Extremely low frequency magnetic exposure attenuates oxidative stress and apoptotic cell death in injured spinal cord of rats

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Secondary spinal cord injury (SCI) sets on immediately after trauma which results in vascular, morphological and biochemical changes at the site of lesion. Amongst these, the crucial events such as oxidative stress and apoptosis result in spreading the injury to adjacent tissues following the initial insult. Exposure to extremely low frequency magnetic field (ELFMF) is reported to modulate oxidative stress and cell death in vivo. Here, we investigated the influence of ELFMF on oxidative stress and cell death after SCI. Adult male Wistar rats were divided into Sham-SCI (Laminectomy only), Sham-SCI+MF (ELFMF exposure; 17.96 µT intensity, 50 Hz frequency, 2 h duration/day to Sham-SCI rats), SCI (complete transection of T13 spinal cord) and SCI+MF (ELFMF exposure to SCI rats) groups. They were sacrificed on day 2 for antioxidant assay and day 1, 2 and 3 for cell death study. The oxidative stress was assessed by antioxidant enzyme activities and the cell death was assessed by TUNEL assay and Caspase-3 immunohistochemistry. Data revealed significant reduction in the lipid peroxidation and number of cells undergone cell death besides improvement in antioxidant levels in the spinal cord of ELFMF exposed SCI rats. These novel findings demonstrate the potential of ELFMF to attenuate oxidative stress and cell death in the early stage of secondary injury process after SCI.

Keywords: Apoptosis, Spinal cord injury (SCI), TUNEL

Spinal cord injury (SCI) is a complex disability requiring specialized knowledge and expertise to manage multisystem impairments. It causes high rate of morbidity and disability and involve primary and secondary injury processes. The primary injury begins with a sharp penetrating force lacerating the spinal cord. Coupled to primary mechanical trauma, the secondary injury processes that commence immediately, persist for even months after the injury. The latter results in vascular, morphological, biochemical and behavioural changes from the underlying inflammatory events, oxidative stress, excitotoxicity, demyelination, disruption of neuronal pathways, necrosis and cell death at the lesion site. Amongst these, the crucial events viz. oxidative stress and apoptotic cell death spread the injury to adjacent, otherwise uninjured tissues following the initial insult.

Studies on genesis and progression of post-SCI sequelae are still in experimental stage. Currently, the management of SCI patients remains to be symptomatic treatment and not targeted towards alleviating the causative factors of secondary injury events following it. To date there is no FDA-approved pharmacological agent to prevent the development of secondary injury cascades and induce regenerative processes aimed at healing the spinal cord and restoring neurological function. Alleviating oxidative stress and apoptotic cell death minimizes the secondary destruction effect after SCI and may be an effective way of therapeutic intervention in traumatic injury.

Magnetic field (MF) is found to modulate oxidative stress, cell death, release of neurotransmitters, and axonal growth besides promoting angiogenesis, osteogenesis in vivo in experimental models. Moreover, it is a non-invasive, non-pharmacological, and simple to administer therapeutic strategy. Extremely low frequency magnetic field (ELFMF) has been reported to improve the quality of life after SCI by improving locomotor recovery, autonomic control of urinary bladder besides neuroregenerative processes, pain status, sensorimotor function, osteoporosis and eating behaviour. However, its influence on post SCI oxidative stress and cell death is not yet known.

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In the present study, we investigated the effect of exposure to ELFMF (17.96 µT, 50 Hz) on oxidative stress and cell death in spinalized rats, particularly in the early stage of secondary injury process that dictate the outcome of SCI.

Materials and Methods

Animals

The study was approved by Ethical Committee of All India Institute of Medical Sciences, New Delhi, India (No: 618/IAEC/11). Adult male Wistar rats weighing 250-300 g were obtained from experimental animal facility of the Institute and were individually housed in specific pathogen-free conditions. They were maintained at 24±2°C and 14:10 h light-dark cycle and provided with laboratory food pellets and fresh tap water ad-libitum (Ashirwad Industries, Chandigarh, India).

Standardization of antioxidant enzyme assays and TUNEL assay were performed by utilizing 6 (3 for each assay) independent set of rats. Separate groups of rats were used for biochemical assay of oxidative stress markers (n=24; 6 in each group) and cell death study (n=72; 6 in each group). For biochemical assay, the rats were sacrificed on day 2; whereas for cell death study they were sacrificed on day 1, 2 and 3 after SCI.

Spinal cord injury surgery

On the day of surgery, the rats were anesthetized with ketamine hydrochloride and xylazine (60 and 10 mg/kg body wt, respectively). After skin incision and laminectomy, the spinal cord (SC) was completely transected at T13 level with a fine micro scissor under surgical microscope. The injury gap was filled with sterile, haemostatic, absorbable gelatin base foam (2 mm long; Abgel, Sri Gopal Krishna Pvt. Ltd, Mumbai, India) and was placed in alignment with the cut stumps of SC (SCI group). The muscles and skin were sutured in layers with silicon thread (Ethicon, Johnson and Johnson Ltd, India) and topical antibiotic cream (Providone Iodine, Glide Chem Pvt Ltd, Rampur Ghat, India) was applied. Intra and post operative body temperature was maintained by placing the rats on a controlled heating pad (CMA-150, CMA Microdialysis, North Chelmsford, MA, USA). Manual expression of urinary bladder was performed thrice daily until spontaneous voiding was achieved. Utmost care was given to the rats and were monitored for any urinary tract infection by daily examination of urine sample under microscope.

MF exposure chamber

MF exposure chamber is a modified Helmholtz coil. Briefly, it consists of four coils (two outer and two inner coils with 18 and 8 turns, respectively) mounted on a stand, a movable platform for the rat cage and a current regulator for maintaining constant current through the coils (Fig. 1). The coils were connected in series to provide uniform MF on the central movable platform, where six rats were placed in a specially designed polypropylene cage. This cage has six compartments to house rats individually during exposure for 2 h. MF was precisely adjusted to 17.96 µT before exposing rats by utilizing magnetometer (Walker Scientific Inc. Auburn Hills, MI, USA) and current regulator.

Fig. 1 — Diagrammatic representation of magnetic exposure chamber. (A) side view; and (B) front view. [Diameter of coils are 1,000 mm with 45 mm width. Distance of outer coil from center of structure is 470 mm and of inner coil is 122 mm. Current in electromagnetic coil is 1 A at 50 Hz frequency]
Assessment of cell death

Spinal cord tissue preparation

The rats were deeply anesthetized with ketamine before transcardial perfusion with 4% paraformaldehyde (PFA) in 0.1M PBS (Phosphate buffer saline). About 15 mm long SC tissue block with lesion epicenter was isolated and kept in the same fixative for 24 h at 4°C followed by graded sucrose (15 and 30%) treatment until it sank. SC was then embedded in optimal cutting temperature compound (Sakura Fine technical Co. Ltd, St. Torrance, CA) before taking its serial coronal sections (5µM thick) by cryostat microtome (Microm HM 550, Thermo Scientific, Kalamzoo, MI, USA). SC sections were taken at 2 and 4 mm from both rostral and caudal ends of lesion epicenter. Four SC sections from each rat were taken for analysis. i.e., two sections from caudal side and two sections from rostral side of lesion center of SC. The sections were then transferred onto poly-L lysine coated slides.

TUNEL assay

Post SCI apoptotic cell death was studied by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The SC sections were first rinsed with PBS followed by incubation in blocking solution (10 min at 15°C). The sections were then rinsed with PBS and incubated in permeabilization solution for 2 min on ice (2°C). Later, the area around the samples were air dried and incubated with TUNEL reaction mixture (Roche Diagnostics, Mannheim, Germany) for 60 min at 37°C in a humidified, dark atmosphere. The sections were then rinsed in PBS to stop the reaction. After air drying the section, it was incubated with converter-POD in a humidified chamber for 30 minutes at 37°C. It was then rinsed with PBS and treated with diaminobenzidine (Vector Laboratories, Burlingame, CA, USA). Cells were considered immunoreactive, if they contained dark brown colour cytoplasmic staining.

Assessment of oxidative stress

Spinal cord tissue preparation

The rat was sacrificed under deep anesthesia as described above. SC segment (T12 through L1) of 1.2 cm long containing lesion center was immediately frozen in liquid nitrogen and carefully transferred to −80°C in pre weighed vial. On the day of assay, SC was homogenized in 10 mM phosphate buffer (pH 7.4) having 10 µL/mL protease inhibitor to get 5% (w/v) homogenate using Potter-Elvehjem homogenizer. The homogenate was filtered through muslin cloth and centrifuged at 800×g for 30 min at 37°C by centrifuge (REMI Sales and Engineering Ltd, Mumbai, India) to separate the nuclear debris. The supernatant (S1) thus obtained was used for the estimation of lipid peroxidation by MDA (malondialdehyde) assay. The remaining S1 was further centrifuged at 10,500×g for 30 min at 4°C to get the post mitochondrial supernatant (PMS), which was used for the assays of reduced glutathione (GSH) and antioxidant enzymes viz. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR).

Biochemical assays

Estimation of lipid peroxidation (LPO)

Lipid peroxidation was determined by estimation of thiobarbituric acid reactive substance (TBARS) content which largely includes MDA. SC tissue were treated with normal serum and then incubated overnight at 4 °C with the primary antibody; anticaspase-3 (1:300, Neomarkers, Cat #RB-1197-R7, Fremont, CA, USA). The sections were then incubated with a biotinylated rabbit secondary antibody for 1 h at room temperature. Bound secondary antibody was amplified during 30 min incubation of the sections in the avidin–biotin complex (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Antibody complexes were visualised by immersing the tissues in 2% diaminobenzidine (Vector Laboratories). This reaction was stopped by rinsing the sections in 50 mM Tris-HCL (pH 7.4). Sections were then covered with a cover slip using mount quick (Daido Sangyo, Saitama, Japan). They were inspected through a microscope (Eclipse 80i, Nikon, Tokyo, Japan), and Caspase-immunoreactive cells were counted by using software (NIS ELEMENTS, Version AR3, Nikon). Cells were considered immunoreactive, if they contained dark brown colour cytoplasmic staining.

Caspase-3 immunohistochemistry

Caspase immunoreactive profile in SC sections of various groups on post surgery day-2 was also assessed to corroborate the findings of apoptosis. After blocking endogenous peroxidase, the sections were treated with normal serum and then incubated overnight at 4 °C with the primary antibody; anticaspase-3 (1:300, Neomarkers, Cat #RB-1197-R7, Fremont, CA, USA). The sections were then incubated with a biotinylated rabbit secondary antibody for 1 h at room temperature. Bound secondary antibody was amplified during 30 min incubation of the sections in the avidin–biotin complex (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Antibody complexes were visualised by immersing the tissues in 2% diaminobenzidine (Vector Laboratories). This reaction was stopped by rinsing the sections in 50 mM Tris-HCL (pH 7.4). Sections were then covered with a cover slip using mount quick (Daido Sangyo, Saitama, Japan). They were inspected through a microscope (Eclipse 80i, Nikon, Tokyo, Japan), and Caspase-immunoreactive cells were counted by using software (NIS ELEMENTS, Version AR3, Nikon). Cells were considered immunoreactive, if they contained dark brown colour cytoplasmic staining.
homogenate (0.25 mL) was mixed with 0.025 mL of BHT (butyl-hydroxy toluene). One mL of thiobarbituric acid (TBA) was added to the mixture and after vortexing, 3 mL of OPA (o-phosphoric acid) was added. The resultant mixture was incubated at 90°C for 45 min. The tubes were then removed and cooled at room temperature (29°C). The absorbance of each aliquot was measured at 535 nm. The rate of lipid peroxidation was expressed as nmol TBARS formed/hg of tissue using a molar extinction coefficient (MEC) of 1.56 × 10^5 M^1 cm^−1.

**Assay of reduced glutathione (GSH)**

PMS was mixed with 4% sulfosalicylic acid in 1:1 ratio. The samples were incubated at 4°C for 1 h and centrifuged at 4000 rpm for 10 min at 4°C. The assay mixture contained 0.4 mL of supernatant, 2.2 mL of 0.1 M phosphate buffer (pH 7.4) and 0.4 mL of dithio-bis-2-nitrobenzoic acid (4 mg/mL) making a total volume of 3 mL. The optical density of reaction product was read immediately at 412 nm on the spectrophotometer (Lambda EZ201; Perkin Elmer). The GSH content was calculated as nanomoles GSH/g tissue using a MEC of 1.36 × 10^4 M^1 cm^−1.

**Assay of glutathione Reductase (GR)**

The reaction mixture consisted of phosphate buffer (0.1M, pH 7.4), NADPH (1.0 mM), EDTA (0.5 mM) and oxidised glutathione (3 mM) and 0.1 mL of PMS. The enzyme activity was quantified at room temperature by measuring the disappearance of NADPH at 340 nm and calculated as nmoles NADPH oxidised/min/mg protein using molar extinction coefficient of 6.22 × 10^3 M^1 cm^−1.

**Assay of superoxide dismutase (SOD)**

SOD activity was measured by monitoring the autooxidation of epinephrine at pH 10.4 for 3 min at 480 nm. The reaction mixture contained 50 mM glycine buffer (pH 10.4) and 0.18 mL of PMS. The reaction was initiated by the addition of epinephrine. The enzyme activity was calculated in terms of nmoles epinephrine protected from oxidation/min/mg protein using molar extinction coefficient of 4.02×10^3 M^1 cm^−1.

**Assay of glutathione peroxidase (GPx)**

The reaction assay consisted of phosphate buffer (0.1M, pH 7.4) EDTA (1.0 mM), sodium azide (1.0 mM), glutathione reductase (1 EU/, mL), Glutathione (1 mM), NADPH (0.2 mM), Hydrogen peroxide (0.25 mM) and 0.1 mL of PMS in the final volume of 1.0 mL. The enzyme activity was calculated a nanomoles NADPH oxidised/min/mg/protein using molar extinction coefficient of 6.22 × 10^3 M^1 cm^−1.

**Assay of catalase (CAT)**

The assay mixture consisted of 1.95 mL of phosphate buffer (pH 7.4), 1.0 mL hydrogen peroxide and 0.05ml PMS in a total volume of 3 mL. The change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of nmoles H2O2 consumed/min/mg protein using MEC of 39.6 M^1 cm^−1.

**Study plan**

The rats were randomly divided into 4 groups such as Sham-SCI, Sham-SCI+MF, SCI and SCI+MF. Sham-SCI+MF and SCI+MF rats started receiving exposure to MF for 2 h from the next day of SCI. Separate groups of rats were sacrificed at post surgery day-1, 2 and 3 for cell death study. Whereas, oxidative stress was studied in different groups of rats sacrificed on day-2 of SCI. SC tissues were collected as described above. SC tissues were collected as described above. Post injury oxidative stress was assessed by antioxidant enzyme activities and cell death was assessed by TUNEL assay and Caspase-3 immunohistochemistry.

All measurements were performed by two observers blinded to the identity of different rat groups. Two observers blinded to the identity of different rat groups were performed the counting of TUNEL positive cells and spectrophotometry for various biochemical assays. However, when there were any discrepancies in the data of these observers, a third person was allotted and repeated these tests to avoid any potential bias.

**Statistical analysis**

Statistical analysis was performed using Statistical Package for Social Sciences version 16 software (SPSS Inc, Chicago, IL, USA). All values are presented as mean±SD. Inter group comparison of oxidative stress and cell death parameters were analyzed among the groups by ANOVA with post hoc analysis using Bonferroni correction. P values <0.05 were considered statistically significant.

**Results**

**Assessment of cell death**

In contrast to the Sham-SCI rats in our study, which was virtually free from any TUNEL positive cells, the overall data of SCI rats showed more number of TUNEL positive cells in the WM compared to GM rostrally and caudally at all time points. For instance, the number of TUNEL positive cells was 394.17±21.54 in the GM while it was 366±28.01 in the GM at 2 mm rostral site on day 2. Moreover, the number of TUNEL positive cells was
more at 2 mm distance (for eg. 394.17±21.54 in rostral WM) compared to 4 mm (250±21.77 in rostral WM) distance of lesion epicentre (Fig. 1) both rostrally and caudally at all time points. These changes in TUNEL positive cells were significantly more on day 2 and continued to be even more on day 3 when compared with day 1 (Figs 2 and 3). Careful histological examination of TUNEL positive cells showed typical morphological features of cell death i.e., margination of the condensed chromatin, blebbing, segregation and disintegration of the nucleus and shrinkage of both cytoplasm and plasma membrane, thereby creating pericellular space (Fig. 3). They were also characterized by brown granules in the nucleus, while the nucleus of TUNEL negative neurons counterstained with hematoxylin showed blue colour (Fig. 3). In contrast, significant reduction in the number of TUNEL-positive cells were noted in all time points of MF exposed SCI rats (Figs 2 and 3). Moreover, the shrinkage of plasma membrane and the volume of pericellular space were also reduced SC sections of MF exposed SCI rats (Fig. 3).

Greater number of Caspase-3 positive cells were also observed in the white matter of SCI group, while it was significantly reduced in SCI+MF group. The intensity of Caspase-3 immunohistochemical staining was negligible in Sham-SCI and Sham-MF groups (Fig. 4).

**Assessment of oxidative stress**

*Lipid peroxidation and antioxidant system*

MDA concentration in the SC samples was used to assess the status of lipid peroxidation. SCI rats showed higher MDA content than Sham-SCI rats.
However, MF exposure to SCI rats restored it (Fig. 5). As indicators of endogenous antioxidant system, we estimated the activities of SOD, GPx, Catalase and Glutathione reductase besides reduced Glutathione content in the spinal cord samples collected around the injury site at T13 spinal segment. Significant decrease of all of these antioxidants was observed in SCI rats. However, MF exposed SCI rats showed significant increase in SOD and Catalase activity; while restoration of glutathione content, activities of glutathione reductase and Gpx (Fig. 5).

**Discussion**

The management strategies after SCI are primarily directed towards promoting repair of the damage and/or minimising the secondary injury processes. Besides several pharmacological and invasive procedures, we selected MF as management strategy since it has the advantages of being non-invasive, non-pharmacological and simple to administer. Electromagnetic fields (EMFs) have long been considered relevant for human health, and recent studies have indicated that EMFs exhibit protective effects in spinal cord injury including multiple pathologies like Alzheimer's disease, stroke, osteoporosis and pain.

In the present study, ELFMF with 50 Hz frequency and 17.96 µT amplitude was found to be beneficial for functional recovery including attenuation of hyperalgesia; reduction in lesion volume and inflammation; facilitation of angiogenesis; increased neuronal regeneration and myelination at the lesion site; increased osteogenesis; improvement in locomotion, food intake, water intake and body weight; and restoration of tonic pain following SCI in rats as observed by others.

Production of reactive oxygen species (ROS) and subsequent oxidative stress is an important consequence occurring during the course of secondary injury. Superoxides, hydroxyl radicals, hydrogen peroxides and peroxynitrites are the principal components of ROS. They can directly interact with proteins, lipids and nucleic acids, leading to cellular and molecular damage, consequently neurological dysfunction. However, the body has an in-built enzymatic and non enzymatic antioxidant system which control ROS production and oxidative stress. Major antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (Gpx), glutathione reductase (GR) and catalase (CAT) which differ from each other in their structure, tissue distribution and cofactor requirement. Damage of the spinal cord impairs their protective ability.

We have found decrease in the activity of antioxidant enzymes namely, SOD, GPx, glutathione reductase, catalase and non enzymatic antioxidant namely, glutathione after two days of SCI. In contrast, improvement in these were noted in MF exposed spinalised rats. A previous study also demonstrated the antioxidant protective effects of ELFMFs in neuronal cell lines by elevating endogenous antioxidant properties, which was in accordance with the current study. ELFMF (50 Hz, 0.5 mT) has been reported to reduce oxidative stress in the brain of gerbils submitted to global cerebral ischemia.
Inhibition and scavenging of ROS production have been demonstrated to increase functional recovery following traumatic CNS injuries. An increase in free radicals causes overproduction of malondialdehyde (MDA) and is one of the final products of polyunsaturated fatty acids peroxidation in the cells. MDA is commonly known as a marker of oxidative stress and the antioxidant status. In our study, ELFMF treatment reduced spinal cord tissue MDA content in spinalised rats, which indicated its efficacy in reducing the lipid peroxidation following SCI. MF has been found to cause the interconversion of singlet to triplet state of free radicals, thereby reducing their reactivity and interaction with bio molecules (Fig. 6). Low frequency electromagnetic field has been reported to reduce the levels of reactive oxygen species, and upregulate the expression of catalase and superoxide dismutase following spinal cord injury.

Acute SCI has been found to cause two peaks of oxidative stress in rats. First peak occurred at 4 hours and a second peak was observed from 24 hours to 5 days; 2nd day being the maximum after SCI.
Therefore, we have assessed the lipid peroxidation and antioxidant assay on 2nd day after SCI. Free radicals formed by SCI possibly damages DNA and the evidence of apoptotic cell death can be detected in hours to weeks following CNS injury. A time course analysis in rats revealed that apoptotic cell death at the site of lesion occurred as early as 4 h post SCI and could be seen in decreasing amounts as late as 3 weeks after injury. Literature suggest that apoptotic cell death peaked 24-48 h after injury. We, therefore, performed TUNEL assay to assess the effect of MF on post-SCI neuronal cell death on day 1, 2 and 3 after SCI. A significant increase in the number of TUNEL positive neurons in both white and grey matter of SC at 2 and 4 mm above and below the level of injury was observed in SCI rats at all time points. This was also supported by greater Caspase-3 immunoreactivity in their SC sections.

A number of experimental studies have provided evidence of widespread neuronal and glial cell death following injury to the central nervous system (CNS) and was prominent in the white matter in which wallerian degeneration was simultaneously observed. We also have observed more apoptotic cells in the white matter. The present study showed significant reduction in number of apoptotic cells in MF exposed SCI rats at all time points. ELFMF has been found to enhance the survival of newborn neurons by reducing the expression of the pro-apoptotic protein, Bax and increased levels of the anti-apoptotic protein, Bcl-2 in the mouse hippocampus.

Post-SCI inflammation is a precursor to subsequent pathological changes, including oxidative stress, cellular necrosis and apoptotic cell death. Most recent study showed anti-inflammatory property of pulsed magnetic field (PMF) exposure in spinal cord. Moreover, one of our previous laboratory studies revealed a significant decrease in microglia and macrophages population in ELF MF (50 Hz, 17.9 µT) exposed SCI rats. Decrease in the density of microglia indicated a switch from the inflammatory/M1 phase to the final repair phase/M2. Decreased microglial activity was also noticed following electronic signal treatment.

Male rats were selected in our study to eliminate the differential effects of sex hormones on neuroprotection and mode of neuroregeneration. However, we presume that the beneficial effects observed in our MF exposed male rats apply to female rats also as there are no reports on gender based differential effects of MF. We had given ELFMF exposure day-1 next day onwards of SCI so as to circumvent the cascade effect of injurious factors released after the injury. We assume that exposure of MF on SCI rats possibly induced plastic changes at the site of injury and provided conducive environment for functional outcome, which was evident in our previous study.

**Conclusion**
Exposure of spinalized rats to extremely low frequency magnetic field (ELFMF) significantly reduced the number of apoptotic cells in both WM and GM (at 2 and 4 mm rostral and caudal to the lesion site) on days 1, 2 and 3 after spinal cord injury (SCI). Moreover, significant improvement in SOD and catalase activity, while restoration in glutathione content, lipid peroxidation and activities of glutathione reductase and Gpx were noted in the spinal cord of ELFMF exposed SCI rats. This study provides valuable insight into the mechanism of beneficial effect of ELFMF on SCI and better understanding of the efficacy of MF on SCI induced secondary injury.

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**Conflict of interest**
None declared.

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