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Management by Erythropoietin and Nerve Growth Factor on Healing of Spinal Cord Injury in Rats

Amal I. Hassan[1] & Abeer H. Abd El-Rahim[2]

[1] Radioisotopes Department, Nuclear Research Centre, Atomic Energy Authority, Egypt

[2] Genatic Engeneering and Biotecnology, Department of Cell Biology, National Research Centre, Egypt

Correspondence: Amal Ibrahim Hassan, Department of Radioisotopes, Nuclear Research Centre, Egyptian Atomic Energy Authority, Ahmed El-Zyyat Street, ZIP 12311, Dokki, Giza, Egypt. E-mail: aml_h@hotmail.com

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Abstract

Research on the pathology of spinal cord injury (SCI) has been recently focused on oxidative radical-induced stress and inflammation associated neuronal apoptosis. However, the study of neurorepair and the underlying mechanism in SCI model has been limited. In this study, we investigated the effects of erythropoietin plus nerve growth factor (EPO + NGF) on neurologic and histopathologic changes after SCI and explored its anti-apoptotic role after SCI. Intravenous injection four times a week over a period of 4 weeks of EPO plus NGF (5000 & 2000 U /kg respectively) in male rats following SCI by whole body gamma irradiation caused significant decrease in 8 hydroxyguanosine (8-HDG) and heat shock protein 70 (HSP70). To investigate the possible mechanism, anti-oxidant effect of EPO plus NGF was assessed by measuring superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and GSH levels after SCI. EPO plus NGF treatment reversed the decrease of SOD activity and increase of MDA level caused by SCI, suggesting its anti-oxidant role in response to the injury. In addition, EPO plus NGF treatment, after SCI, restored the concentration of neurotransmitters to near normal values seen in control rats after 4 weeks of treatment. Assays for DNA damage in bone marrow cells as well as in spinal cord of rats by chromosomal aberrations, and micronucleus assay and DNA fragmentation techniques were performed in this study. Data showed that the frequency of total chromosomal aberrations and number of micronucleated polychromatic erythrocytes (MNPCE) in animals that received erythropoietin and nerve growth factor after radiation were lower than that in radiated animals that had not been treated with erythropoietin and nerve growth factor. Also, the percentage of DNA fragmentation was reduced after treatment of radiated animals with erythropoietin and nerve growth factor. Further, histopathological alternations were evaluated with H.E. staining that showed a restored SC histology after EPO plus NGF administration.

Keywords: spinal cord apoptosis, nerve growth factor, erythropoietin, healing, apoptosis

1. Introduction

Spinal cord injury (SCI) is a considerable health impact followed by complications that can accompany physical psychological and often result in tremendous economic loss and social burden. However, even after many promising experimental studies, so far there is no effective treatment that can dramatically eliminate the secondary damage after SCI (Shi et al., 2010; Macaya & Spector, 2012). SCI presents a complex regenerative problem due to the multiple facets of growth inhibition that occur following trauma to the SC. Several studies published in recent years have shown that the growth factors can induce regeneration of spinal cord nerve fibers in animals. Nerve growth factor (NGF) can promote the repair of nerve after nerve injury. In the last few years, it has been shown that erythropoietin (EPO) exerts neuroprotective effects in various *in vivo* and *in vitro* models of brain injury and has a significant effect on axonal regrowth of peripheral nerve fibers (Carelli et al., 2011; Ming et al., 2012). Cytokine EPO is an extremely important mediator of injury-related tissue protection in mammals following ischemic and nonischemic injuries (Bianchi et al., 2004). Furthermore, the expression of EPO after SCI has been shown to be part of the physiological response to hypoxia (Grasso et al., 2005).

Recently, some researches on SCI in rat model showed that the administration of a single dose of rhEPO immediately after cord injury improved motor function recovery and increased neuronal regeneration. A slow rate of axonal regeneration is a major preventive measure toward functional recovery after peripheral nerve injury (Heeschen et al., 2003; Wiessner et al., 2001). Recent research has demonstrated neuroprotective role of

erythropoietin (EPO) and EPO receptor in the central nervous system (Glazova et al., 2011). EPO may also have more direct actions on neurons, oligodendrocytes, and astrocytes (Yin et al., 2010). The results of this study demonstrate that EPO plus NGF provide neurotrophic actions in a rat model of SCI. Moreover, EPO and NGF potentiate each other to give positive results on the recovery of SCI in rats. Future studies will also focus on NGF and NGF-mediated events, as this neurotrophin seems to be a key factor that can modulate neuronal pathways regulating.

2. Methods

2.1 Irradiation

Gamma irradiation was performed using the cobalt-60 cell 3500 of Middle Eastern Regional Radioisotope Center for the Arab Countries (MERRCAC) in Dokki, Giza, Egypt.

2.2 Animals

Forty male albino rats weighing 160-180 g were obtained from the animal house of the National Research Center (NRC), Dokki, Giza. They were allowed to acclimate at the animal facilities for two weeks before use. They were housed under standard environmental conditions with free access to food and water throughout the experiment. Animals were randomly divided into four groups each of 10 rats. The first group (G1) was kept without treatment and served as normal control. The second group (G2) (positive control) received intravenous EPO plus NGF (2000 U/kg) plus EPO (5000 U/kg) IV daily for four weeks(Yin et al., 2010). The third group (G3) was exposed to a single dose (10 Gy) of whole body (WB) irradiation delivered from Co-60 source in MERRCAC, in Dokki, A.R.E. The fourth group (G4) constituted irradiated rats that were exposed to 10 Gy WB following 24 hr of receiving EPO plus NGF daily for four weeks. All animal procedures were approved by our local ethical committee of animal care and use.

2.3 Biochemical Parameters

At the end of the experimental period, all rats were sacrificed, spinal cords were isolated and stored in PBS PH 7.4 for determination of total heat shock protein 70 (HSP70) levels (Duo Set IC kit), and 8 hydroxyguanosine (8 -HDG) levels (Blue Gene ELISA kit from Life Science Advanced Technologies Saint Petersburg). Spinal cord monoamine concentrations were determined according by using HPLC (Mitchell et al., 2000). Superoxide dismutase (SOD) levels (Sun et al., 1988). catalase levels (CAT) (Bonaventura et al., 1972) were determined following published protocols. Glutathione dehydrogenase (GHX) and malondialdehyde (MDA) levels, as indicator for antioxidant status, were determined by the method described by Wasowicz et al. (1993).

2.4 Chromosomal Aberration Assay

Cytogenetic analysis of rat chromosomal aberrations (CA) in bone marrow cells was performed according to the technique previously described (Brusick, 1980) with some modifications as recommended by others (Preston et al., 1987). Each rat was injected intraperitoneally (IP) with 1.0 ml of colchicine (3 mg/kg body weight) 2 hr prior to sacrifice. The animals were sacrificed by cervical dislocation and their femoral bones removed and flushed in 2.2% sodium citrate and cells were centrifuged at 1500 rpm for 10 min. Pelleted bone marrow cells were then resuspended in 5 ml of a hypotonic solution (0.075 M KCl) for 20 min at 37°C. The cells were centrifuged again and fixed with three changes of 5 ml each of ice-cold Carnoy's fixative (methanol -acetic acid, 3:1, v/v) for 30 min at 25°C. The cells were then dropped on to clean slides which were air-dried and stained with 5% Giemsa for 15 min.

2.4.1 Scoring of Chromosomal Aberrations Data

Metaphase cells with one or more types of chromosomal aberrations were scored at X1000 magnification. Fifty well-spread metaphase cells per rat were examined and the frequencies of chromosomal aberrations were expressed as mean of the number of aberrations. Aberrations were classified into structural and numerical parameters as already described by Karmakar (1998).

2.4.2 Micronucleus Assay

Animals were sacrificed by cervical dislocation**.** Both the femora were removed and cleaned by flushing the bone marrow out using fetal calf serum and centrifugation at 1000 rpm for 10 min. The cells were washed twice with phosphate buffered saline (PBS) followed by centrifugation at 1000 rpm for 10 min. The supernatant was removed by aspiration and the cells were fixed in cold 3:1 methanol: acetic acid. Slides were prepared by dropping portions of the pellet on slides and then air-dried for 20 min. Slides were stained with 5% solution of Giemsa in 0.01 M phosphate buffer at pH 7.4 according to the method described by Schmid (1975) with slight

modifications by Agarwal and Chauhan (1993). Slides were scored on a fluorescence microscope. The polychromatic erythrocytes (PCE) were determined and the micronuclei were scored in 2000 PCE per animal.

2.4.3 DNA Fragmentation Assay

Samples from spinal cord tissues were obtained from the zone of the lesion and from the rostral and caudal zones. The tissue was homogenized in 9 x volumes of a lysis buffer [5 mM Tris–HCl, 20 mM ethylene diamine tetra-acetic acid (EDTA) and 0.5% (v/v) t-octylphenoxypolyethoxyethanol (Triton-X 100); pH 8.0)]. Two separate samples of 1 ml each were taken from the sample and centrifuged at 25000 g for 30 min to separate the intact chromatin in the pellet from the fragmented DNA in the supernatant. The supernatant was taken out to be saved and the pellet was resuspended in 1 ml Tris-EDTA buffer (pH 8,0) (10 mM:1 mM). Both the supernatant and the resuspended pellets were then assayed for DNA content by the diphenylamine reaction described by Burton (1968). DNA was quantified by a spectrophotometer.

2.5 Statistical Analysis

Results were analyzed statistically using one way analysis of variance (ANOVA) test with subsequent multiple comparisons using Duncan's test. Differences were considered statistically significant at *p* less than 0.05. Results are presented as the mean ± standard error of the mean (SEM) with the number of observations (n). All data obtained were submitted to a computerized statistical treatment using SPSS statistical package, version 17.0

3. Results

As shown in Table (1), whole body gamma irradiation (10 Gy) caused a significant ($p < 0.05$) elevation in 8 HDG and HSP70 in spinal cord tissues 30 days post irradiation as compared to the normal control rats. The data also revealed that administration of EPO combined with NGF after SCI induced a significant decrease ($p < 0.05$) in both 8HDG and HSP70 as compared to SCI group. Concentrations of neurotransmitters 5-hydroxytryptamin **(**5HT), norepinephrine (NE), and dopamine (Dopa) in spinal cord tissues of experimental animals are presented in Table 2. Exposure to irradiation induced a significant decrease ($p < 0.05$) in 5 HT and Dopa levels as compared to the control animals. Intravenous EPO plus NGF induced the concentration of Dopa and 5 HT to be restored to near normal values seen in control rats, after 4 weeks of the treatment. Furthermore, SCI appeared to be detrimental to the redox status in spinal cord tissues as evidenced by a significant rise in MDA level and significant depletion in SOD, GSH and CAT activities of spinal cord tissues when compared to the corresponding controls. As compared to the group treated with EPO plus NGF (5000 & 2000 U/kg respectively) restored the activity of MAD, SOD, GSH and CAT after 4 weeks of treatment.

Table 1. Effect of EPO plus NGF on HSP70 and 8-HDG in SCI rats

Groups	**Control**	**+ve control EPO + NGF**	**SCI**	**SCI & EPO + NGF**
HSP70(pg/g)	66.70^{b}±8.1	46.62^{b}±3.8	343.40^{a}±33.4	283.60^{a}±12.3
8HDG(pg/gm)	37.52^{c}±1.52	36.17^{c}±1.49	104.38^{a}±9.5	79.77^{b}±1.11

Data were expressed as mean ± S.E. Means with different superscript letters are significantly different ($P < 0.05$).

Table 2. Effect of EPO plus NGF on monoamines in SCI rats

Groups	**Control**	**+ve control EPO + NGF**	**SCI**	**SCI & EPO + NGF**
5 HT(ng/gm)	36.68^{a}±1.46	38.02^{a} ±1.47	17.08^{c} ±1.22	29.66^{b}± 1.67
NE(ng/gm)	156.55^{b}±3.52	154.74^{b}±2.85	168.32^{a}±2.10	156.46^{b}±2.13
CAT(U/gm)	8.40^{a}±0.42	7.50^{a}±0.29	2.50^{c}±0.29	5.10^{b}± 0.58

Data were expressed as mean ± S.E. Means with different superscript letters are significantly different ($P < 0.05$).

The spinal cord tissue MDA content in the control group (27.53 ± 1.30 nmol/gm) was significantly less than that in the SCI rats (47.30 ± 1.51nmol/gm, $P < 0.05$); however, EPO plus NGF treatment completely prevented the SCI-induced elevation in tissue MDA levels (29.90 ± 0.64 nmol/gm; $P < 0.05$; Table 2). SCI caused a significant decrease in tissue GSH levels (0.69 ± 0.05 μmol/gm; $P < 0.05$) compared to that of the control group (1.84 ±

0.10 µmol/gm), while in the EPO plus NGF-treated SCI group, spinal cord GSH content was found to be restored to a higher level (1.43 ± 0.09 µmol/gm; P < 0.05). Similarly, SCI caused significant decrease (p < 0.05) in both SOD and CAT levels as compared to those in the control group and reached values comparable to the control levels after treatment with EPO plus NGF (Table 3).

Table 4 shows that chromosomes from irradiated rats have higher frequencies of structural and numerical aberrations compared to the normal (G1) group. Statistical analyses showed significant differences between SCI and normal rats for the frequencies of chromatic gaps, breaks, deletions, and fragments. In contrast, the SCI rats treated with EPO plus NGF (G4) had significantly decreased frequencies of structural and numerical chromosome aberrations compared to G3 group. In the present study, it was observed that structural chromosomal aberrations were increased in SCI rats than in treated groups (Table 4). Numerical aberrations are more frequent in irradiated rats than those found in control and treated rats.

Table 3. Effect of EPO plus NGF on MDA, SOD, GSH and CAT in SCI rats

Groups	**Control**	**+ ve control EPO + NGF**	**SCI**	**SCI & EPO + NGF**
MDA(nmol/gm)	27.53^{b}±1.30	28.27^{b} ±1.44	47.30^{a}± 1.51	29.90^{b}±0.64
SOD(U/gm)	1.92^{a}±0.04	1.74^{a} ± 0.13	0.73^{c}±0.037	1.59^{b}±0.26
GSH(µmol/gm)	1.84^{a}±0.10	1.51^{b}± 0.10	0.69^{c}±0.06	1.43^{b} ± 0.09
CAT(U/gm)	8.40^{a}±0.42	7.50^{a} ±0.29	2.50^{c}±0.29	5.10^{b}± 0.58

Data were expressed as mean ± S.E. Means with different superscript letters are significantly different (P < 0.05).

3.1 Micronucleus Study

As shown in Table 5, the frequencies of micronuclei are significantly higher (P < 0.05) in rats with SCI, induced by radiation (G2 group), than those found in G1 (control) group. On the other hand, the frequencies of micronuclei significantly decreased (p < 0.05) in the SCI group (G3) treated with EPO plus NGF compared to those found in G2. It is likely that EPO and NGF may have contributed for their ability to decrease the irradiation-mediated defects in the micronucleated polychromatic erythrocytes.

3.2 DNA Fragmentation

The rates of DNA fragmentation were significantly increased in irradiated rats than in control group (Figure 1). On the contrary, treatment with EPO and NGF in combination led to a decrease (P < 0.05) in the rates of DNA fragmentation in SCI rats.

Table 4. Chromosomal aberrations in SCI and EPO in combination with NGF in rats

Total aberrations	**Total Numerical aberrations**	**Total structural aberrations**	**Different types of structural chromosomal aberrations**			**Treatment**
			Deletion	**Break**	**Gap**	
$3.25±1.11^{d}$	$1.75±0.48^{d}$	$1.75±0.75^{d}$	$1.0±0.41^{c}$	$0.25±0.25^{b}$	$0.5±0.29^{d}$	**Control**
$24.25±1.11^{a}$	$8.50±0.65^{a}$	$15.75±0.63^{a}$	$8.25±0.48^{a}$	$2.5±0.29^{a}$	$5.0±0.41^{a}$	**SCI**
$17.25±0.25^{b}$	$6.50±0.56^{b}$	$10.75±0.48^{b}$	$6.0±0.0.41^{b}$	$1.75±0.48^{a}$	$3.0±0.41^{b}$	**SCI &EPO+NGF**
$12.25±1.11^{c}$	$4.50±0.65^{c}$	$7.75±0.48^{c}$	$4.75±0.48^{b}$	$1.25±0.48a^{b}$	$1.75±0.48^{c}$	**+ve control (EPO+NGF)**

Table 5. Frequencies of micronucleated polychromatic erythrocytes in rat bone marrow cells exposed to radiation and treated with erythropoietin and nerve growth factor

Mean value of MNPCE	Treatment
4.75±0.48[c]	**control**
21.50±0.65[a]	**SCI**
13.75±0.85[b]	**SCI &EPO+NGF**
12.25±0.85[b]	**+ve control (EPO+NGF)**

Mean value of MNPCE based on 2000 PCEs assessed per animal. Data were expressed as mean ± S.E. Means with different superscript letters are significantly different ($P < 0.05$).

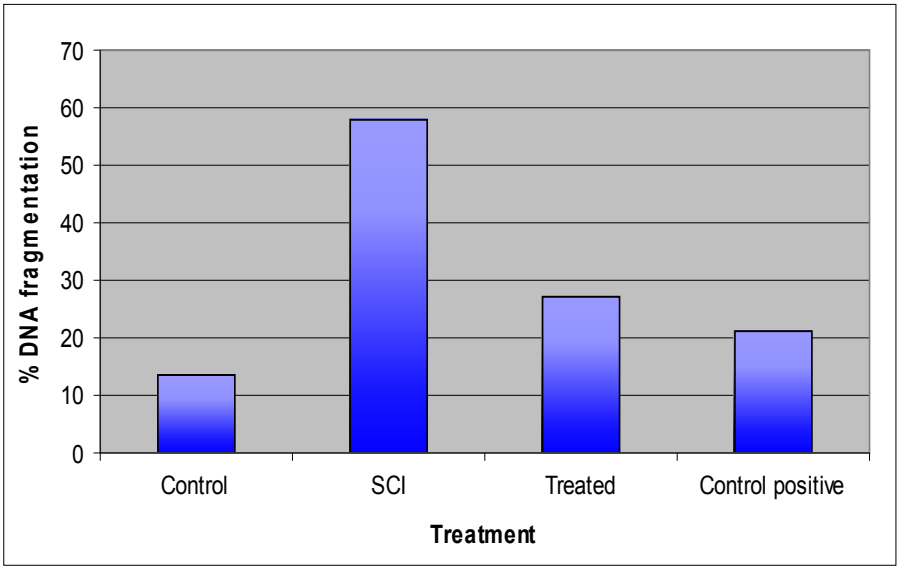

Figure 1. The percentage of DNA fragmentation in spinal cord of rats exposed to radiation and treated with erythropoietin and nerve growth factor

Next, we compared histopathological alterations in the spinal cord before and after injury. Hematoxylene/eosin (H.E) staining illustrated in Figure (2A) showed that spinal cord in the control group had normal histological structure of the central canal (c), neuron in gray matter (g) and nerve fibers in white matter (w).Neuronal degeneration with nuclear pyknosis were observed in the gray matter in SCI by irradiation (Figure 2B). Patches of necrosis was seen in the gray matter and also liquefaction surrounded the damaged tissues. In addition, gaps between cells and blood vessels became remarkably larger. A portion of neurons were found with condensed nucleus, darkly red stained cytoplasm and also appearance of apoptotic bodies (Figure 2B). The grey matter showed congestion in blood vessels in control group was administered EPO plus NGF (Figure 2C). According to histopathological results, treatment with EPO plus NGF the blood vessels and central canal also exhibited normal morphology. No neuronal, apoptosis and glial. Proliferation was observed in the treatment group with EPO plus NGF (Figure 2D).

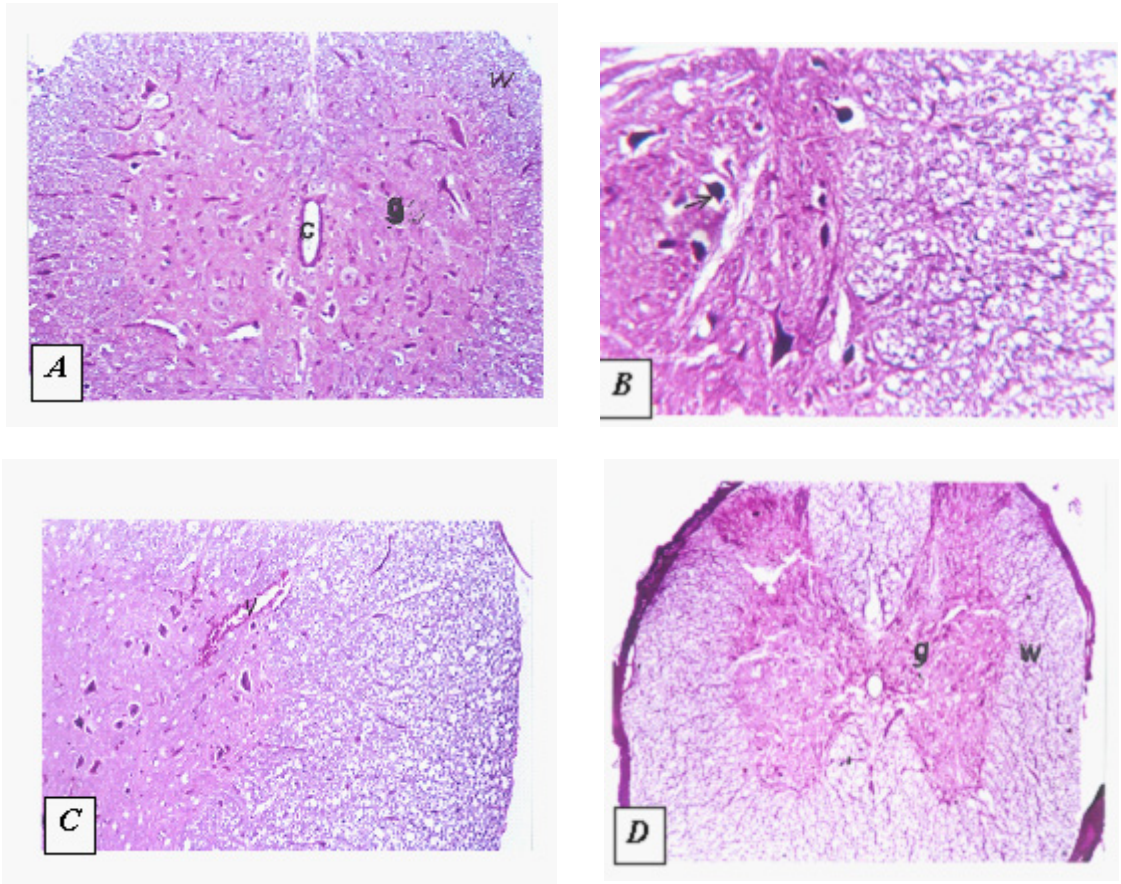

Figure 2. Histopathological in spinal cord. A: Control of SC. B: SCI. C: control group was administered EPO plus NGF. D: SCI group treated with EPO plus NGF

4. Discussion

Spinal cord injuries can be caused by traumatic insults or disease to the spinal cord, which subsequently cause injury to sensory and motor function (Pfister et al., 2011). After spinal cord injury (SCI), a complex cascade of events leads to tissue degeneration and number of cell death (Kang et al., 2009).

The experimental results demonstrated that after SCI, there were many dramatic changes occurring in the injured cord tissue, such as neuronal degeneration with nuclear pyknosis were observed in the gray matter, activation of lipid peroxidation, chromosomal aberration and increase of DNA fragmentation which were obviously related to the extent of SCI. The present study demonstrated that treatment with EPO and NGF was able to promote recovery in rats subjected to spinal cord injury.

Erythropoietin (EPO) is typically known for its role in erythropoiesis but is also a potent neuroprotective factor for spinal motor neurons (Tator, 2006).

The observed improvement in the spinal cord injured is in agreement with the results of the previous experimental studies and shows that EPO reduces neurologic deficits in a variety of peripheral nerve injury models (Pallet et al., 2010; Brines et al., 2000).

In vitro study by Bianchi et al. (2004) showed that the administration of EPO had a neurotrophic effect on the growth of neuronal processes *in vitro* (Kumral et al., 2005). The present work was performed to examine the outcome of EPO plus NGF treatment on SCI recovery in rats. The results of this study demonstrate that EPO + NGF provide neurotrophic action in a rat model of SCI. NGF was selected in the current study because of its separately well-known function in sensory axon development (Zhang et al., 1994; Bianchi et al., 2004), regeneration (Burton, 1968) , and sprouting (Tang et al., 2007).

Recent reports show that the hematopoietic protein erythropoietin (EPO) and EPO receptors, which play a role in central nervous system (CNS) development, are upregulated in the brain and spinal cord within minutes to hours after injury (Yin et al., 2010; Siren et al., 2001).

The exact neuro-protective and neuro-repair mechanisms of EPO after SCI are still unclear, but it is postulated that mechanisms may include reduction of ischemic damage, reduction of inflammation, and saving of white matter tracts in the spinal cord (Kaptanoglu et al., 2004; Grasso et al., 2007). In the present study, it is postulated that EPO + NGF may contribute a reduction in demyelination within the spinal cord and reduce the inflammatory and decrease apoptosis. These results are shown in histopathological study (Figure D). The present data indicated that treatment of EPO and NGF caused an increase in the activity of antioxidant enzymes. Therefore, a significant increase in the MDA concentration occurred indicating increased lipid peroxidation caused by irradiation. Treatment with EPO plus NGF could effectively treat against the spinal cord lipid peroxidation induced by irradiation. Spinal cord injury (SCI) induces a series of endogenous biochemical changes that lead to secondary degeneration, including apoptosis (Kotipatruni et al., 2011). Oxidative stress may play a pivotal role in arsenic carcinogenesis, possibly by generation of adducts in DNA. 8-HDG has long been utilized as a DNA oxidative stress marker (Kundu et al., 2011). In the present study SCI induced significant increase of 8-HDG and EPO plus NGF modulated this increase. This increase may be due to free radicals after SCI. Oxygen radicals, oxidize lipid and protein molecules, generating intermediates, which can react with DNA and form adducts which can cause DNA strand breakage (Shiota et al., 2001). Increased levels of oxidative damage can result not only from oxidative stress, but also from failure of repair or replacement systems.

In the present study we investigated the frequencies of various types of chromosomal aberrations and micronuclei in bone marrow cells as well as DNA fragmentation in damaged spinal cords of rats induced by exposure to 10 Gy radiation. These results are in compliance with reports that suggested that radiation produces DNA double- strand breaks, DNA single-strand breaks, base damages and DNA-protein cross-links as well as chromatid breaks (Wiseman & Halliwell, 1996; Peter et al., 2010) and whole chromosome aberrations determined as dysenteric and deletion frequencies (Hall, 2000; Sudpraserta et al., 2006). In the current study, we demonstrated that, DNA damage in male rats induced by radiation can be modulated by treatment with a combination of erythropoietin and nerve growth factor. Erythropoietin is a hormone that induces differentiation and mitosis in erythropoietic stem cells in the bone marrow (Conti et al., 2007) as well as improvement the chromosomal aberrations (Singbrant et al., 2011). Erythropoietin may act in a coordinated fashion at multiple levels, including limiting the production of tissue-injuring molecules such as ROS, stimulation of angiogenesis to promote epithelial cell proliferation (Koroglu et al., 2006; McPherson & Juul, 2007). The results of our study also indicated that DNA damage decreased in the presence of nerve growth factor treatment. This may due to the fact that nerve growth factor stimulates tropomyosin receptor kinase receptors which blocks DNA fragmentation (Nguyen et al., 2010; Demont et al., 2011). Also, Satoh et al. (1999) reported that nerve growth factor protected cells from superoxide anion (O^{2-})-induced cell death through a novel regulation of reactive oxygen species (ROS) which increased O^{2-} and decreased hydrogen peroxide (H_2O_2), indicating that decreasing conversion from O^{2-} to H_2O_2 is a critical process for the protection by nerve growth factor. In the present study, HSP70 still increased after EPO plus NGF treatment. HSP70 expression, reportedly, reduces apoptosis of neural cells and improves recovery after SCI (Kim et al., 2010).

5. Conclusions

Our findings show that EPO plus NGF promotes the recovery and enhances nerve regeneration after spinal cord injury induced by WB irradiation in rats. Furthermore, the combination of NGF and EPO has better repairative ability in spinal cord injury. It may potentially reduce neuronal apoptosis, and promote the growth of endothelial cells of vessels in white matter, and therefore, could have a significant synergistic effect.

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