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#### ARTICLE

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## Extremely low frequency magnetic field protects injured spinal cord from the microglia- and iron-induced tissue damage

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#### ABSTRACT

Spinal cord injury (SCI) is insult to the spinal cord, which results in loss of sensory and motor function below the level of injury. SCI results in both immediate mechanical damage and secondary tissue degeneration. Following traumatic insult, activated microglia release proinflammatory cytokines and excess iron due to hemorrhage, initiating oxidative stress that contributes to secondary degeneration. Literature suggests that benefits are visible with the reduction in concentration of iron and activated microglia in SCI. Magnetic field attenuates oxidative stress and promotes axonal regeneration in vitro and in vivo. The present study demonstrates the potential of extremely low frequency magnetic field to attenuate microglia- and iron-induced secondary injury in SCI rats. Complete transection of the spinal cord (T13 level) was performed in male Wistar rats and subsequently exposed to magnetic field (50 Hz,17.96  $\mu$ T) for 2 h daily for 8 weeks. At the end of the study period, spinal cords were dissected to quantify microglia, macrophage, iron content and study the architecture of lesion site. A significant improvement in locomotion was observed in rats of the SCI + MF group as compared to those in the SCI group. Histology, immunohistochemistry and flow cytometry revealed significant reduction in lesion volume, microglia, macrophage, collagen tissue and iron content, whereas, a significantly higher vascular endothelial growth factor expression around the epicenter of the lesion in SCI + MF group as compared to SCI group. These novel findings suggest that exposure to ELF-MF reduces lesion volume, inflammation and iron content in addition to facilitation of angiogenesis following SCI.

#### Introduction

Secondary tissue damage following traumatic spinal cord injury (SCI) is the consequence of a series of vascular, biochemical, molecular and cellular events that begin as early as few seconds and extend for as long as weeks/months following the primary injury (Conta and Stelzner, 2008; Tator and Fehlings, 1991). Resident microglia get activated and release inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6), reactive oxygen and nitrogen species (ROS, RNS) (Carlson et al., 1998; Davalos et al., 2005; Popovich et al., 1997). Macrophage-derived proinflammatory monocytes (ED-1) infiltrate into the primary injury site and further deteriorate the condition leading to secondary damage. Iron (Fe<sup>2+</sup>) sequestrate into microglia through phagocytosis of RBCs that flood the injury site. Later, iron released from these cells through lipid peroxidation of the plasma membrane contributes to gradual free radical induced tissue damage of the remaining spinal cord tissue (David and Kroner, 2011). Thus,

Spinal cord injury; extremely low frequency magnetic field; microglia; secondary injury; iron

microglia and iron at the site of lesion are the main facilitators that promote secondary injury.

Extremely low frequency magnetic field (ELF-MF) exposure has been shown to provide a microenvironment conducive to neural repair by stimulating the release of neurotrophic factors and neurotransmitters; decreasing apoptosis and promoting axonal growth (Fanelli et al., 1999; Lai et al., 1993; Macias et al., 2000; Mert et al., 2006; Pal et al., 2013). We have earlier reported beneficial effect of ELF-MF (50 Hz, 17.96 µT) on locomotor, sensorimotor, autonomic functions, neurotransmitter profile and osteoporosis in rat model of complete and hemisected spinal cord, following daily MF exposure of 2h/day till 8 weeks (Das et al., 2012; Manjhi et al., 2013; Kumar et al., 2016, 2010, 2013). However, the mechanism of this improvement remains to be elucidated. Given the fact that there is data supporting the involvement of microglia in pathophysiology of SCI and that ELF-MF stimulation has beneficial effect in SCI, we hypothesise that ELF-MF exposure

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**KEYWORDS** 

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will protect the injured spinal cord by preventing the microglia/macrophage- and iron-induced secondary tissue damage following SCI.

As any insult to the spinal cord directly gets reflected through altered locomotion, we have assessed it every week following SCI to observe any spontaneous or MFinduced recovery. The extent and morphology of the lesion, presence of viable neurons (cresyl-violet staining) and the area of scar tissue formation (trichrome staining) were also assessed as they are indicative of recovery. Finally, we determined total microglia and proinflammatory macrophage population (CD11b, ED-1), iron and angiogenesis (VEGF) within the injured segment of the spinal cord to understand the mechanism of recovery due to MF intervention, if any.

#### Materials and methods

#### Animals

Adult male Wistar rats (N = 44; body weight 230–250 g) were divided into three groups-control, SCI and SCI + MF. They were housed individually in polypropylene cages at the Experimental Animal Facility maintained at 24 ± 2°C with a light: dark cycle of 14:10 h and provide food (Ashirwad industries, Haryana, India) and water ad libitum. The study was performed in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), New Delhi, Animal Welfare Division under Ministry of Environment, Forest and Climate Change (Govt. of India) and approved by the Institutional Animal Ethics Committee (File No. 665/IAEC/12).

#### Surgery and magnetic field exposure

The rats were anesthetised using ketamine 60 mg/kg and xylazine 10 mg/kg (i.p.). Laminectomy (T10-T12 vertebrae) was performed to expose the spinal cord before complete spinal cord transection (T11 vertebra corresponding to T13 spinal cord) with micro-scissors. The rostral and caudal stumps of the spinal cord were gently lifted and 2.0 mm long sterile hemostatic, absorbable gelatin base foam (Abgel, Sri Gopal Krishna Pvt Ltd, India) was gently placed in the gap which was aligned with the cut ends of the spinal cord. The muscle, fascia and skin were sutured in layers and rats were returned to their home cages. After surgery, the rats were administered a bolus of Lactate Ringers solution (5 ml, i.p.) to compensate for blood loss along with a dose of antibiotic (systemic, gentamycin 50 mg/ kg bw, i.m.; local neosporin ointment). Manual evacuation of bladder was done 3 times/day until reflex micturition was restored.

Rats in the SCI + MF group were exposed to magnetic fields of 17.96µT, 50 Hz, 2 h daily for 8 weeks using an indigenously manufactured magnetic field chamber (Kirschvink, 1992; Manjhi et al., 2013) (Figure 1). The chamber consists of four parallely placed circular coils of equal diameter (1.0 m) wound on the same cylindrical surface. For experiments, which require only a small intensity of uniform magnetic field, this design is highly recommended (Lee-Whiting, 1957). Although the ideal current ratio between the outer and inner pairs of coils was 2.2604, a simple turn ratio of 9/4/4/9 is within 0.5% of this ideal value and yielded eighth-order uniform MF of 17.96 µT in the center of the chamber. The rats were kept in a Plexiglas enclosure (All India Institute of Medical Sciences, New Delhi, India) and placed on the Plexiglass platform in the middle of the MF chamber.



**Figure 1.** The original photograph (A), schematic side view (B) and front view (C) of the magnetic field chamber. It contains four coils, 2 outer (OC) and 2 inner (IC) on stands (CS). A **plexiglass** plate (P) is placed at the center of the coils for keeping rats during ELF-MF exposure. The diameter of the coils is 1000 mm having 45 mm width. The distance of outer coil from center of structure is 470 mm and of inner coil is 122 mm. The number of turns in outer coil is 18 and in inner coil is 8.

At the end of the experiment period of 8 weeks, the rats were sacrificed by intraperitoneal injection of sodium pentobarbitone (120 mg/kg); transcardially perfused with cold saline (4°C) followed by 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; pH 7.4); spinal cord tissue was excised and preserved in 4% paraformaldehyde at 4°C for histology and immunohistochemistry studies. For flow cytometric study, the rats were sacrificed by decapitation and their spinal cords were carefully removed and processed for analysis.

#### Assessment of locomotor functions

The quality of locomotion was assessed by Basso, Beattie and Bresnahan (BBB score) locomotor rating score performed on a weekly basis. The test rat was placed in the center of an open field (Coulbourn Instruments, USA). The locomotion of the rat was video monitored for offline reassessment of the footage in slow motion. The data was scored and analyzed off-line by two independent observers unaware of the rat groups (Basso et al., 1995).

#### Tissue processing for histological analysis

The spinal cord was isolated from T5 to L5 before fixation in paraformaldehyde for 24 h and then cryoprotected in 15% and 30% sucrose in 0.1M phosphate buffer (72 h) at 4°C. The lesion site was visually located and a 10 mm long spinal cord tissue block containing the lesion epicenter was selected and processed for cryosectioning (Thermo HM550, USA). Horizontal sections (20 µm) were obtained on poly-L-lysine-coated slides. All the sections of the tissues obtained from all the groups were preserved and sequentially mounted for different staining procedures namely; cresyl violet, Masson's trichrome, Prussian blue and CD11b, vascular endothelial growth factor (VEGF) immunohistochemistry. Every sixth section was processed for the same staining procedure. Ten sections were stained for each staining/immunohistochemistry from each rat.

Cresyl violet staining was used to assess the extent of the lesion. The total area of lesion was traced and measured using a Nikon eclipse 80i digital light microscope and NIS element version 3.0 software. The area marked by dense gliosis, cysts or void of tissue was considered for calculation of the total area damaged. To calculate the total lesion volume, the total area of a single section was multiplied with the number of sections per spinal cord and the thickness of the single section. Assessments were made by an investigator blinded to treatment allocation.

#### Masson's trichrome staining

Masson's trichrome staining was done to identify the presence of collagenous connective tissue at the injury site using an Accustain Trichrome Stain (Masson's Kit, Catalog number HT15, Sigma-Aldrich, USA). The standard procedure recommended by the manufacturer was followed.

#### Prussian blue iron staining and quantification

Prussian blue staining was done to stain the iron ( $Fe^{3+}$ ) particles at the lesion site. Briefly, the frozen tissue sections were thawed and dipped in distilled water for 10 min for rehydration. Hydrated tissue sections were then dipped in a 1:1 solution of 10% potassium ferrocyanide (Merck) and 20% HCl (Merck) for 15 min. The slides were rinsed for 5 min in distilled water and counterstained with nuclear fast red (Vector, USA). The sections were then dehydrated through an increasing ethanol concentration (95%, 100%), and xylene (with two changes each for 5 min). The sections were mounted with DPX medium (Sigma-Aldrich, USA).

Tissue sections were scanned under a digital microscope using the 20x objective lens. Six fields were selected at the lesion epicenter from every section. The relative area of the section covered by Prussian blue staining was calculated in NIS-element v3.0 software by dividing the positively stained, i.e., iron-containing, areas by the total area of each section. The percentage of area obtained for each section was averaged for each animal.

#### Immunohistochemistry

Spinal cord sections were incubated in blocking solution (PBS containing 10% normal goat serum, 0.1% Triton X-100) for 2 h at room temperature, followed by overnight incubation at 4°C in a humidified chamber with mouse anti-CD11b/OX 42, (1:100; BD Biosciences) to visualize microglia and rabbit anti-VEGF (1:500; Abcam) to visualize blood vessels, fluorescent secondary antibodies were either Alexa 488 conjugated goat anti-rabbit IgG (1:200; Abcam) or Alexa 594 conjugated goat anti-mouse IgG (1:200; Abcam) prediluted in PBS containing 0.5% NGS and 0.1% Triton X-100. Sections were incubated with 0.1% DAPI (4', 6-diamidino-2-phenylindole dihydrochloride; Sigma Aldrich) for staining the nuclei. Slides were cover-slipped with Fluoroshield mounting medium (Sigma Aldrich) and images were captured with a confocal microscope (SP2, Leica, Germany). Quantitative analysis of CD11b and VEGF positive cells were performed by a blinded observer from images captured surrounding 1 mm length of the lesion epicenter using 40x objective lens. Image J 1.49 software (NIH, USA) was used for cell counting. The numbers of positively labelled cells were automatically counted using a mark and count tool and then the mean of each section was calculated.

#### Flow cytometric analysis

Proinflammatory ED-1<sup>+</sup> macrophages were quantified by flow cytometry. Briefly, the spinal cord segments were minced with fine scissors in Hank's balanced salt saline (HBSS). Cells were retrieved by centrifugation and enzymatically dissociated with trypsin and collagenase in Dulbecco's Modified Eagle's Medium (DMEM) for 20 min at 37°C followed by trituration with a Pasteur pipette. The pellet was resuspended in HBSS and overlayed on an OptiPrep gradient. Dissociated spinal cells in HBSS were layered on top of the OptiPrep gradient and centrifuged (15 min, 1900 rpm, 20°C). The debris layer was carefully aspirated. The remaining cells were washed and resuspended in HBSS for immunolabelling. Cells were blocked for 30 min in goat serum, and incubated for 1 h with FITC conjugated mouse anti-rat ED-1 antibody (AbD Serotec, USA). Samples were analyzed on a fluorescence-activated cell sorting Calibur (Becton-Dickinson, USA) flow cytometer using Cell Quest software. Ten thousand events were read for all samples. Flow cytometric gates were set using control IgG isotype labelled cells or spinal cord cells from uninjured control animals to set baseline values for normalization across time point.

#### Statistical analysis

Statistical analysis was performed using Sigma Plot 12.0 software (Chicago, IL, USA). Data is presented

as mean  $\pm$  SD. The within group data of BBB score was analyzed using repeated measures of ANOVA. The inter-group data of BBB score, lesion volume, iron content, number of microglia and amount of VEGF expression was compared by one-way ANOVA while the post hoc analysis was done by Bonferroni test. Statistical significance was accepted as p < 0.05.

#### Results

A total 44 rats were used in this study, with 12 rats in the control group and 16 each in SCI and SCI + MF. The data of six rats from the SCI group and four rats from the SCI+MF group were excluded and the rats were sacrificed because of self-biting/mutilation, urinary tract infection and/or blockage, death. No significant difference in pre-injury baseline data was observed between the three groups.

#### Locomotion (BBB score)

The basal BBB score in control, SCI and SCI + MF groups was  $21 \pm 0$ . It remained same from week one through eight in the control group, but significantly decreased in SCI and SCI + MF groups from week 1 (0.00  $\pm$  0.00 in both groups) through 8 (2.83  $\pm$  0.41 and 8.17  $\pm$  0.82, respectively) as compared to their basal values (p < 0.001).

The BBB score decreased significantly (p < 0.001) from post-SCI week 1 till 8th week in SCI and SCI+MF groups as compared to the control group, whereas, it gradually improved (p < 0.001) from week 3 through 8 in the SCI+MF group in comparison to the SCI group (Figure 2).



**Figure 2.** Depicts the BBB score of control, SCI and SCI+MF groups (n = 6). In control group BBB score remained same (21 ± 0) throughout 8 weeks. The BBB score was significantly higher in the SCI+MF group than the SCI group from post-surgery week 3 onwards. \* indicates comparison of SCI/SCI+MF versus the control group and # sign indicates comparison of the SCI versus the SCI+MF group. Single sign denotes p < 0.001. SCI, spinal cord injury; MF, magnetic field.

#### Histological analysis and lesion volume

In the control group, cresyl violet staining revealed intact spinal cord morphology. The white matter and grey matter with large number of viable neurons were clearly evident. However, in SCI and SCI+MF groups, bilateral tissue loss from the rostral and caudal extent of the injury to the epicenter was observed. At the site of lesion number of cysts, infiltrated cells, gliosis and dead/dying neurons were apparent in both the groups. No viable neurons were visible in SCI group, though a few viable motor neurons were observed surrounding the epicenter in the grey matter of the SCI+MF group (Figure 3A).

The total tissue damage volume in the SCI group was  $15.23 \pm 3.89 \text{ mm}^3$ , while in the SCI+MF group, it decreased significantly (p < 0.05) to  $8.74 \pm 1.64 \text{ mm}^3$ , suggesting attenuation in the extent of damage (Figure 3B).

#### Masson's trichrome staining

No collagen scar tissue was observed in the control group, while at the lesion epicenter dense collagen scar tissue interspersed with cystic cavities encircled by a thin layer of collagen was noted in the SCI group of rats. The collagen tissue was found to be rather diffuse and very less in the SCI+MF group as compared to SCI group (Figure 3C and D) when observed under high power.

### Prussian blue iron (ferric; fe<sup>3+</sup>) staining

Prussian blue staining in the SCI group revealed dark blue colored iron particles, present in a diffused and scattered manner throughout the lesion core, making it easily identifiable (Figure 4A). However, in the SCI+MF group iron particles were more prominent, less diffuse and present in clusters around the lesion epicenter (Figure 4B). The iron particles were located extracellularly. Quantitative analysis of Prussian blue content in these areas revealed significantly higher (p < 0.01) iron accumulation at the lesion epicenter in the SCI+MF group (49.68 ± 23.3 µm<sup>2</sup>) as compared to the SCI group (22.39 ± 6.12 µm<sup>2</sup>) (Figure 4C).

#### Immunohistochemistry

OX-42 immunoreactivity revealed moderate expression of resident microglia in the Control group, exhibiting the "resting phenotype" characteristics (Figure 5A). On the



**Figure 3.** The extent of lesion in the cresyl-violet stained images of spinal cord from control, SCI and SCI+MF groups is shown in 3A. In the control and SCI+MF groups, viable motor neurons are indicated by arrowheads. In the SCI group, the viable motor neurons are absent while dense inflammatory cells are observed. Scale bar for 2x magnification is 1000  $\mu$ m; for 20x it is 100  $\mu$ m. (3B) shows total tissue damage volume (Mean ± SD) of SCI and SCI+MF groups. Volume was significantly less in the SCI+MF group in comparison to the SCI group. # denotes *p* < 0.05. (3C) shows representative images of spinal cord stained with Masson's trichrome stain for collagen scar tissue. Dense collagen scar staining was observed in the SCI group which was decreased in the SCI+MF group. Scale bar 100  $\mu$ m for 3C.



**Figure 4.** Prussian blue stain for iron particles in the SCI (A) and SCI+MF group (B) (Scale bar 1000  $\mu$ m; for magnified images 100  $\mu$ m). The total amount of iron (Mean ± SD) is higher in SCI+MF as compared to SCI group (C). # denotes p < 0.05.

contrary in SCI and SCI+MF groups, microglia acquired "activated phenotype" characteristics liked marked cellular hypertrophy and retraction of cytoplasmic processes (Figure 5B and C). The microglia were predominately located surrounding the lesion site and decreased towards both the rostral and caudal sites. In the SCI group, the microglia were predominant, densely aggregated and appeared in patches, while in the SCI+MF group they were less dense and aggregated. Quantitative analysis revealed a significant increase the number of microglia cells surrounding the lesion epicenter in the SCI group  $(115 \pm 37)$  as compared to both the SCI+MF group  $(45 \pm 19; p < 0.05)$  and uninjured spinal cord of the control  $(14 \pm 6; p < 0.001)$  group. Further, in the SCI+MF group microglia population was significantly less as compared to the SCI group (p < 0.01) (Figure 5N).

VEGF is an angiogenic factor that stimulates proliferation of endothelial cells, formation of new blood vessels and enhances vascular permeability. The expression of VEGF was almost negligible surrounding the lesion epicenter in the SCI group, whereas in the SCI +MF group VEGF expression was more prominent (Figure 6). Quantitative analysis revealed a significant increase in the VEGF positive cell population in the SCI +MF (54 ± 8; p < 0.001) group as compared to SCI group (22 ± 5).

#### Flow cytometric analysis for ED-1 macrophages

In Figure 7, macrophages in the spinal cord homogenate are defined by ED-1 expression and side-scattered light in a dot plot and their relative changes in different groups are shown. The percentage of microglia population in SCI (11.51%  $\pm$  0.61%) (p < 0.01) and SCI+MF (6.24%  $\pm$  0.48%) (p < 0.05) groups were significantly higher as compared to the control group (2.21%  $\pm$ 0.66%). However, in comparison to the SCI group, the percentage was significantly lower in the SCI+MF (p < 0.05) group.



**Figure 5.** Shows the photomicrograph of immunohistochemistry of microglia cells in control, SCI and SCI+MF rats. Figure (5B-C) shows resting microglia with small compact soma bearing long thin ramified processes in the control group (arrowheads) and activated microglia with marked cellular hypertrophy and retracted cytoplasmic processes (arrowheads) in SCI and in SCI+MF groups; however, their density, aggregation and number were reduced in the latter. In SCI and SCI+MF groups, the activated microglia are mainly distributed surrounding the lesion area compared to rostral and caudal areas from the lesion site. The microglia count was significantly higher in the SCI group compared to both control and SCI+MF groups. \* indicates comparison of SCI/SCI+MF versus the control group and # sign indicates comparison of SCI versus SCI+MF group. \*\* p < 0.01; \*\*\* p < 0.001, # p < 0.05. Scale bar 1000 µm for images A, D, I; for images B and C 50 µm; for images G, H, L, M 30 µm.

#### Discussion

The present study demonstrates a significant deficit in locomotion, qualitative and quantitative changes in microglia, macrophages, presence of iron particles, high lesion volume and collagen scar tissue in complete spinal cord transected rats (T13). Daily exposure (2h/d) to ELF-MF for 8 weeks significantly reversed these detrimental effects of SCI. Attenuation of secondary damage in the SCI+MF group was indicated by decrease in lesion volume and collagen scar tissue, concomitant with reduction in activated microglia and macrophages and significantly higher amount of angiogenesis at the epicenter, contributing to a significant improvement in locomotion. Complete transection model of SCI was selected because both the axons and neurons are severed under vision to produce highly reproducible injury that is a prerequisite for studying the efficacy of intervention. Moreover, the outcome of SCI is dictated by the temporal sequence of injury response and the survivability of different neurons, which itself is under the influence of inherent factors (Basso et al., 1995; Koda et al., 2007). This crucial factor of similar dimensions of the injury was reflected in the locomotor behavior (nadir BBB score) during week 1 in all the rats of the SCI and the SCI+MF groups.



**Figure 6.** ELF-MF exposure increases VEGF immunoreactivity in the region surrounding lesion epicenter of injured spinal cord in the SCI+MF group as compared to the SCI group, thereby indicating angiogenesis. The VEGF+ cell count was significantly higher in the SCI+MF group in comparison to the SCI group. # sign indicates comparison of SCI versus SCI+MF group. ### p < 0.001. Scale bar 1000µm; for magnified images 75 µm.

#### Microglial repertoire after SCI

We report a high density of activated microglia at the injury site that persisted even after 8 weeks of complete transection injury of spinal cord. The morphological characteristics of microglia were indicative of their activated phenotype, which is in line with the existing literature. The activation of microglia has been well documented within 12-24 h of SCI irrespective of the mode of injury (contusion/compression) (Beck et al., 2010; Clark et al., 2010; Hains & Waxman, 2006; Qu et al., 2012; Yang et al., 2005). Microglia express mRNA for proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  within 12 h of SCI (contusion), which further promotes apoptosis of neurons and oligodendrocytes, thereby enhancing lesion volume (Ferguson et al., 2008; Pineau & Lacroix, 2007; Yang et al., 2005). In trichrome-stained sections, thick interconnected bundles of collagen tissue were observed at the lesion site suggesting thick scar formation. The injury site did not reveal viable cells and there was negligible angiogenesis.

Free radical-induced secondary injury is significantly contributed by the iron particles after primary injury. Blomster et al. (2013) reported the presence of iron in acute (1 week) and chronic (4 weeks) contusion model of SCI in mice. In our SCI rats at the end of 8 weeks Prussian blue staining revealed presence of extracellular iron. The major source of iron is believed to be hemoglobin of RBCs that are initially phagocytosed by resident microglia/macrophages. These ferrous iron particles promote free radical-induced oxidative stress and apoptosis (David & Kroner, 2011; Rathore et al., 2008).

#### Effect of ELF-MF

Pulsed-MF exposure (4 h/day for 12 weeks) in cats; rTMS in human (20 min/day for 9 days) and in rodents (1.5T, 1 Hz; 7.5 min/day for 24 days) have been reported to promote recovery in locomotion after SCI (Ahmed et al., 2011; Ahmed & Wieraszko, 2008; Crowe et al., 2003; Kumru et al., 2010). The authors have attributed this recovery to the modulation of serotonergic pathways innervating locomotor central pattern generator (CPG) of lumbar spinal cord by MFs (Poirrier et al., 2004). Moreover, it also improved the deficits in food intake, water intake, body weight, pain, osteoporosis (bone mineral content, bone mineral density, bone microarchitecture) and neurochemical profile (Das et al., 2012; Kumar et al., 2010, 2013; Manjhi et al., 2013). We



**Figure 7.** Left panel of A, B, C, D shows representative dot-plot of ED-1 expression in cells obtained from spinal cord homogenates of unstained, control, SCI and SCI+MF groups. The square delimits the ED-1+ population of cells used in this study. The right panel depicts representative histogram where populations of CD11b+ cells (microglia) were defined from control, SCI and SCI+MF groups. (E) Mean values of macrophage population in control, SCI and SCI+MF groups. The macrophage count was significantly higher in the SCI group compared to both control and SCI+MF groups. \* indicates comparison of SCI/SCI+MF versus control group and # sign indicates comparison of SCI versus SCI+MF group. \*\* p < 0.01; SCI, # p < 0.05.

reported an improvement in the local concentration of 5-HT, in addition to glutamate, glycine, nor-adrenaline in our similar complete transection model of SCI by ELF-MF (17.96µT for 2 h/day X 8 weeks) (Kumar et al., 2016, 2013). The mechanism of action is not yet clear. Nevertheless, several possibilities have been suggested (Kumar et al., 2016). ELF-MF stimulation creates electric fields that can penetrate soft tissue and bone to reach deep structures including nervous system. If the created electric field is of appropriate intensity, amplitude and duration, the neural microenvironment can be altered besides being stimulated through depolarization (Grissom, 1995). This may help in the maintenance of functional integrity of the injured neurons and axons. In addition, the restoration of the neurochemical (BDNF, GDNF) profile complements and provides functional conducive microenvironment for the perpetuation of enhanced regeneration and limitation of lesion processes (Sharma, 2006; Zhang et al., 2009) The present study provides further mechanistic support to the beneficial effect of ELF-MF on SCI.

Significant reduction in collagenous scaring and total damaged tissue volume indicates attenuation of secondary damage in SCI+MF rats. Since, Prussian blue stains the ferric iron (Fe<sup>3+</sup>); we can conclude that more ferric iron is present in the SCI+MF group. The exposure to MF may have facilitated the conversion of toxic ferrous iron to non-toxic ferric iron. In addition, ELF-MF may promote the magnetization of iron particles and more iron particles become magnetite which further contributes towards the attenuation of secondary injury. ELF- MF possibly reduces the free radical generation and restores the equilibrium in ROS/antioxidants by molecular alignment (Pal et al., 2013; Zaporozhan and Ponomarenko, 2010).

Besides free radical damage, neural inflammation significantly contributes towards the secondary injury. Ongoing inflammation is indicated by the overwhelming numbers of activated microglia and macrophages in the SCI group of rats as late as week 8. Analysis by both the immunohistochemistry and flow cytometry techniques revealed a significant decrease in microglia and macrophages population in our SCI+MF rats. Decrease in the density of microglia indicates a switch from the inflammatory phase to the final repair phase. MF-induces conformational changes in G-proteins and stabilizes other integral membrane proteins which lead to decreased microglial activity (Odell and Sorgnard, 2008).

It is apparent that the number of viable cells was more in SCI+MF rats, which is supplemented by increased angiogenesis at the lesion site. VEGF is a potent stimulator of angiogenesis and a mediator of vascular permeability (Herrera et al., 2009). VEGF is also considered to be neuroprotective and thus may be a critical mediator in the recovery after SCI (Herrera et al., 2009; Svensson et al., 2002). We observed a significant increase in VEGF expression in SCI+MF group, this enhanced angiogenesis seems to have contributed enormously to behavioral and morphological recovery in them.

In conclusion, the present study provides a mechanistic overview of the ELF-MF potential in promoting recovery and in the management of SCI. It has opened up new vistas for more experimental cellular and molecular evidences with translational aspects.

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#### **Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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