were then neutralized with 0.4 M tris-HCl buffer (pH 7.4) and they were stained with silver staining method.[24] The dried microscope slides were covered with a coverglass prior to analysis with 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 Programme, 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%, T%) or in tail moment [Tail DNA%xTail Length (percent of DNA in the tail, TM)x(tail length)] or tail length (length of the comet tail measured from the right border of the head area to end of tail in pixels, TL). Three samples per rat were assayed implicating 50 cells per sample. The mean±standard error of means (SEM) of the three samples was calculated for each rat.

STATISTICAL ANALYSES

Comet scores of tissue samples were expressed as median, min-max values. Statistical analyses of the urine oxidative DNA products were performed using the Pearson's correlation test, Kruskal Wallis and Mann Whitney U tests; a p value of less than 0.05 was considered significant. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Version 13.0 for windows (SPSS Inc, USA).

RESULTS

NEUROLOGICAL FINDINGS

The BBB open-field locomotor scoring was performed to determine the functional outcome of ENSC grafts to injured spinal cords.[21] For all groups, the preoperative baseline BBB score was 21. After injury, BBB score dropped to 0-2 and then gradually recovered. Twenty-eight days after hemisection, BBB scores reached a plateau in the ENSC groups of acute and chronic injury groups. In the chronic injury group (28 days after ENSC transplantation) the animals had an average BBB score of 11.2±2.9, which was significantly higher ($p<0.01$) when compared to the acute injury group (7 days after ENSC transplantation) (4.2±0.8). There were no significant differences between the acutely and chronically injured rats (1.1±0.4 and 1.3±0.6, respectively), ($p>0.05$).

RESULTS OF TISSUE COMET ASSAY

Comet assay parameters (including T%, TL and TM) in the lesioned region of the sham operated, acute and chronic SCI groups and ENSC therapy rats were shown in Figure 1.

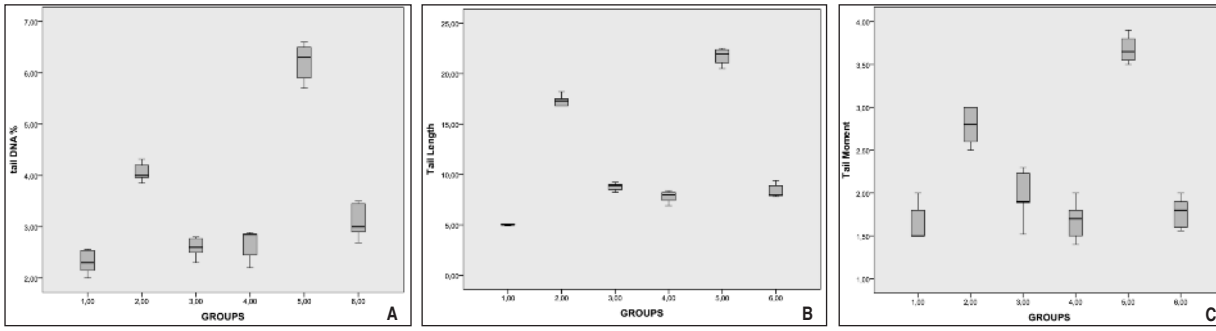

FIGURE 1: DNA damage assessed by the comet assay (pixel) in lesioned region of SC on days 7 and 28 after embryonic neural stem cells (ENSCs) transplantation. Effects measured as **(A)** percentage of DNA in the tail (tail DNA %), **(B)** tail length, and **(C)** tail moment are shown for acute sham (1), acute spinal cord injury (SCI) (2), acute SCI with ENSCs therapy (3) chronic sham (4), chronic SCI (5), and chronic SCI with ENSCs therapy (6) study groups [number of sample (n)=5].

Results of acute study groups: T%, TL and TM levels were significantly higher in the acute injury group when compared to the acute sham operated group (p=0.009, p=0.009, and p=0.009, respectively). T% (p=0.094), TL (p=0.009) and TM (p=0.009) levels decreased in the acute injury+ENSC therapy group.

Results of chronic study groups: T%, TL and TM levels were significantly higher in the chronic injury group when compared to the chronic sham operated group (p=0.009, p=0.009, and p=0.009, respectively). When the results of the chronic SCI+ ENSC therapy groups were compared with the chronic injury group, significant differences in terms of TL, TM, T% levels (p=0.009, p=0.009, p=0.009, respectively) were determined (Table 1).

RESULTS OF URINE HPLC ANALYSIS

Urinary oxidative DNA damage products (including 5HMU and 2dU) of the sham operated, acute and chronic SCI groups and ENSC therapy rats were shown in Figure 2.

Results of acute study groups: The animals of the acute injury group exhibited 1.15-fold and 1.3-fold increase in 5HMU and 2dU levels in the spinal cord tissues compared to the sham operated animals (p=0.009 and p=0.009, respectively). The levels of 5HMU (1.55-fold) and 2dU (1.17-fold) markedly decreased (p=0.009 and p=0.009, respectively) in the acute SCI+ENSC therapy group compared to the acute injury group.

Results of chronic study groups: Urinary 5HMU (1.30-fold) and 2dU (1.46-fold) levels were

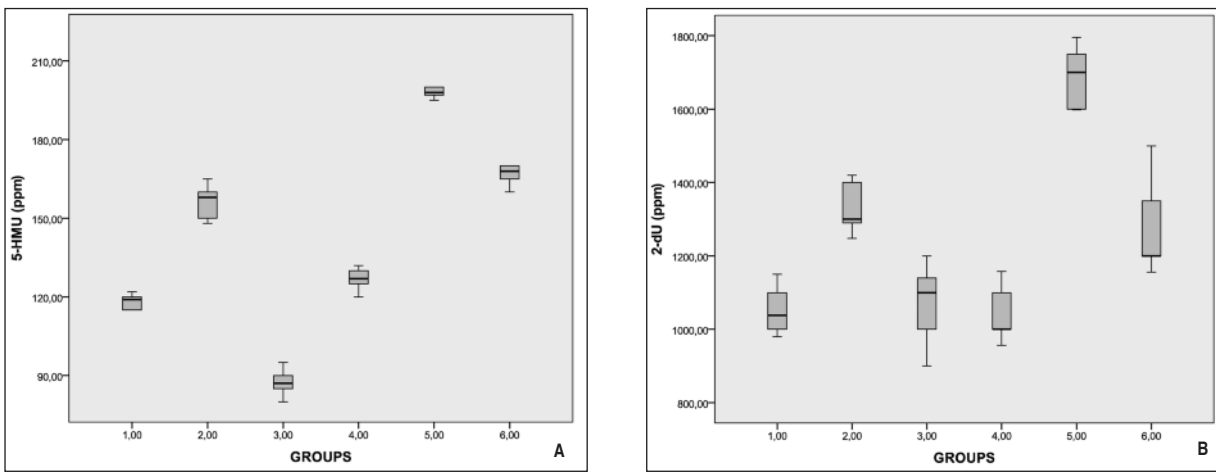

FIGURE 2: DNA damage assessed by the HPLC in urine on days 7 and 28 after embryonic neural stem cells (ENSC's) therapy. Levels of 5HMU **(A)** and 2dU **(B)** are shown for acute sham (1), acute spinal cord injury (SCI) (2), acute SCI with ENSCs therapy (3) chronic sham (4), chronic SCI (5), and chronic SCI with ENSCs therapy (6) study groups.

TABLE 1: Median (M) and Min-Max values of study groups (G) are shown for acute sham (1), acute SCI (2), acute SCI with ENSCs therapy (3) chronic sham (4), chronic SCI (5), and chronic SCI with ENSCs therapy (6) study groups.

G	T DNA%		TL		TM		5HMU		2dU	
	M	Min-Max	M	Min-Max	M	Min-Max	M	Min-Max	M	Min-Max
1	2.3	2.0-2.6	5.0	4.9-5.3	1.50	1.0-2.0	119.0	115.0-122.0	1038.0	980.0-1150.0
2	4.0	3.8-4.3	17.3	16.8-18.2	2.80	2.5-3.0	158.0	148.0-165.0	1300.0	1248.0-1420.0
3	2.6	2.3-2.8	8.9	8.2-9.2	1.90	1.5-2.3	87.0	80.0-95.0	1100.0	900.0-1200.0
4	2.8	2.2-2.9	8.0	6.9-8.4	1.70	1.4-2.0	127.0	120.0-132.0	1000.0	956.0-1158.0
5	6.3	5.70-6.6	22.3	20.5-23.9	3.60	3.0-3.9	198.0	195.0-210.0	1700.0	1598.0-1796.0
6	3.0	2.7-3.5	7.9	7.8-9.4	1.80	1.56-2.0	168.0	160.0-170.0	1200.0	1156.0-1500.0

Tail DNA %: Percentage of DNA in the tail; TL: Tail length; TM: Tail moment.

significantly higher (p=0.009) in the chronic injury group compared to the sham operated groups. After the ENSC transplantation, 5HMU (1.22-fold) and 2dU (1.17-fold) levels markedly decreased (p=0.009) in the spinal cord tissue (Table 1).

DISCUSSION

Although several treatment strategies are being explored, no effective therapy is available at present for spinal cord injury.[4,25] However, use of experimental models of SCI and advances in the understanding of the secondary mechanisms of injury may lead to greater progress in this area.[1,2,26] Stem cell therapy can be defined as part of a group of new techniques that rely on replacing diseased or dysfunctional cells with healthy, functioning ones. These techniques are being applied experimentally in a wide range of human disorders, including neurological diseases such as spinal cord injuries.[26,27]

The ROSs cause extensive DNA damage, including base and sugar modifications, and covalent crosslinks, with often single- and double-stranded breaks as the end result of such reactions.[5,12,13] The oxidative DNA damage products 5HMU and 2dU were determined as the predominant forms of radical induced DNA damage, and have therefore been widely used as biomarkers for oxidative stress.

The comet assay can be used to detect DNA damage caused by double strand linking with DNA or protein. The comet assay is also used to monitor DNA repair by living cells. According to the results of tissue comet assay, DNA damage levels increased in the acute and chronic SCI. However, the ENSC therapy improved comet scores in these groups.

There are few studies assessing the DNA damage products in SCI after ENSC therapy. In our study, we observed a significant increase in DNA damage in the spinal cord tissue and urine of acute and chronic SCI animal groups. These results have shown conformance with the results of our previous study. After ENSC therapy, comet scores (Tail-DNA %, TL and TM) clearly decreased. The ENSC treatment reduced the 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 SCI.[28]

When tested using linear regression analysis, 5HMU and 2dU concentrations in urine showed a positive correlation with tail length, tail % and tail moment in the acute and the chronic SCI groups. 5HMU concentration in the acute SCI group had a stronger correlation with tail moment compared to the chronic group (r=0.9039, p=0.02). In the chronic injury group, 5HMU and 2dU concentrations correlated significantly with tail length (r=0.9990, p=0.001 and r=0.9272, p=0.016, respectively). While 5HMU seems to be a better biomarker for acute injury, both HMU and 2dU excretions increase in chronic damage. However, levels of other oxidative DNA damage products should also be investigated in the monitoring of the damage caused by SCI.

Among the acute and the chronic study groups, there was a positive correlation between the comet scores and results of urinary oxidative DNA damage products. As a result, our data show that the level of excretion of the free base 5-HMUra in SCI rats urine is higher than 2dU, supporting biological relevance for this DNA modification. The present study focused on only two types of oxidative DNA damage products. However, we planned to assess the alterations in other urinary oxidative DNA damage products in the same study groups. Finally correlation analysis indicates that the HMU (r=0.9039, p=0.02) in the acute SCI group, and HMU and 2dU (r=0.9990, p=0.001 and r=0.9272, p=0.016, respectively) in the chronic SCI group are significantly associated with increased comet scores.

This is the first report investigating both the urinary oxidative DNA damage products and the lesioned region comet parameters in acute and chronically injured rats with SCI+ENSC therapy. In a previous study, we showed that the presence of APE/Ref-1 decreased after acute and chronic SCI. After the ENSC therapy, DNA damage improved and elevated APE/Ref-1 expression levels.[28,29] On the other hand we used the Nissl stain technique to demonstrate an increase in the number of neurons in the lesioned region after embryonic neuronal cell therapy.[30]

In conclusion, our study suggests that the ENSCs transplantation has neuroprotective and restorative effects on secondary pathobiochemical events (such as edema, hemorrhage, inflammatory reaction, ischemia and oxidative stress) after SCI in rats. These restorative effects were observed mainly in neurons affected by oxidative stress detrimentally. Our results reflect the need for further and detailed experimental studies to better delineate the effects of ENSCs on the detrimental results of secondary SCI in rats.

REFERENCES

1. Azbill RD, Mu X, Bruce-Keller AJ, Mattson MP, Springer JE. Impaird mitochondrial function oxidative stress and altered antioxidant enzyme activities following traumatic spinal cord injury. Brain Res 1997;765(2):283-90.
2. 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.
3. 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.
4. Kabatas S, Teng YD. Potential roles of the neural stem cell in the restoration of the injured spinal cord: review of the literature. Turk Neurosurg 2010;20(2):103-10.
5. Cooke MS, Olinski R, Evans MD. Does measurement oxidative damage to DNA have clinical significance? Clin Chim Acta 2005;365 (1-2):30-49.
6. Bohr VA, Dianov GL. Oxidative DNA damage processing in nuclear and mitochondrial DNA. Biochimie 1999;81(1-2):155-60.
7. Kobayashi S, Susa T, Tanaka T, Wada Y, Okuda S, Doi M, et al. Urinary 8-hydroxy-2′ deoxyguanosine reflects symptomatic status and severity of systolic dysfunction in patients with chronic heart failure. Jap Euro J Heart Fail 2011;13(1):29-36.
8. Wang D, Kreutzer DA, Essigmann JM. Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. Mutat Res 1998;400(1-2):99-115.
9. Li KM, Rivory LP, Clarke SJ. Rapid quantitation of plasma 2-deoxyuridine by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry and its application to pharmacodynamic studies in cancer patients J Chromatogr B Analyt Technol Biomed Life Sci 2005;820(1):121-30.
10. Evans MD, Saparbaev M, Cooke MS. DNA repair and the origins of urinary oxidized 2′-deoxyribonucleosides. Mutagenesis 2010;25(5): 433-42.
11. Faure H, Mousseau M, Cadet J, Guimier C, Tripier M, Hida H, et al. Urine 8-oxo-7,8-dihydro-2-deoxyguanosine vs. 5-(hydroxmethyl) uraci as DNA oxidation marker in adriamycin-treated patients. Free Radic Res 1998; 28(4):377-82.
12. Foksinski M, Rozalski R, Guz J, Ruszkowska B, Sztukowska P, Piwowarski M, et al. Urinary excretion of DNA repair products correlates with metabolic rates as well as with maximum life spans of different mammalian species. Free Radic Biol Med 2004;37(9):1449-54.
13. Lodovici M, Casalini C, Cariaggi R, Michelucci L, Dolara P. Levels of 8-hydroxydeoxyguanosine as a marker of DNA damage in human leukocytes. Free Radic Biol Med 2000;28(1): 13-7.
14. Kasai H. A new automated method to analyze urinary 8-hydroxydeoxyguanosine by high-performance liquid chromatography-electrochemical detector system. J Radiat Res (Tokyo) 2003;44(2):185-9.
15. Peoples MC, Karnes HT. Recent developments in analytical methodology for 8-hydroxy-2-deoxyguanosine and related compounds. J Chromatogr B Analyt Technol Biomed Life Sci 2005;827(1):5-15.
16. Chuang CH, Hu ML. Use of whole blood directly for single cell gel electrophoresis (comet) assay in vivo and white blood cells for in vitro assay. Mutat Res 2004;564(1):75-82.
17. Ellahueñe MF, Pérez-Alzola LP, Farfán-Urzua M, González-Hormazabal P, Garay M, Olmedo MI, et al. Preliminary evaluation of DNA damage related with the smoking habit measured by the comet assay in whole blood cells. Cancer Epidemiol Biomarkers Prev 2004;13 (7):1223-9.

18. 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.
19. 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.
20. 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.
21. Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 1995;12(1):1-21.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.
23. Shigenaga MK, Gimeno CJ, Ames BN. Urinary 8-hydroxy-2'-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proc Natl Acad Sci U S A 1989;86(24):9697-701.
24. Nadin SB, Vargas-Roig LM, Ciocca DR. A silver staining method for single-cell gel assay. J Histochem Cytochem 2001;49(9): 1183-6.
25. Tran KD, Ho A, Jandial R. Stem cell transplantation methods. In: Jandial R. Frontiers in Brain Repair. 1st ed. Vol. 671. New York: Springer Science; 2010. p.41-57.
26. Kan EM, Ling EA, Lu J. Stem cell therapy for spinal cord injury. Curr Med Chem 2010;17 (36):4492-510.
27. Sieber-Blum M. Epidermal neural crest stem cells and their use in mouse models of spinal cord injury. Brain Res Bull 2010;83(5):189-93.
28. Dagci T, Konyalioglu S, Keser A, Kayalioglu G. Effects of embriyonic neural stem cell transplantation on DNA damage in the brain and spinal cord following spinal cord injury. Neurophysiology 2009;41(6):409-15.
29. Dağcı T, Armagan G, Konyalioglu S, Yalcin A. 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. Physiol Res 2009;58(3):427-34.
30. Konyalioglu S, Ozgonul M, Dağcı T. Effect of embriyonic neural stem cell therapy on oxidative and nitrosative stress biomarkers in acute and chronic spinal cord injured rats. Turkiye Klinikleri J Med Sci 2009;29(2):429-37.