

Therapeutic Potential of Bone Marrow Derived Mesenchymal Stem Cells in Modulating Astroglyosis of Surgical Induced Experimental Spinal Cord Injury

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Abstract

Background: Spinal cord injury (SCI) unsuccessful regeneration was due to glial scar development. It was a major obstacle to axonal restoration. Safe therapeutic intervention by the use of bone marrow derived stem cells (BMMSCs) transplantation applied in the present study could reduce spinal disability. **Material and methods:** Forty male albino rats were divided into four groups: GI: negative control (n = 10 rats); GII: positive control after SCI (n = 10 rats); GIII: SCI + BM – MSCs intravenous injected and GIV: SCI + BM – MSCs intra lesion injected (n = 10 rats in each group). The samples were taken from spinal cord tissues around the region of injury and were subjected to histological, immunohistochemical assessment. RNA extraction and real time PCR for detection of nerve regeneration and astrocyte response to the injury were also performed. **Results:** Clinical improvement occurred by the enhancement in the Basso, Beattie and Bresnahan (BBB) score after SCI. Histological examinations showed positive regenerative responses in GIV compared to GIII. **Conclusion:** BM-MSCs transplantation has a promising role in enhancing the microenvironment for nerve regeneration through stumbling the glial scarring formation and inflammatory response after chronic spinal cord injury especially by using intra-lesion route injection.

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Keywords

Spinal Cord Injury—Bone Marrow Mesenchymal Stem Cells, Rats and Glial Tissue

1. Introduction

In the last couple of years, spinal cord injury (SCI) was the most devastating ailment afflicting males. A complete injury results in: permanent paralysis with complete loss of sensation in the affected limbs and trunk, as well as loss of bowel, bladder, and sexual functions [1].

SCI does not only cause disabilities. It also has a deep impact on the social and economic prospects of the individual and the whole community. The high costs of treatment and prolonged hospitalization are an economic load on individual, the family and the society [2].

The main cause for SCI especially at a young age was found to be accidents. As of now, there is no established therapeutic intervention capable of restoring significant neurological functions after SCI [3].

Recent advances in stem cell research have bestowed great hope amongst researchers for the development of new treatments for many serious diseases. Finding out the potential of these cells demands significant scientific and medical evidence to derive beneficial ways which may then be offered as a clinical alternative to other existing present therapies [4].

SCI is followed by secondary events including a strong inflammatory reaction which results in secondary tissue destruction and additional loss of the tissue function [5]. The unembellished lesions become dominated by the deposition of scar tissue composed of connective tissue and fluid-filled cysts, surrounded by dense, astroglial scars [6].

Several studies on experimental animals exhibit how this scar represents a physical and molecular barrier, which isolates the lesion site from adjacent, healthy tissues. Meanwhile, it is also considered a center for the lack of axonal regrowth after injury [7].

In our study, we aimed at focusing on the role of BM-MSCs and some growth factors in increasing or decreasing SCI.

Under normal physiological conditions, there is a dynamic balance between synthesis and degradation of tissue using the help of Matrix Metalloproteinases (MMPs)—a family of proteolytic enzymes—which is largely controlled by chemokines and cytokines secreted by inflammatory cells such as: Transforming Growth Factor- β (TGF- β) [8].

The role of TGF- β in scar formation post-spinal cord injury explains why we use it as a parameter to observe the healing effect pronounced after BM-MSCs injection [9] [10].

In previous experiments of SCI, TGF- β isoforms were shown not only to be involved in the early inflammatory response, but also to play an essential role in the formation of the scar at the lesion site. However, the extent to which experimental SCI induced changes of TGF- β expression, reflecting the events taking place following human SCI; remains unknown. Thus, the present investigation was undertaken to determine the pattern of TGF- β expression in samples, taken from animals of different groups following severe SCI and treatment [11]-[13].

It was postulated that the functional recovery after SCI is not simply a significance of the mechanical destruction of tissue, but also ascribed to the appearance of complex secondary events leading to the regenerative failure of injured axons and formation of a glial scar.

The glial scar consists mainly of reactive astrocytes that arrest axon regeneration by secretion of chondroitin sulfate proteoglycans (CSPGs) which chemically arrest the regrowth [14].

The glial scar formation took place due to astrocyte migration in the area of the lesion [15] [16]. The proliferative action of astrocytes depends on: actin cytoskeleton mediated by the small GTPases [17] [18], integrity of the intermediate filament including vimentin and glial fibrillary acidic protein (GFAP), cell adhesion [19] [20], and water invasion through the cells' plasma membrane [21].

Matrix metalloproteinases (MMPs) is a family of zinc-dependent endopeptidases that can destroy a variety of protein constituents in the extracellular matrix (ECM) and thus remodel per cellular microenvironment for cell translocation [22]. MMPs modulate numerous pathological and tissue-repair processes in the nervous system; particularly those that require cell migration [23].

In the injured spinal cord, it is likely that MMPs may exert both constructive and disadvantageous effects, depending upon which members of the family are involved and where they are actively expressed [24] [25].

Many investigators observed that MMP-2 and MMP-9 are expressed in reactive astrocytes after SCI, raising

the possibility that these MMPs may participate in the formation of the glial scar. Recent studies have shown that astrocyte migration is modulated by MMPs [26] [27].

The multipotent properties of MSCs and their remarkable malleability -to the extent that they can differentiate into lineage other than the tissue origin-sorted them into a suitable choice for studying their therapeutic effects in different injuries [28].

2. Materials and Methods

Forty male Albino rats were of 6 weeks old, weighing between 200 and 250 g. Rats were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions. All experimental animals have ethical approval from the institutional animal care committee. They were subjected to a normal cycle and allowed to balance diet and water. All experimental protocols followed the guidelines of the Animal Committee of the Faculty of Medicine of Cairo University.

2.1. Experimental Design

Rats were divided into the following groups:

- 1) G I (Control group): 10 rats not exposed to SCI and served as -ve control.
- 2) GII (Spinal cord Injured group): 10 rats have surgical induced SCI and served as +ve control.
- 3) GIII (Spinal cord injury/BM-MSCs Intravenous group): 10 rats have surgical induced SCI. The rats were infused with 10^7 MSCs/rat intravenously (through tail vein) and scarified after 6 weeks.
- 4) GIV (Spinal cord injured/BM-MSCs Intra-lesion group): 10 rats have surgical induced SCI. The rats were infused with 10^7 MSCs/rat intra lesion and scarified after 6 weeks.

All rats were sacrificed and spinal cord tissue was harvested for histopathological, immunohistochemical examination and real time PCR analysis.

2.2. Induction of Spinal Cord Injury

We performed surgical SCI induction according to Erbayraktar, Gokmen and Yilmaz [29]. In brief, Aseptic T9-T10 laminectomy under anesthesia (pentobarbital sodium; dose 50 - 80 mg/kg IP; Hospira, Lake Forest, IL) was performed. Spinal cord contusion was induced using a weight-drop apparatus, where a guided 10 g rod was dropped 12.5 or 25 mm onto the exposed dura mater, representing moderate or severe SCI, respectively.

2.3. Isolation and Culture of BM-MSCs

Bone marrow cells were flushed from tibia and fibula of rat bones with phosphate-buffered saline (PBS) containing 2 mM EDTA. Over 15 ml Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY), 35 ml of the diluted sample was carefully layered, centrifuged for 35 minutes at 400 xg rpm and the upper layer was aspirated leaving undisturbed mononuclear cell (MNC) layer at the interphase. This MNC layer was aspirated, washed twice in PBS containing 2 mM EDTA and centrifuged for 10 minutes at 200 xg rpm at 10°C. The cell pellet was re-suspended in a final volume of 300 µl of buffer. Isolated MSCs were cultured on 25 ml culture flasks in minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS) and incubated for 2 hours at 37°C and 5% CO₂. Adherent MSCs were cultured in MEM supplemented with 30% FBS, 0.5% penicillin, streptomycin and at 37°C in 5% CO₂ in air [12]. Cultured MSCs was confirmed by morphology and Florescent Analysis Cell Sorting (FACS) by detection of CD29⁺ and CD44⁺ specific to MSCs.

2.4. Labeling Stem Cells with GFP

At 4th passage, MSCs were harvested and labeled with GFP (amaxa GmbH, amaxa Inc. Europe/World USA Scientific Support). MSCs were nucleofected using the MSC Nucleofector Kit and a plasmid encoding the fluorescent protein GFP. Cells were centrifuged, washed twice in serum free medium, pelleted and suspended in nucleofector solutions. A final concentration of $(4 - 5) \times 10^5$ cells/100 µl nucleofector solutions was applied. The sample was placed in cuvette of electroporation transfection instrument at program U-23 (for high transfection efficiency) or C-17 (for high cell survival). 24 hours post-nucleofection cells were analyzed by light and fluorescence microscopy. Transfection efficiencies of around 80% can be reached with GFP. Labeled cells were injected intravenously in rat with surgical induced SCI. After 6 weeks, spinal cord tissue was examined with a

fluorescence microscope (Leica, Germany) to detect and trace the cells stained with GFP.

2.5. Histopathological Examination

Spinal tissues were collected and divided into two sections. The first section was assessed for tracing of injected labeled cells with GFP. The second section was washed with PBS and fixed overnight in 40 g/L paraformaldehyde at 4°C. Serials (μm sections of the dissected spinal cord tissues were stained with hematoxylin and eosin (H&E) [30].

2.6. Immunohistochemistry

Immunohistochemical staining was performed on 5- μm , formalin-fixed, paraffin-embedded sections using the glialfibrillary acidic protein (GFAP) antibodies and anti myline basic protein (MBP) antibody at 1:50 dilution (DAKO, Carpinteria, CA). Antigen retrieval was performed in all cases by steam heating the slides in a 1-mmol/L solution of EDTA (pH 8.0) for 30 minutes. After blocking of endogenous biotin, staining was performed using an automated immunostainer (DAKO) followed by detection by using a streptavidin-biotin detection system (DAKO). Positive and negative control sections were used for each assay [31].

2.7. Computer Assisted Digital Image Analysis (Digital Morphometric Study)

Slides were photographed using Olympus digital camera installed on microscope with 1/2 X photo adaptor, using 40 X objective. The result images were analyzed by using (Russia) software with a specific built-in routine for stain quantification. 5 slides from each case were prepared, 5 random fields from each slides were analyzed. Integrated density of the immunostain was quantified and exported to an excel sheet for statistical analysis.

2.8. RNA Extraction

Tissue of all studied groups was homogenized and total RNA was isolated with RNAeasy Mini Kit (Qiagen) and further analyzed for quantity and quality with Beckman dual spectrophotometer (USA).

2.9. Real Time PCR (qRT-PCR)

For quantitative expression of TGF- β , MMP-2 and MMP-9 genes; the following procedure were assessed. 200 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C and 30 second at 72°C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the $\Delta\Delta\text{Ct}$ method. We used 1 μM of both primers specific for each target gene. Primers sequence and annealing temperature specific for each gene demonstrated in **Table 1**.

2.10. Behavioral Assessment

The functional status of the hindlimbs was assessed 6 weeks after SCI, using the Basso, Beattie and Bresnahan

Table 1. Primers sequence and annealing temperature specific for each gene.

Target gene	Primer sequence: 5'-3'	Gene bank accession number
TGF- β	Forward: TGCGCCTGCAGAGATTCAAG Reverse: AGGTAACGCCAGGAATTGTGCTA	NM021578.2
MMP-2	Forward: CTATTCTGTCAGCACTTTGG Reverse: CAGACTTTGGTTCTCCAACCT	NM031054.2
MMP-9	Forward: AAATGTGGGTGTACACAGGC Reverse: TTCACCCGGTTGTGGAAACT	NM031055.1
GAPDH	Forward: CACCCTGTTGCTGTAGCCATATTC Reverse :GACATCAAGAAGGTGGTGAAGCAG	NG028301.2

(BBB) locomotor rating scale [32].

2.11. Statistical Analysis

Measurement data are expressed as the mean \pm SD. Statistical analyses were performed with SPSS 12.0 software (SPSS, Chicago, IL, USA). For cell viability assays, analysis of variance (ANOVA) was applied to identify significant differences. BBB locomotor rating scores and cell counts at different time points were carried out using repeated measures analysis of variance. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Morphological, Phenotype Characteristics and GFP-Labeling Identification of Expanded Undifferentiated (BMSCs)

Under an inverted microscope (Leica, Germany). Undifferentiated (BMSCs) were typical of adherent spindle and fibrocyte-like at one week culture and reached 80% - 90% confluence at 2 weeks culture (**Figure 1(a)** and **Figure 1(b)** respectively). After plastic adherence selection, BMSCs were culture over four passages. Flow cytometric analysis of the MSCs at the 4th passage showed that these cells were positive for CD90 (96.2%) and CD 73 (98.7%) and negative of hematopoietic/ endothelial markers CD45 (0%) (**Figure 1(c)**). These results indicated that relatively purified (BM-MSCs) were isolated. Before cells injecting, GFP-labelled BM-MSCs were analyzed and confirmed for their green auto fluorescence for *in vivo* cells tracing (**Figure 2(a)**).

3.2. BMSCs Homing and Florescence Assessment

Frozen florescence microscopy of the section of the cell treated spinal cord tissue of all groups indicated that the GFP-transduced injected cells were assimilated within spinal cord tissue (**Figure 2(b)**).

3.3. Behavior Assessment

At 5th week after injection of mesenchymal stem cells, there are improvements in locomotor function ($p > 0.01$)

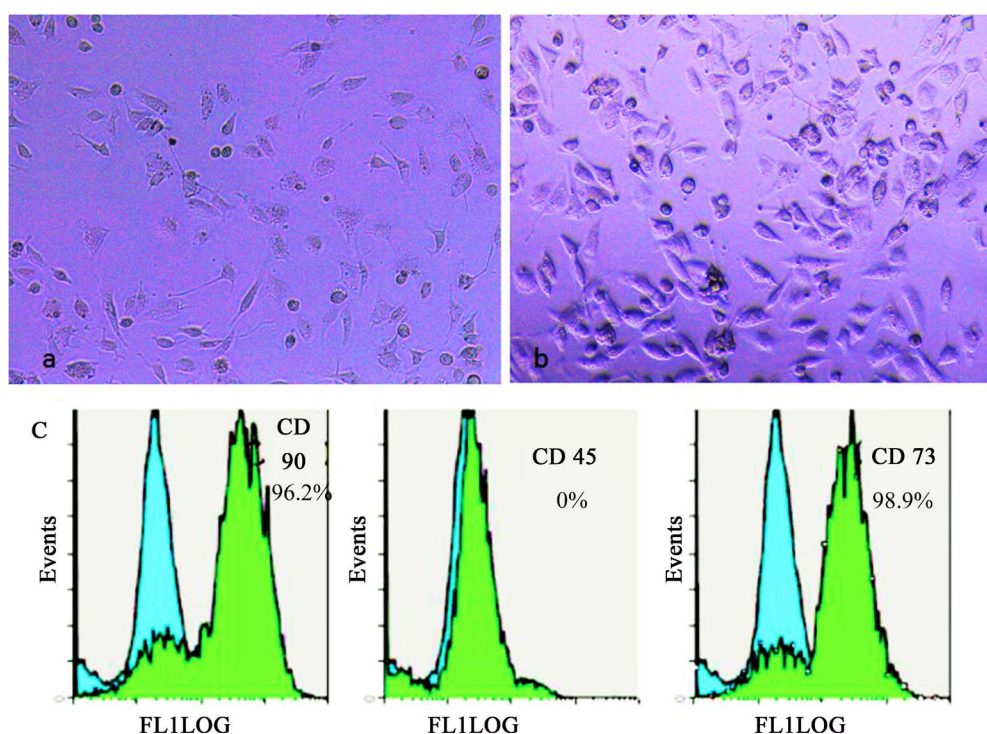


Figure 1. (a) BMSCs at one week of culture (30% - 40% confluent), (b) BMSCs at two weeks of culture (80% - 90% confluent), (c) it shows CD markers expressed for mesenchymal stem cells.

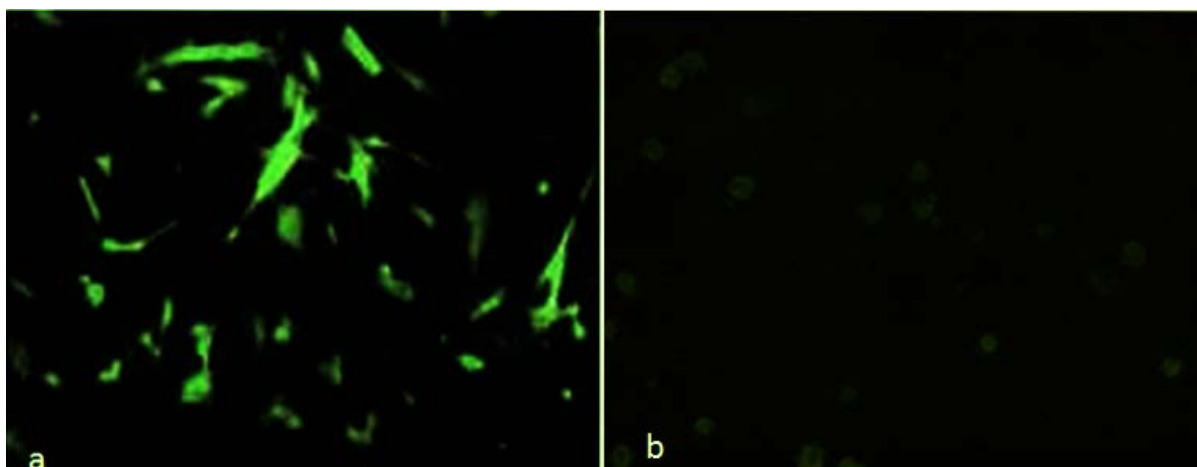


Figure 2. (a) Labeling of BMSCs with GFP fluorescent dye (*in vitro*), (b) homing of the injected labeled BMSCs by detecting GFP fluorescent dye in the rat spinal cord tissue (*in vivo*).

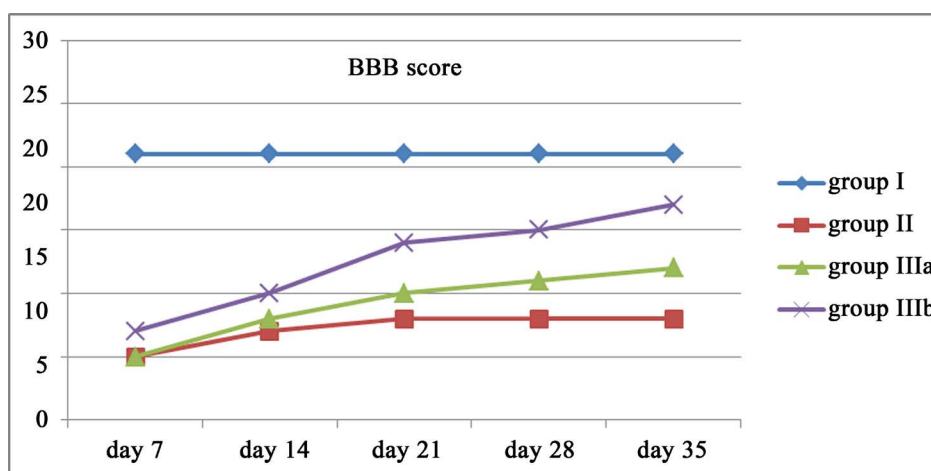


Figure 3. Showed histogram for the assessed motor and sensory functions of rats in all studied groups.

such that GIII and GIV showed a significant increase in BBB score compared with control group $p > 0.01$.

This trend indicates that mesenchymal stem cells injection is necessary for locomotor function recovery. At the 1st week after injection GIV showed significant increase in BBB score compared with GI but at 1st week after injection intravenous GIII showed significant decrease in BBB score compared with GI $p > 0.01$ which was gradually increased showing increased locomotor function at the fifth week post stem cells injection. Motor and sensory functions were demonstrated at **Figure 3**.

4. Histopathological Results

GI (Control group): Spinal cord sections showed normal architecture where the grey matter surrounded by white matter. The ventral horn with number of processes extends into the white mater. Large multipolar cells that are relatively large compared to the nerve fibers which surround these cells. The surrounding nerve fibers are neuroglial cells, although the cytoplasm of these cells is not visible (**Figure 4(a)**, **Figure 4(b)**).

Meanwhile Untreated GII: spinal cord sections 36 days post injury showed hemorrhagic emphasis in the grey and white matters with complete destruction of the tissue, this was represented in neuron dissolution giving liquefied appearance in grey matter (**Figure 5(a)**). Sections of this injured group showed as well eosinophilic (degenerating) neurons and pyknotic nuclei. Swollen cells and many vacuoles were demonstrated. Some dark shrunken neurons were also detected (**Figure 5(b)**). There was deceptive cellular destruction and necrosis as compared to all other groups (**Figure 5(c)**, **Figure 5(d)**).

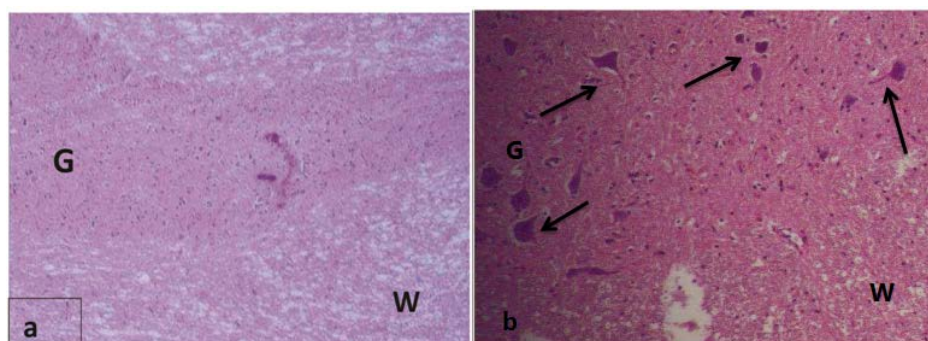


Figure 4. (a) Photomicrograph GI: showing normal view of the grey matter (G) of the spinal cord which is clearly surrounded by white matter (W). The ventral horn has a number of processes which extend into the white matter (H&E $\times 100$). (b) Photomicrograph GI: normal spinal cord tissue displaying number of multipolar cells (arrow) that are relatively large compared to the nerve fibers which surround these cells (H&E $\times 200$).

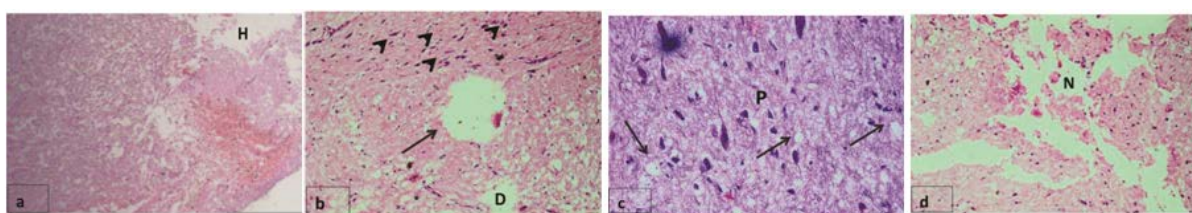


Figure 5. (a) photomicrograph of spinal cord tissue section of GII (untreated group): showing hemorrhagic focus (H) in the grey and white matters (H&E $\times 100$). (b) photomicrograph of spinal cord tissue section GII :showing neuron dissolution giving liquefied appearance in grey matter (arrow), degenerated areas and well eosinophilic degenerating neuron (arrow heads) (H&E $\times 100$). (c) Photomicrograph of spinal cord tissue section GII: showing swollen cells and many vacuoles (arrow) some dark irregular shrunken neurons with pyknotic nuclei (P) were demonstrated (H&E $\times 200$). (d) Photomicrograph of spinal cord tissue section GII: showing apparent cellular destruction and necrosis (N) as compared to all other groups (H&E $\times 100$).

Group III in which animals were intravenously treated with BM-MSCs spinal cord sections showed, apparently more glia cells and less dark neurons as compared to both normal control and untreated groups (**Figure 6(a)**). In addition, some areas showed inflammatory infiltrates, pyknotic cells surrounded by diffused glial cells with the presence of some vacuoles (**Figure 6(b)**).

Group IV animals were treated intra lesion with BM-MSCs. spinal cord sections showed normal structure of spinal cord tissue: the neurons are well observed, the outer limits of the grey & white matters are marked with no cystic spaces (**Figure 7(a)**, **Figure 7(b)**). However, a small hemorrhage aggregation was still observed in some sections as well as slight gathering of gliocytes were still denoted (**Figure 7(c)**).

5. Quantitative Genes Expression

Regarding gene expression, TGF- β , gene was highly expressed in GII and expression was significantly decreased after stem cells administration. Least expression was in GI. Nevertheless, there was significant difference between GIII and GIV where GIII showed higher expression than GIV. As regard to gene expressions of MMP-2 and MMP-9; they were highly expressed in spinal cord tissues in the GII, while significant decrease in their expression in both GIII & GIV compared to GII. MMP-9 and MMP-2 genes in GIV were significantly highly expressed compared to control group GI (**Table 2**).

6. Immunohistochemistry Results

Anti-GFAP stained spinal cord sections: spinal cord sections of GI exposed brown immunostaining in the cytoplasm and processes of astrocytes in all regions in white and grey matter. The immunoreactive cells were detected mainly in a regular form in both regions (**Figure 8(a)**, **Figure 8(b)**). GII showed highly significant immunoreactivity as compared to GI and all other groups; the immunoreactive astrocytes were detected mainly in all spinal tissues closely related to site of the lesion The dense packing of intensely GFAP-positive astroglial

processes between the spaces made it extremely difficult to comment on changes in astrocytic cell body density (Figure 9(a), Figure 9(b)). Comparing both GIII intravenous and GIV intra lesion treated groups to the untreated group, Anti-GFAP stained spinal cord sections showed moderate reactivity which was restricted to the place of lesion as going further away from the lesion site, no significant astrocytic reaction could be detected. There was no indication of increased GFAP immunoreactivity, and the astrocytic processes and cell bodies appeared normal in both grey matter and white matter tracts (Figure 10(a), Figure 10(b)). Still GIV showed better results rather than GIII. Table 3 showed the mean area percent of GFAP and myelin basic protein immunopositive cells in control and all experimental groups.

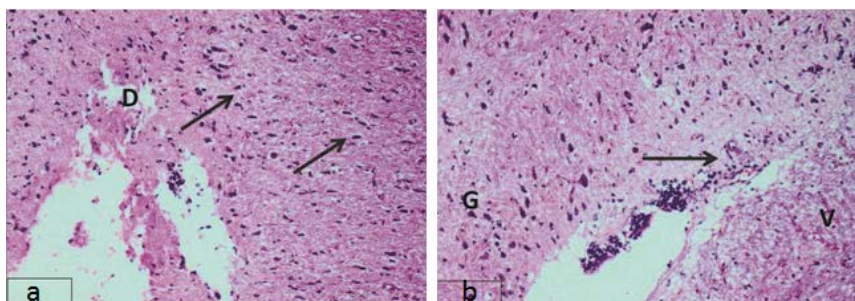


Figure 6. (a) photomicrograph of spinal cord tissue section of group III (intravenously treated with BMSCs) showing apparently more glia cells and less dark neurons (arrow) and degenerated areas (D) (H&E ×100). (b) photomicrograph of spinal cord tissue section of group III intravenously treated with BMSCs showing some areas inflammatory infiltrates (arrow), pyknotic cells surrounded by diffused glial cells (G) &with mild vacuolation (V) (H&E ×100).

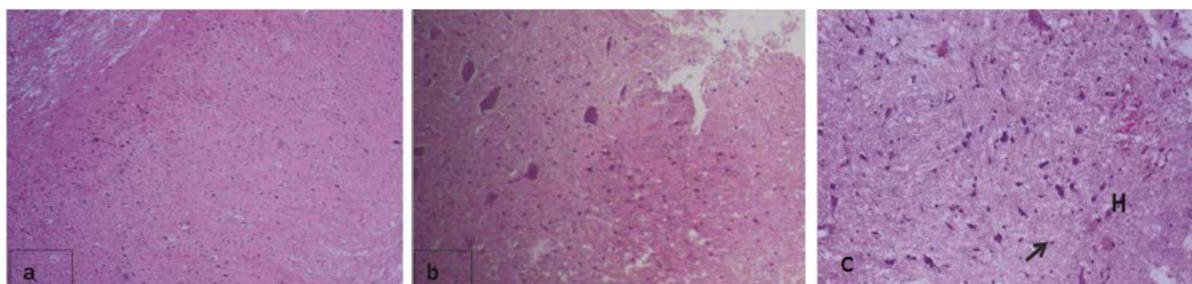


Figure 7. ((a), (b)) Photomicrograph of GIV: (intra lesion injection BMSCs treated group) spinal cord tissue sections showing normal structure the neurons are clearly visible, the outer limits of the grey & white matters are marked and there were no cystic spaces (H&E ×100 (a), H&E ×200 (b)). (c) Photomicrograph of spinal cord tissue section of group IV showing a small amount of tissue hemorrhage (H) a slight gathering of gliocytes (arrow) (H&E ×200).

Table 2. Showing quantitative expression of all studied genes.

Gene symbol	Mean ± SD	95% CI	F test	p value
TGF-β				
Group I	0.56 ± 0.17	0.45 - 0.66	7.54	0.001**
Group II	1.83 ± 1.28	1.02 - 2.65		
Group III	0.97 ± 0.55	0.61 - 1.32		
Group IV	0.71 ± 0.33	0.5 - 0.92		
MMP2				
Group I	0.69 ± 0.25	0.53 - 0.85	5.01	0.004**
Group II	1.51 ± 0.72	1.05 - 1.97		
Group III	1.14 ± 0.50	0.82 - 1.46		
Group IV	0.99 ± 0.52	0.66 - 1.32		
MMP9				
Group I	0.78 ± 0.25	0.62 - 0.94	6.64	0.001**
Group II	1.55 ± 0.60	1.17 - 1.94		
Group III	1.13 ± 0.45	0.84 - 1.41		
Group IV	1.03 ± 0.36	0.80 - 1.26		

p value highly significant (p < 0.001) -: p value significant (p < 0.05).

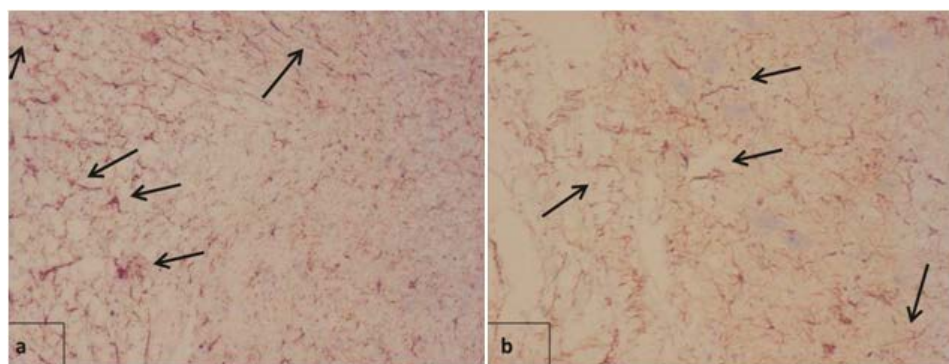


Figure 8. Arrows showing brown immunopositive staining in the cytoplasm and processes of astrocytes in all regions in white (a) and (b) grey matter (Anti-GFAP $\times 200$).

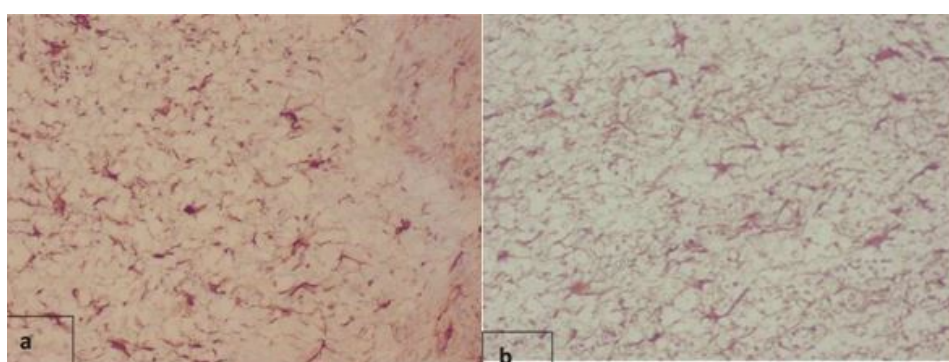


Figure 9. ((a), (b)) Photomicrograph of spinal cord transverse sections group II (untreated group) revealed apparently strong immunopositive staining detected mainly at the site of lesion (Anti-GFAP $\times 200$).

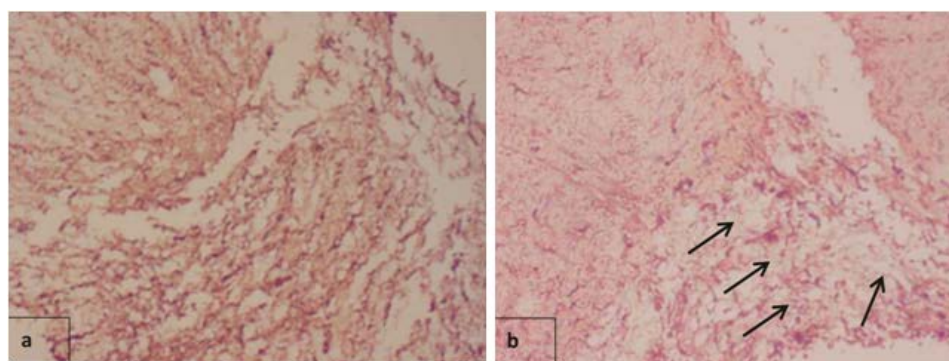


Figure 10. (a) Photomicrograph of spinal cord transverse sections of group III spinal cord sections showed moderate GFAP immunopositive staining in astrocytes (Anti-GFAP $\times 200$). (b) Photomicrograph of spinal cord transverse sections of group IV spinal cord sections showed less significant GFAP immunopositive staining (arrows) in astrocytes compared to group III (Anti-GFAP $\times 200$).

Table 3. Showed mean area percent of immunopositive cells in all studied groups.

	GFAP stain mean \pm SD	Myelin basic protein stain mean \pm SD
Intra-lesional group	113.2 \pm 26.32	146.74 \pm 4.87
Intra-venous group	148.45 \pm 18.3	128.74 \pm 10.18
Control group	195.44 \pm 18.23	103.05 \pm 10.7
F test	44.68	49.26
p value	0.001**	0.001**

p value highly significant ($p < 0.001$) -: p value significant ($p < 0.05$).

Anti-MBP staining established a decrease in density of myelin rings in the degenerating white matter in group II compared to normal control and both treated groups (**Figure 11(a), Figure 11(b)**). GII overall showed a heterogeneous and shapeless structure, which most likely represented degenerated myelin sheath.

On the other hand number of apparently intact MBP-positive myelin rings visible in GIII shown in (**Figure 12(a), Figure 12(b)**) with lots of myelin degenerated areas. Group IV showed deep positive MBP staining indicating the good sign of tissue healing to somehow approaching normal spinal cord tissue (**Figure 13**).

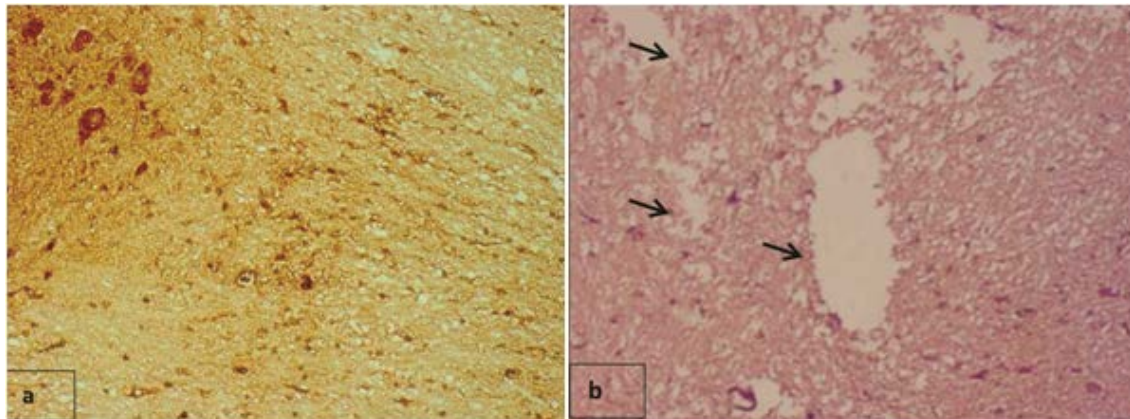


Figure 11. (a) Photomicrograph of spinal cord transverse sections of GI: showing positive myline basic protein staining with normal appearance of white and grey matter. Myline basic protein ($\times 200$). (b) Photomicrograph of spinal cord transverse sections of GII: showing negative myline basic stain and degeneration in white matter (arrows). Myline basic ($\times 200$).

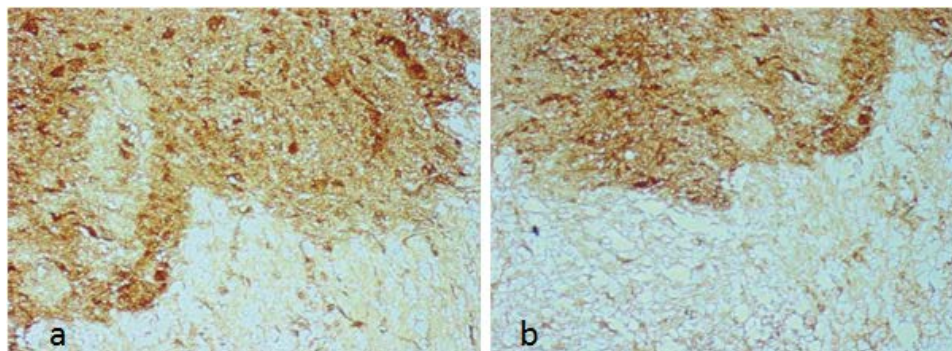


Figure 12. ((a), (b)) Photomicrograph of spinal cord transverse sections GIII: showing moderate reactivity of myelin basic indicating the reformation of myelin sheath. Myelin basic ($\times 200$).

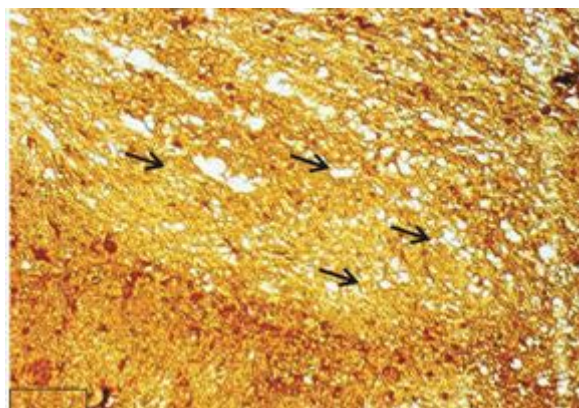


Figure 13. Photomicrograph of spinal cord transverse sections of intra Lesion treated group IV showing strong myelin basic reaction with some Weillerrings (arrows) were observed indicating the reformation of myelin sheath. Myelin basic ($\times 200$).

7. Discussion

Spinal cord injuries (SCI) result in everlasting incapacities. They persist throughout the person's life, with no chance of recovery. Spinal cord injury is not simply a significance of the initial mechanical destruction of tissue, but also accredited to the evolution of complex secondary events that contribute to early, along with, delayed cell injury. In our study, we aimed at focusing on the role of TGF- β , MMP-9 and MMP-2 involvement in increasing or decreasing tissue repair after induced SCI. Previous studies showed that TGF- β is a major mediator influencing collagen turnover, thus increasing the potential of scar formation in SCI [33].

TGF- β has already been known to possess receptors for alpha granules of platelets and macrophages, which in turn increase their accumulation at the sight of injury leading to scar formation [10]. This illustrates its highly expressed potential in untreated group II and its diminished expression in both treated groups III and IV in the present study.

Proteinases like matrix metalloproteinases (MMPs) is a family of extracellular zinc- and calcium-dependent endopeptidases [34], essential for remodeling of the extracellular matrix, tissue morphogenesis, and wound healing. In our study, MMPs are shown to be highly expressed in GII rather than other treated and control groups. However, some investigators perceived that excessive proteolytic activity of MMPs can lead to numerous pathologic conditions, including disruption of the blood-brain barrier [22] [35], and inflammation [36] [37]. MMP-9 reduces collagens, elastin, vitronectin, myelin basic protein and other substrates [38]. This protease is predominantly expressed by inflammatory cells, including macrophages, lymphocytes, and neutrophils, as well as endothelial cells [39] [40]. MMP-9 is expressed in microglia, astrocytes, and hippocampal neurons, and can be induced in astrocytes [41].

We have focused on the role of MMP-9 and MMP-2 because of their beneficial effects in glial scarring [43]. Moreover, the increased migration capacity of white matter astrocytes on laminin is mediated by MMP-9 and MMP-2, thus increasing the reformation of tissue scar [42].

Through our study, we find that in the injured spinal cord untreated GII there is an increase in the level of MMP-9, MMP-2 and GFPA while there is a decrease in the level of MBP. But in GIII and GIV, treated with stem cells, there is a decrease in the level of MMP-9, MMP-2 and GFPA while there is an increase in MBP.

Our findings are concurrent with previous studies where astrogliosis observed in the untreated tissues was attributed primarily to increased migration of astrocytes toward the site of a lesion [43] [44]. Astrocytes-derived from ependymal cells-migrate into the lesion to participate in glial scar formation after spinal cord injury in the adult [45]. Thus, proliferation of migratory astrocytes was involved in astrogliosis; considered to be non-suitable media for tissue regeneration.

The increase in GFAP in GII is due to the alterations in the intermediate filament cytoskeleton which have been occupied in astrocyte motility. Investigators had stated that astrocytes express GFAP as intermediate filament re-expressed in reactive astrocytes, which display abnormal morphology and severely compromised motile behavior [46].

The increase in MBP in our study was attributed to the repairing effect of stem cells which affect how MMPs facilitate the migration of bipotential oligodendrocyte-type 2 astrocyte progenitors on CNS myelin—a strongly inhibitory substrate that can be inactivated by proteolytic degradation mediated by metalloproteases [47]. Hence, decreased Weillerring and reformation of myelin sheath were well observed in group III and group IV in the present study.

Also we found that injection of BM-derived MSC improved the outcome of spinal cord injury as documented clinically by the BBB scoring system for spinal cord injured rats.

Our study results coincided with how other researchers stated that BMSC subpopulations, namely mesenchymal-stem cells (MSCs), accelerate motor recovery when grafted intraspinally in mice with moderate SCI [47]. Additionally, peripheral blood monocytes are required for tissue repair after SCI, likely after being primed into an anti-inflammatory phenotype in the choroid plexus. This hypothesis would then confirm recent data showing that BMSCs have beneficial effects in spinal cord injured mice by modulating the recruitment or the activity of alternatively activated macrophages, therefore, enhancing tissue repair and motor behavior [48].

A previous study conveyed that BMSCs can migrate into the injured part of spinal cord tissue and replace damaged and lost cells, resulting in improved motor function [49]. Another study reported that BMSCs can promote axonal regeneration throughout SCI lesions and improve functional recovery by restoring circuitry motor control [50]. Our results indicate that the immunoreactivity of MBP was stronger in the intraslesional GIV compared to the intravenous GIII and GII. These data suggest that BMSCs may provide a supportive matrix for

intraspinal axonal growth, which is essential for functional recovery from SCI [51] [52].

Further supporting of this finding was the BBB score elevation in GIV compared to that of GIII and GII. It indicated that the presence of BMSCs in the site of the lesion can increase BBB scores and improve functional recovery after SCI [53].

Transplantation of BMSCs has shown great promise in the treatment of SCI [52]. The identification of an optimal method of cell transplantation is essential for the successful treatment of SCI. Local injection [53] or intravenous injection [54] [55] are currently the most common methods of cell transplantation in animal models of SCI. Although direct injection can deliver a large number of BMSCs into the spinal cord tissue, the entry of the needle itself causes further injury. Intravenous injection and lumbar puncture methods are far less invasive, but have a low efficiency, as few transplanted cells ultimately migrate to the SCI lesion [56]. Therefore, increasing the number of cells delivered to the lesion after transplantation is the key to improving functional recovery after SCI [57]. In this study the injected MSCs may create an auspicious environment for functional recovery through modulating the post-SCI inflammatory response and by having neurotrophic activity [58] [59].

BM-MSC transplantation on the animal SCI models have been evidently confirmed by a large number of studies which employed several strategies including: pre-neural replacement, neural differentiation, neurotrophic gene transduction, glial cell co-transplantation, and tissue engineering [60].

The above mentioned results were interpreted by the observed movement in the treated rats hind limbs at the end of the experimental duration. Our surveillance was similar to how Song *et al.* [61] have demonstrated improvement of hind limbs' motor function after allograft of bone MSCs in rats with acute injury to their spinal nerve.

Yin *et al.* [62] have reported the antiapoptotic effect of MSC transplantation in adult rats after spinal cord ischemia-reperfusion injury. This suggests that MSCs may affect cell regeneration and repair, through control of apoptosis following SCI [63].

Gelatin sponge scaffold used has also been reported to support rat BM-MSC adherence, survival, and proliferation in the rat SCI model. The seeded scaffolds were shown to decrease inflammation, promote angiogenesis, and reduce cavity formation [64] [65].

8. Conclusions

BM-MSCs transplantation has a promising role in functional restoration of the spinal cord in rats after chronic spinal cord injury especially with the intralesional route of injection.

Sources of MSCs other than BM have also been known by researchers, such as adipose tissue amniotic fluid, placenta, umbilical cord blood, and in several fetal tissues including liver, lung, and spleen. These sources could be established in SCI

Also combination of anti-matrix metalloprotenases with stem cells will improve the result of MSC transplantation.

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