Original Article

Evaluation of Early Therapeutic Effect of Bone Marrow Mesenchymal Stem Cells Transplantation on Injured Spinal Cord in Albino Rats by Two Routes

Sherehan Fikry Othman[1], Mohammad Abd-El Monaem Mahmoud[1], Somia Hassan Abd-Aallah[2] and Hanan E. L. Mokhtar[1]

[1]Department of Human Anatomy and Embryology, [2]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Zagazig University, Egypt

ABSTRACT

Background: Stem cells have created hope for treatment of obstinate cases as injured spinal cord (ISC).
Objective: Aims to assess possible early therapeutic effect of bone marrow mesenchymal stem cells (BM-MSCs) on regeneration of ISC and to estimate effective transplantation routes in animal models.
Material and Methods: 62 adult male albino rats were used; two rats for BM-MSCs extraction and 60 rats were allocated into three groups. Control group: subjected to ISC, intralesional (IL) group: injected with BM-MSCs via intralesional injection and intravenous (IV) group: injected with BM-MSCs via intravenous injection at 3rd day post cord injury (PCI). Rats were examined to assess motor functional recovery. SC specimens were extracted for histological, morphometric, chemical, Immunological and statistical studies.
Results: Tracing BM-SCs of SC, (IL) group versus (IV) group showed higher number of engrafted stem cells at all studied ages with gradual decrease from 1st to 6th week PCI. The MSCs counts in (IL) versus (IV) groups were highly significantly increased at 1st, 3rd weeks but only significantly increased at 6th week. They were concentrated at injury site of SC in (IL) group then extended out rostrally and caudally. In (IV) group, they were dispersed over wider area including injury site indicating their capability to "home". The mean cavitation areas in treated versus control groups showed highly significant decrease and the smallest cavitation areas observed were in (IL) group. Also (IL) group showed highest neurotrophic factors expressions than other groups with increasing pattern from 1st to 3rd week then decreased at 6th week. Locomotor recovery scores in treated versus control groups showed highly significant increase with highest improvement in (IV) group.
Conclusion: This work concluded that early transplantation of BM-MSCs in ISC gave outstanding improvement. Intravenous route therapy provided highest locomotor recovery while intralesional route gave best histological and neuroprotective outcomes.

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Key Words: BM-MSCs, injured spinal cord, neurotrophic factors, transplantation routes.

Corresponding Author: Hanan E.L. Mokhtar, MD, Department of Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt, **Tel.**: +20 11195 32265, **E-mail:** lotfyann@gmail.com, HAMokhtar@medicine.zu.edu.eg

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INTRODUCTION

The belief that the central nervous system (CNS) is not capable of repair and regeneration like other tissues such as the skin or the liver has historically restricted the management of CNS disorders[1]. However, it has become apparent, over the past few years, that the adult CNS has the capability of repair and regeneration[2,3].

Neuronal replacement treatments are built on the belief that neurological function might be enhanced by presenting new cells that can differentiate and incorporate suitably to substitute the function of missing neurons. One mechanism that may play a key role in neuro-regeneration is cellular transplantation therapy[4].

Stem cell-based treatment is an encouraging method for the medication of various disorders of CNS including injury of spinal cord (ISC) which is a deterioration event that can cause severe motor and sensory loss. For transplantation therapy for ISC, progenitor and neural stem cells (NSCs) have been revealed to be particularly appropriate due to their ability to deliver an indefinite source of nerve cells for cell replacement[5,6], in addition to trophic maintain for endogenous neuro-regeneration[7-9].

The treatment of ISC has been extensively studied by transplantation of various kinds of cells. The adult stem cells of bone marrow are classified into two forms; mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs). In clinical trials, the cell population which have been highlighted and most investigated was the mesenchymal stem cells (MSCs). The therapeutic potency of MSCs is built on their ability to be easily isolated, cultured, harvested, directly implanted and differentiated into various cell types. Moreover, the most essential reason for their use was because of their low immunity and their releasing to biologic factors that reduce tissues damage[10-13].

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Transplantation of stem cells have created hope that they will be able not only to repair but also to rebuild damaged body tissues and regain their function[14]. This capability would be particularly suitable for injuries of spinal cord where enhancing caring has been the base rather than any try for repair or recovery of function[15,16].

The timing of stem cells transplantation in ISC is a significant factor, since the biology of repair in chronic damage varies from repair in acute injury[17]. The best route of administration BM-MSCs is still under search. Several groups have checked different routes of transplantation of BM-MSCs for spinal cord repair including; intralesional, intraventricular, intrathecal, and intravenous routes. Intravenous injection of BM-MSCs in rats with ISC was used in many studies which showed promising functional outcome with MSCs detected in the injured spinal cord[4,17,18]. However, few studies comparing these approaches have been issued[4,19].

This work aimed to evaluate possible early therapeutic effect of BM-MSCs on regeneration of spinal cord injuries (subacute stage). Also, to estimate the beneficial transplantation routes whether by intralesional (IL) or by intravenous (IV) injection in the adult male albino rats. The effect was measured by clinical evaluation of locomotor function, histological, chemical, morphometric and immunological changes. Finally, statistical analysis of results was followed.

MATERIAL AND METHODS

Animal models

Sixty-two adult male albino rats with average 300-350 gm body weight were obtained from animal house faculty of medicine, Zagazig University. The animals were housed under standard conditions of light and dark cycles with free access to food and tap water under controlled temperature (28ºC- 30ºC). The animals were fed a commercial diet and given water ad libitum. They were observed in this environment for seven days prior to surgery ensuring adequate adaptation. Animal housing conditions and all experimental dealings were done according to animal protocols of national institutes of health guidelines of animal care[20].

Experimental design

Two rats were used for extraction of BM- MSCs, the remaining 60 rats were randomly allocated into three groups (20 rats for each): as follows:

Group A: (positive control group): 20 animals of this group were subjected to surgical induction for injury of spinal cord (ISC) only.

Group B: (intralesional injected (IL) group): 20 animals of this group were subjected to ISC by surgery and then injected with 20 µl of 1.5×10^6 MSCs/µl of PKH26-labelled MSCs through intralesional area (IL route) at the 3rd day PCI.

Group C: (intravenous injected (IV) group): 20 animals of this group were subjected to ISC by surgery and then injected with same dose of PKH26-labelled MSCs by intravenous injection through the tail vein of rats (IV route) at the 3rd day PCI.

1-Induction of ISC

Rats from groups A, B, C, were deeply anesthetized by intraperitoneal injection of ketamine (7.5 mg/kg) and xylazine (60 mg/kg). After putting the rats in prone position on the surgical table, their dorsal area between neck and hind limbs was shaved and sterilized with betadine 10% solution. Under the surgical loupe magnification (Karl-Ziess 4X surgical loupe), the spinal cord was exposed by a midline skin incision over T8-T11 region of the vertebral column with laminectomy at the T8-9. Using a clip compression technique[21], a moderate contusion model SC was performed by clamping it with curved Sugita aneurysm clip (35g closing force) for 30 seconds. Following compression injury, the spinal cord was observed swollen with surface contusion after de-clamping. The deep and superficial muscles and subcutaneous layers were sutured, and the skin was closed with 4/0 prolene simple interrupted sutures using stainless-steel wound clips (Figure A). Enrofloxacin (5 mg/kg sc. injection every 12 hours) and, Ketoprofen (2mg/kg sc. injection every day) were given for 7 days after surgery for pre and postoperative care. Maintaining of body temperature persistent at 37 °C using warming pad was done during surgery and the recovery time. Manual emptying of the urinary bladder for removal of urine was done twice daily for 7–14 days PCI until the bladder reflex was well returned.

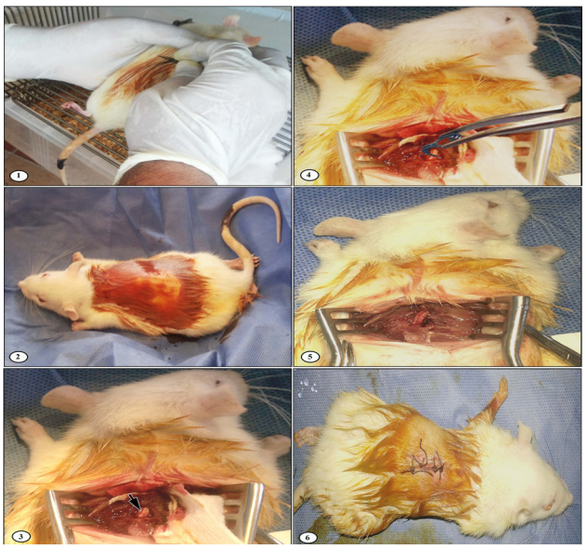


Fig. (A): Photographs of rats subjected to surgery for induction of ISC. [1]: showing shaving process of the dorsal area between the neck and hind-limbs using number 15 scalpel blade. [2]: showing the shaved area was painted with betadine 10% sterilizing solution. [3]: showing the spinal cord (arrow) exposed after careful T8 and T9 laminectomy. [4]: showing compression injury of the spinal cord using curved Sugita aneurysm clip for 30 seconds. [5]: showing the spinal cord looked swollen with surface contusion after de-clamping. [6]: showing suturing of the skin with simple interrupted sutures.

2- Assessment of motor function recovery

Five rats from each group following therapy by stem cell were examined once weekly by two viewers to evaluate the recovery of motor function in the experimental rats for six weeks through the Basso Beattie and Bresnahan (BBB) locomotor scale[22]. One of the frequently used scales to measure motor functional rehabilitation is the BBB motor scale. The scale of BBB score is an ordinary list of definitions built on enhanced functional activity. Scores ranging from 0 to 21 were analyzed, with higher scores reflecting higher levels of motor control.

An open-field box approximately 90 cm2 area was designed as a testing device. Its walls around the perimeter of box, should be between 7 and 10 cm tall. Rats were placed in the box and noticed by two inspectors who evaluated the motor function based on stability, paw placement during weight support and stepping, trunk and tail positions, joints movements of hind-limb, forelimb-hindlimb coordination, etc. The left and right sides of the subject were given different scores during four-minute observation.

3-Preparation, of bone marrow-mesenchymal stem cells (BM-MSCs)

A-Isolation, separation and culture of BM-MSCs

Preparation of MSCs was done in the unit of stem cells research at biochemistry department, faculty of medicine, Zagazig University according to the method of previous literatures[23]. After isolation of femurs and tibias from rats, they were sterilized in low glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) [Lonza Bioproducts, Belgium]. Their bony epiphysis was cut opened and flushed with 10 ml syringes including DMEM-FBS into a 100 mm Petri dish with 1ml of heparin (heparin 2000 IU/0.2 ML). According to the previous protocol[24], the bone marrow cells were re-suspended in basal medium and was cultured in complete culture medium; high glucose DMEM, 4.5 g/L glucose with L-glutamine, containing 1% penicillin streptomycin -10% FBS, -Amphotericin B Mixture (Lonza Bioproducts, Belgium). The cells were incubated in Co2 incubator at 37°C in 5% humidified Co_2 (Heraeus, Germany). To remove non adherent cells, the media was changed every 3 days for 12- 14 days, until large colonies of mononuclear cells were reached 70-80 % confluence as confirmed by the inverted microscope. When large colonies were developed (80-90% confluence), the cultures were washed twice with PBS. To dislodge the adherent cells from the flask, the cells were trypsinized with 0.25% trypsin/ethylene diamine tetra acetic acid (EDTA) (Lonza Bioproducts, Belgium). The third passage of MSCs was utilized in this experiment. Before culture, the MSCs were tested to detect their viability by trypan blue test (0.4% trypan blue/PBS)[25]. Counting of stem cells in *vitro* was done by the hemocytometer[26].

B-Determination of immune-phenotype of the cultured MSCs by flow cytometer

For determination of their phenotype as MSCs by expressing their specific cell surface antigen markers, the cells were detached by 0.25% trypsin-EDTA solution and suspended at a concentration of 1×10^6 cells per milliliter. The cells were immuno-labelled and incubated with fluorescein iso-thiocynate (FITC)-conjugated and phycoerythrin (PE)-conjugated monoclonal antibodies against CD 34, CD 105 and CD 44 (Flanklin Lakes, NJ, USA). A flow cytometer machine was used for evaluating the positive expression of CD105 and CD 44 and the negative expression of CD 34 surface markers[27].

4-Transplantation of PKH26-labeled BM-MSCs by two routes

Before stem cells transplantation, labeling of stem cells with red fluorescent membrane-intercalating dye PKH-26 was done (Sigma-Aldrich, St. Louis, USA) and observed under fluorescent microscope according to the manufacturer's protocol[4,28]. Six rats of control group were left untreated. However, 6 rats (2 at each time point) of (IL) group at 3rd day PCI, were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital, with intramuscular injection of 0.8 mg/kg atropine sulfate. To prevent extra cord damage, the original wound was carefully reopened. Twenty µl of 1.5×10^6 MSCs/µl were injected slowly over a period of 5 min (as a single injection approach) through a 10-µl Hamilton syringe connected to a 25-gauge cannula and implanted through the dura mater to the lesion area at a depth of 2 mm (Figure B). The wound was closed layer by layer. However, the rats of (IV) group (n=6 rats, 2 at each time point) were injected by PKH26-labelled MSCs via (IV) route as a single dose at 3rd day PCI. A 26-gauge scalp vein needle connected to a 25-µl Hamilton syringe was inserted into the vasodilated tail vein of rat by applying a heat lamp or warm towel over the tail for 1–2 min. The same previous dose of MSCs/µl was injected slowly over a period of 10 min after the release of venous blood, followed by injection of 10 µl normal saline.

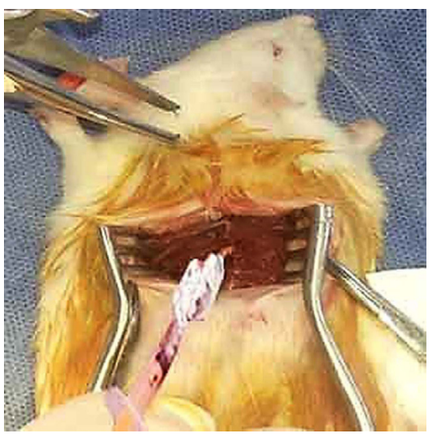


Fig. (B): Photograph showing the method of intralesional injection of stem cells at the site of lesion (to protect stem cells from light, the syringe wrapped in aluminum foil).

5-Tracing of transplanted PKH-26-labeling of BM-MSCs in vivo

After stem cells transplantation, 6 rats/treated group (2 rats at each time point) were sacrificed and specimens from spinal cord and spleen were extracted at 1st, 3rd, and 6th weeks PCI for fluorescence examination. Specimens from spleen and from injured spinal cord (10 mm long segment centered at the injury site) were fixed in 10 % formalin and embedded in paraffin. Six sagittal sections (50 µm thick) of spinal cord and spleen were taken from each rat at 500 µm intervals and were examined by Leica IX71 inverted fluorescent microscope coupled to Leica DP72 digital camera (Leica, Tokyo, Japan). On each sagittal section, six non overlapping fields at ×200 magnification were randomly carefully chosen and high-definition (HD) images were taken by digital camera. PKH-26 positive cells were counted in each image by Image J software. The total number of cells in each section was calculated as the sum of cells counted in the six fields. Then, the mean number of counted cells for each rat were recorded from 6 sagittal sections of the spinal cord and that of the spleen. Then, the mean number of counted cells for each treated group (2 rats/ experimental week) was recorded and compared statistically at 1st, 3rd, and 6th weeks PCI.

6-Evaluation of neurotrophic factors expressions by Reverse Transcription Polymerase Chain Reaction (RT-PCR) Method[29,30]

To estimate the degree of expression level of neurotrophic factors as neuronal growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in the tissue of spinal cord, RT-PCR test was used. The excised segments of injured spinal cord after transplantation from 9 rats/group (3 at each time point) were processed into the following steps of semi-quantitative RT-PCR protocol method[31]. A-Total RNA was extracted from spinal cord tissue homogenate using RNeasy Mini Kit according to manufacture's instructions (Qiagen, California, USA). B- Determination of concentration and purity of extracted RNA were done using Spectrophotometer (Quawell, California, USA). The extracted RNA concentration was >50 µg/ml according to QuantiTect Reverse Transcription Kit manufacturer's instructions (Qiagen, California, USA). and its purity ≥ 2.0. C- Reverse Transcription of RNA into complementary DNA (cDNA) was done by two-step method. This method involves creating cDNA first by means of a separate reverse transcription reaction and is then amplified through PCR. After genomic DNA (gDNA) elimination, the RNA sample was equipped for reverse transcription using master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix. D- The Semi-quantitative RT-PCR was done for expression of mRNAs of target genes (BDNF) & (NGF) in cord tissue with the housekeeping gene, GAPDH (glyceraldehyde-3-phosphate dehydro-genase) as an internal reference. The reaction was performed using Taq PCR Master Mix (QIAGEN, USA). Gene-specific primers were used for amplification of the target and the housekeeping genes from purified cDNA produced from previous step. These Primers as shown in (Table 1) were manufactured by In*vitro*gen Company (California, USA). The following thermal cycling (Perkin Elmer DNA thermal cycler 480, England) was planned to perform 35 cycles of amplification (DNA denaturation at 94 °C for 40 s, annealing at 59–62 °C for 40 sec (as seen in primer table), extension at 72 °C for 1 min) with initial denaturation at 94 °C for 3 min, and final extension at 72 °C for 10 min. E- Agarose gel electrophoresis reagents were established according to previous protocol[32]. PCR products were electrophoresed on 1.5% agarose gels. Assessment of BDNF and NGF gene expression was done by comparing the band intensities scoring system by using UV transilluminator in different groups in relation to the control group as follows; + (normal), + + (mild), + + + (moderate), + + + + (high) and + + + + + (very high).

Table 1: List of primers, sequence and annealing temperatures (Ta) of each primer and product size are shown (In*vitro*gen, California, USA).

Gene	Primer type	Sequence (5' to 3')	Ta	Product size
BDNF	Forward	5′-GCGGCAGATAAAAAGACTGC-3′	60°C	238 bp
	Reverse	5′-GCCAGCCAATTCTCTTTTTG-3′		
NGF	Forward	5'-CAGACCCGCAACATCACTGTA-3'	62°C	189 bp
	Reverse	5'-CCATGGGCCTGGAAGTCTAG-3'		
GAPDH	Forward	5'-AGTTCAACGGCACAGTCAAG-3'	59°C	496 bp
	Reverse	5'-TACTCAGCACCAGCATCACC-3'		

7-Histopathological and morpho-metric studies of the spinal cord[33]

Six weeks after surgery, 5 rats were sacrificed from each group and the injury site in the spinal cord was dissected and post-fixed in 10% buffered formalin. After being dehydrated using a series of ethanol washes, spinal cord tissues were embedded in paraffin and cut into 5-µm sections. The injury site of SC in each rat was sectioned into ten sequential transversal sections with an interval of 300 um. Sections were deparaffinized in xylene, hydrated using a series of ethanol washes then stained with Hematoxylin and Eosin. Slides were then examined by Optika B-350 light microscope coupled with Optika digital camera (Optika, Italy) at the pathology department, Faculty of medicine, Zagazig University. Cavitation areas (lytic areas with cystic cavity formation caused by compression injury of the spinal cord) in each slice picture was measured using the Image J software (National Institute of Health,

Bethesda, MD, USA) and then for each rat was determined (Figure C).

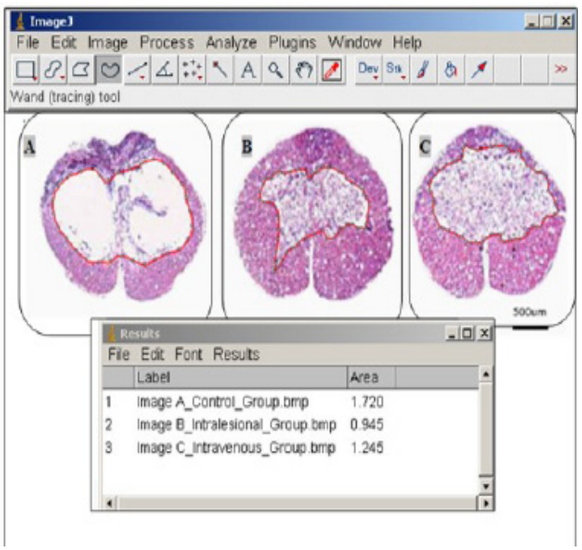


Fig. (C): A screenshot from Image J software during calculation of cavitation areas in 3 images of the spinal cord.

8- Statistical analysis

variable numbers and ratios data of this study were available as means ± standard deviations after being collected, arranged, summarized and then analyzed using statistical package of social sciences (SPSS) version 19 (SPSS Inc., Chicago, Illinois, USA). The results were evaluated by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. In addition, comparison of groups regarding relative mRNA expression level by the scoring system (+, ++, +++, ++++, +++++) used for gel band intensity was done by the Chi square test. A Probability (*P value* < 0.05) was considered statistically significant.

RESULTS

Results of stem cells

Morphology of cultured stem cells: (Figure 1)

Proliferation of mesenchymal stem cells during culture was demonstrated by repeated examination under inverted microscope. At 3rd day of cell culture, the stem cells appeared circular in shape (Figure 1a). At 5th day of culture, MSCs were characterized by their adhesiveness, increasing number and showed different sizes and shapes (may be small rounded, oval, & elongated) and their nuclei were apparent (Figure 1b). However, from 10-14 days of culture, the cells form small colonies progressively enlarged to reach 80-90 % confluence (Figures 1c-1d). By trypan dye blue test, the viable cells appeared with variable sizes and intact cell membranes while the dead cells showed disturbed cell membrane and stained blue in color. The cell counting was done by hemocytometer (Figure 1e).

Immunophenotype of cultured MSCs by flow cytometer

To confirm their phenotype as mesenchymal stem cells (MSCs), flow cytometric analysis of cell surface markers of the cultured cells was used. MSCs have been characterized by their positive expression of CD105 and CD44 and by their lack of expression of CD34 (negative CD 34) surface markers. This surface markers expression pattern corresponds to BM- MSC (Figure 2).

The counting and distribution of PKH26-labeled BM-MSCs in spinal cord and spleen

By tracing the transplanted PKH26-labelled MSCs of spinal cord by fluorescent microscopic examination, they were detected around the injury site of the spinal cord in the 2 treated groups but with variable degrees. The (IL) group showed higher number of engrafted stem cells in the spinal cord comparative to the (IV) group at all studied ages PCI (Figure 3). In contrary in the slpeen, the (IV) group showed higher number of engrafted stem cells if compared to the (IL) group at all studied ages PCI (Figure 4). As regarding the distribution of transplanted PKH26-labelled BMSCs inside the spinal cord of the (IL) group; at 1st week PCI, the transplanted BMSCs were concentrated at the site of injury of spinal cord. However, at 3rd and 6th weeks PCI, the cells were observed to extend out the injury site in rostral and caudal directions (Figure 5). On the other hand, in the (IV) group, BMSCs of the spinal cord were dispersed from the start over a wider area of the spinal cord including the injury site (Figure 6). By observing the six high magnification field images of each experimental week, the numbers of PKH26-labelled MSCs in the 2 treated groups were seen gradually decreased from 1st to 6th weeks PCI with their marked reduction at the 6th week PCI (Figures 7,8).

Statistically, the counts of transplanted BM-MSCs of injuried spinal cord in (IL) versus (IV) groups were highly significantly increased (P=0.001) at 1st, 3rd weeks but only significantly increased (P=0.041) at 6th week. Percentages of cell counts detected at the 6th week in comparison to their count at the 1st week PCI were 43% and 42% for (IL) and (IV) groups of spinal cord respectively (Table 2 and Histogram 1). However, the engrafted stem cell counts in spleen showed high significant difference (P=0.001) between the 2 treated groups at all studied weeks PCI with higher number of stem cells observed in (IV) group than (IL) group (Table 2).

Expression of NGF, BDNF mRNA in the spinal cord

By Bonferroni's post hoc analysis after one-way ANOVA, in all studied ages, the mean relative optic densities of NGF and BDNF bands showed statistical significant increase (P<0.05) between; (IL) group versus control group and (IV) group versus control group. The (IL) group showed the highest NGF and BDNF expressions than other groups in all studied weeks with increasing pattern of expression from the 1st to the 3rd week then

the levels were decreased at the 6th week (Tables 3,4 and Histograms 2,3).

By using UV transilluminator, the NGF band intensities were higher in the 2 treated groups versus control group. At 1st w PCI, the NGF band intensity level in (IL) group was high (++++), in (IV) group was moderate (+++) in comparison to control group (+). However, at 3rd week PCI, the band intensity level was very high (+++++) in (IL) group, high (++++) in (IV) group comparative to control group (+). At 6th week, NGF band intensity level in (IL) group was moderate (+++), in (IV) group was mild (++) compared to control group (+) (Figures 9,10). As regard NGF relative expression level by statistical analysis using the Chi square (X2) test, there was statistically significant difference in 2 treated groups versus control group in all different ages [(X^2 =13.5, *P*=0.031 at 1st w) (X^2=13.5, *P*=0.020 at 3rd w) (X^2 =14, *P*=0.042 at 6th w)] (Table 5).

As regard BDNF band intensities by using UV transilluminator, there were higher expression in the 2 treated groups in comparison to control group. At 1st week, the BDNF band intensity level in (IL) group was moderate (+++), in (IV) group was mild (++) in comparison to control group (+). At 3rd week, BDNF band intensity level was very high (+++++) in (IL) group, high (++++) in (IV) group in comparison to control group (+). At 6th week, BDNF band intensity level was high (++++) in (IL) group, moderate (+++) in (IV) group in comparison to control group (+) (Figures 11,12). Statistically using the Chi square (X2) test, the BDNF relative expression level showed statistically significant difference between stem cell-treated groups versus control group (X^2=13.5, *P*=0.046 at 1st w) (X^2=13.5, *P*=0.015 at 3rd w) (X^2=13.5, *P*=0.030 at 6th w) (Table 6)].

Histopathological examination of the spinal cord in three groups

By histological examination of midsagittal and axial sections of injured spinal cord after transplantation, areas of cavitation with variable degrees were apparent in the centers of site of lesion in all groups. The two stem cells-treated groups showed notable small cavitation areas as compared to control group. The (IL) group had the smallest cavitation area comparative to other groups. The mean cavitation area was 1.79 ± 0.08 mm2, in control group, 1.20 ± 0.27 mm2 in (IL) group and 1.47 ± 0.28 mm2 in (IV) group as measured by Image J software (Figure 13).

As regard statistical analysis of histopathological results, the mean cavitation areas in (IL) and (IV) groups versus to control group showed highly significant decrease (*P*=0.001) (*P*=0.002) respectively. However, the mean cavitation area in the (IL) group versus (IV) groups showed only statistically significant decrease (*P*=0.011) (Table 7, Histogram 4).

Functional locomotor assessment

As regard functional locomotor assessment, BBB (0) score indicated complete hindlimb paraplegia with flaccid position in all injured rats immediately after the operation. Gradual locomotor recovery of hindlimb with variable degrees was detected in rats of all groups during the six-week periods of observation. The BBB scores in stem cells treated (IL and IV) groups were higher than those in the control group. The (IV) group recorded the highest BBB scores of improvements compared to other groups during all weeks of observation period as detected from limb positions with weight bearing ability (line chart 1, Figure 14). Statistically, the (IV) group versus control group showed significant increase (*P*<0.05) in BBB scores except at 3rd and 4th week was highly significant increased (*P*=0.002). However, the BBB scores of (IV) group versus (IL) group, showed insignificant differences at 1st week (*P*=0.054) but revealed significant increase (*P-value* <0.05) from 2nd week thereafter. In addition, the (IL) group versus control group recorded statistically significant increase in BBB scores from the 3rd week thereafter (Table 8 and Histogram 5).

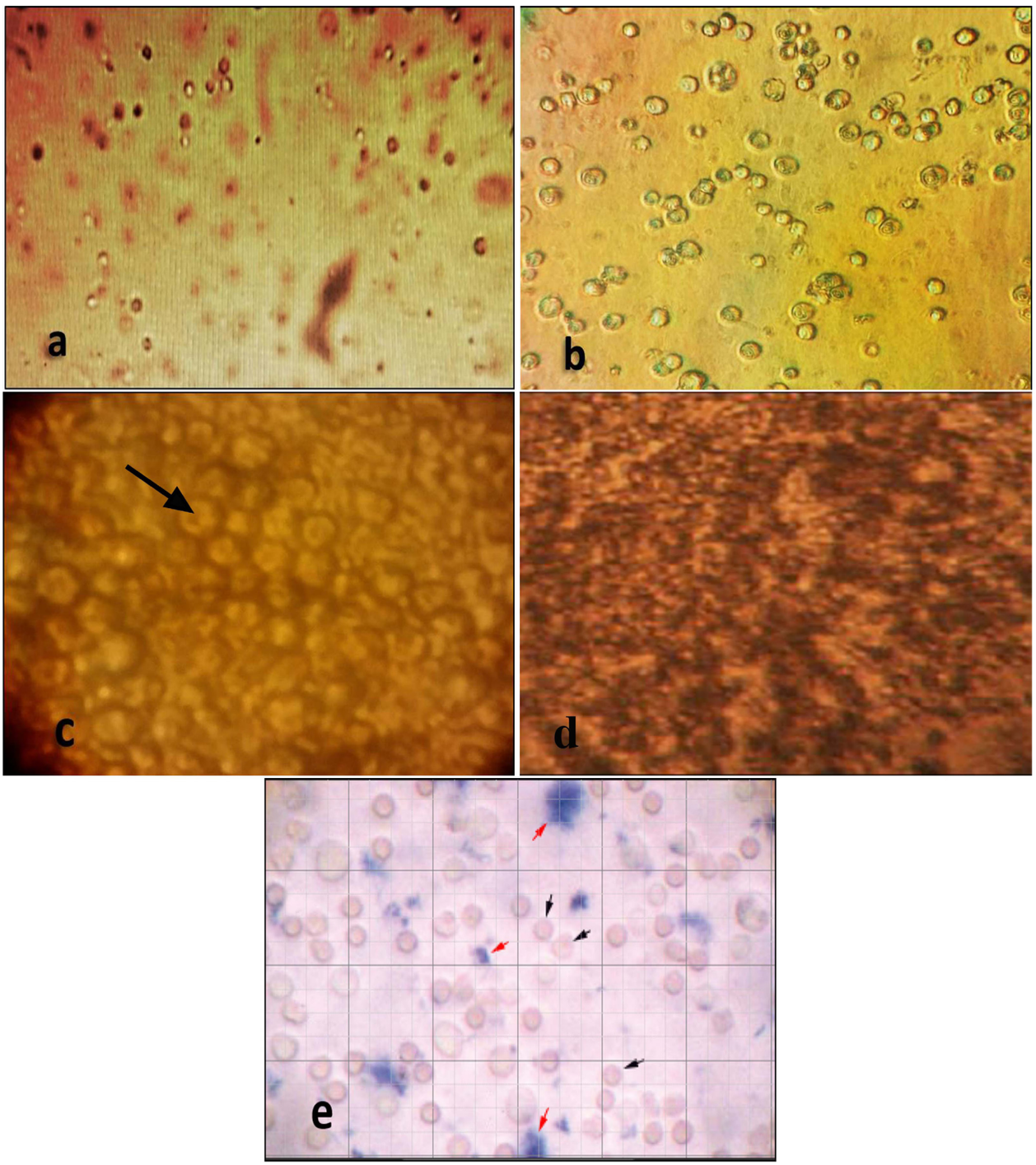


Fig. 1 (a-e): photomicrographs showing the proliferation of mesenchymal stem cells during their culture. [a]: At 3rd day of cell culture, the cells were circular in shape (× 100). [b]: At 5th day, cells are increased in number, adherent and show different sizes and shapes (oval, rounded & elongated) and their nuclei are noted (× 200). [c]: At 10th day of cell culture, the proliferated stem cells form cell colonies (arrow). (×200) [d]: At 14th day of cell culture, the stem cells show colonies with 90% confluence (×100). [e]: Trypan blue test showing the viable cells with variable sizes and intact cell membrane (black arrows) while dead cells have disturbed cell membrane and get blue color (red arrows). Cell counting by hemocytometer is done (× 200).

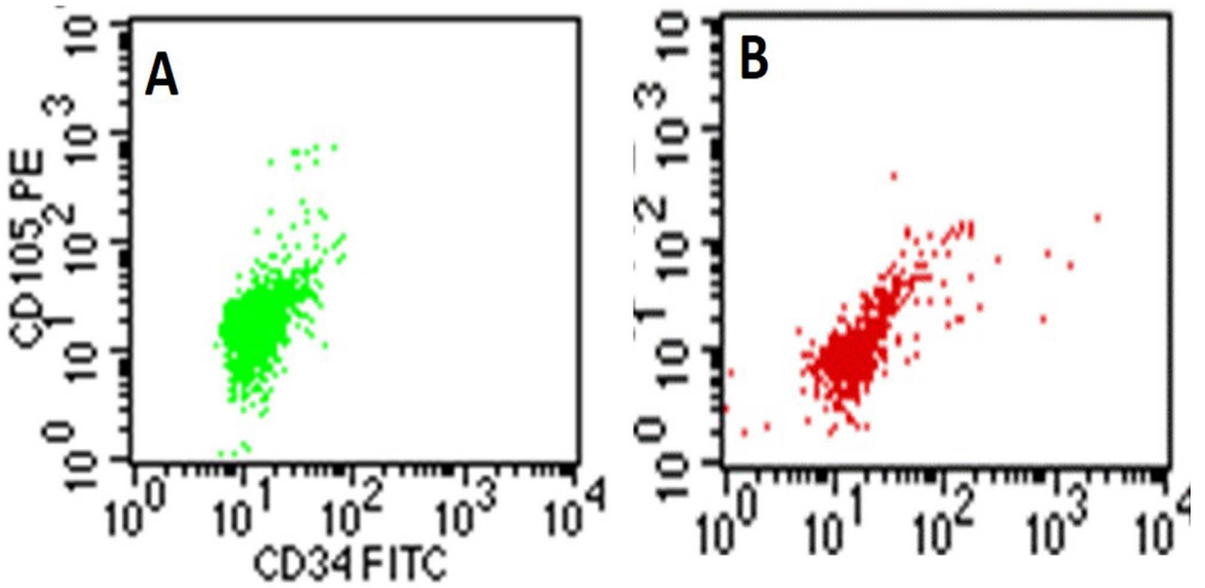


Fig. 2: Flow cytometric analysis of cell surface markers in mesenchymal stem cells (MSCs). A: showing CD 105 (positive) & CD 34 (negative). B: showing CD 44 (positive)

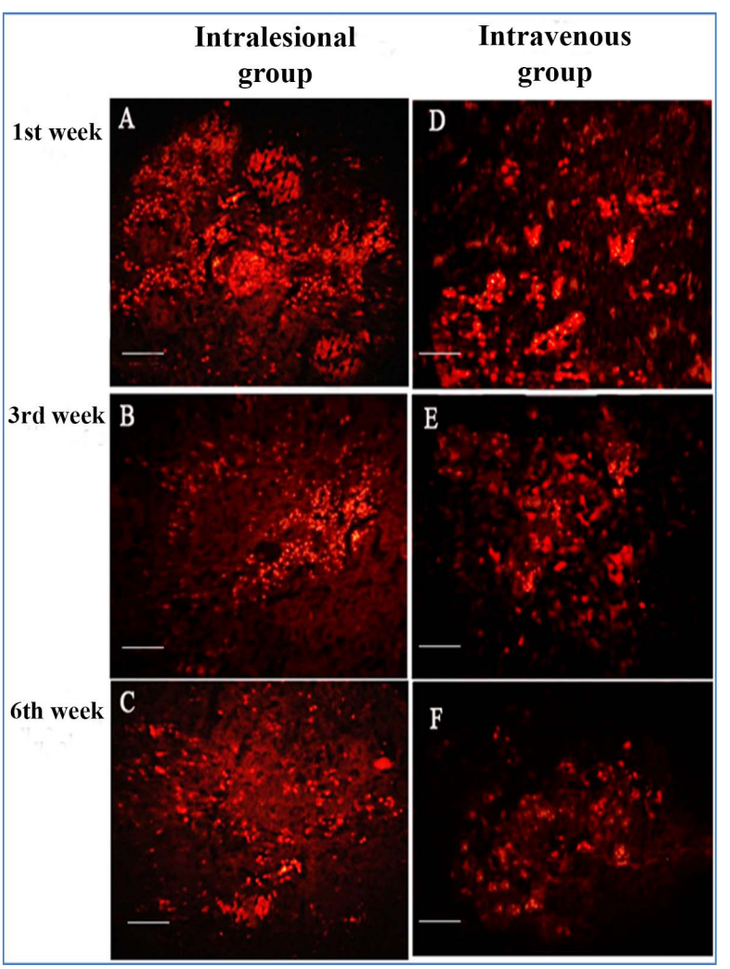


Fig. 3: Photographed Images by fluorescent microscopy showing comparison of the numbers of PKH26-labeled stem cells engrafted in the spinal cord between (IL) group (A, B and C) and IV group (D, E and F). The (IL) group shows higher number of engrafted cells as compared to the (IV) group at 1st, 3rd, 6th weeks PCI. (X 100, scale bar= 500µm)

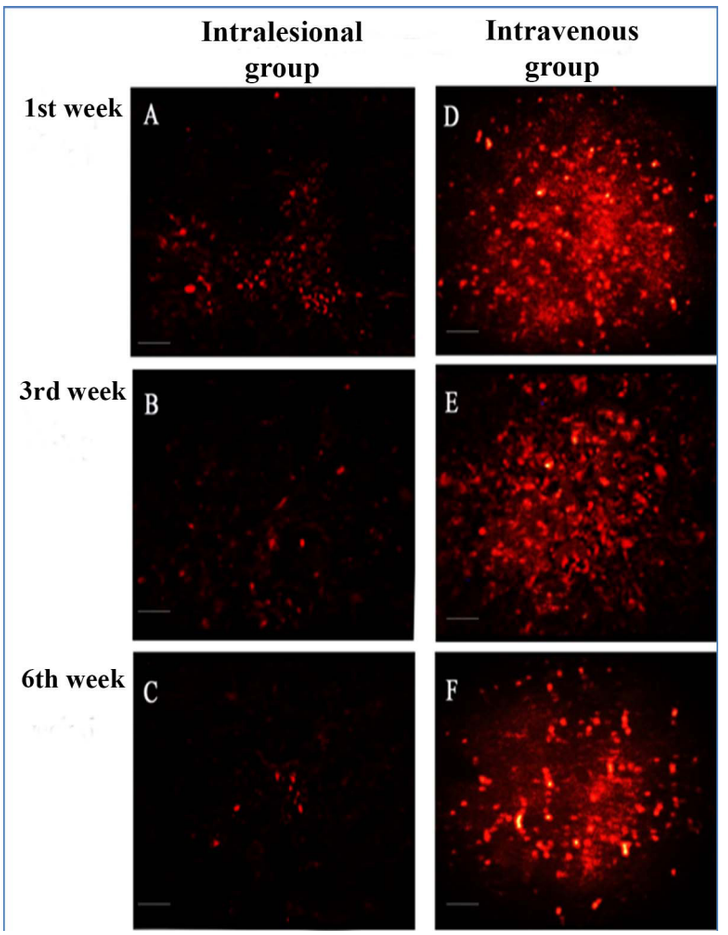


Fig. 4: Photographed Images by fluorescent microscopy showing comparison of numbers of PKH26-labeled stem cells engrafted in the spleen between (IL) group (A, B and C) and (IV) group (D, E and F). The (IV) group shows higher number of engrafted stem cells as compared to the (IL) group at 1st, 3rd, 6th weeks PCI. (X 100, scale bar= 500µm)

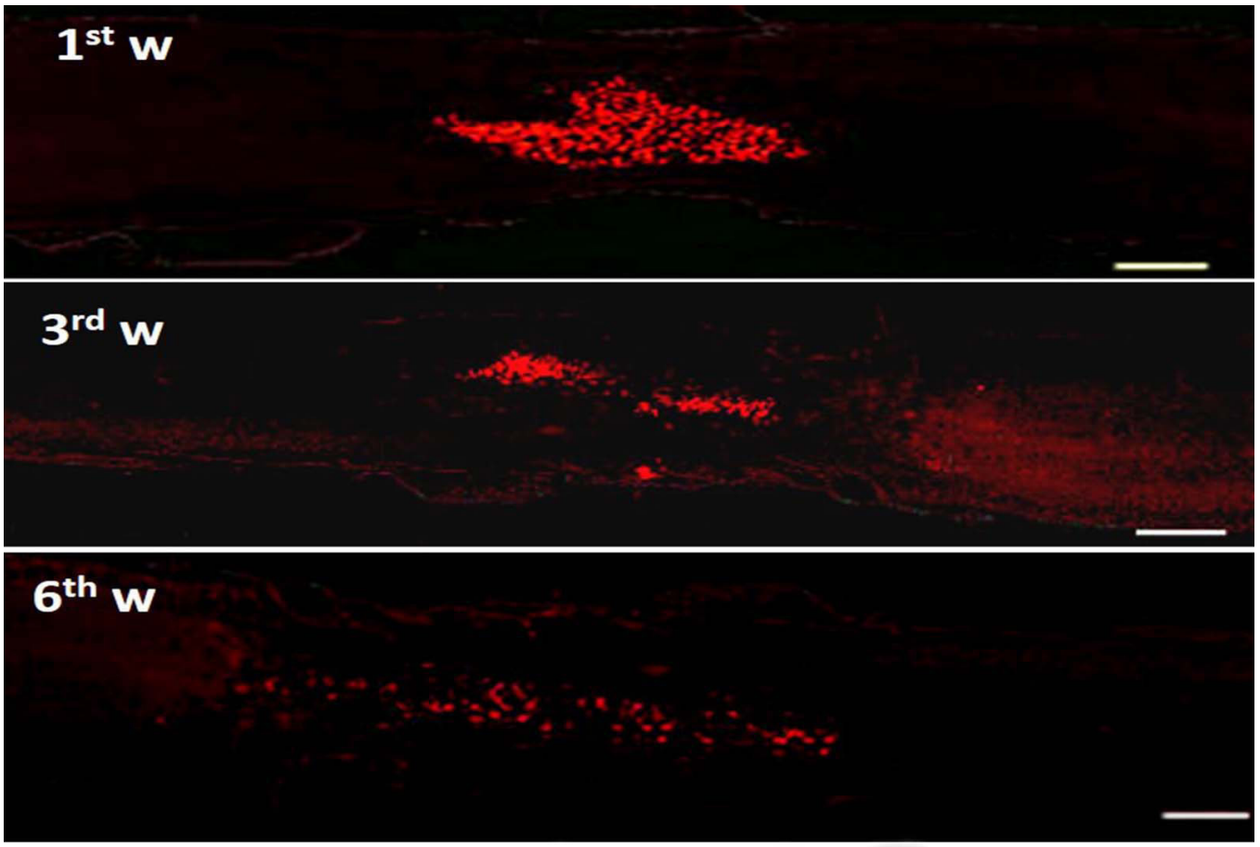


Fig. 5: Photographed Images by fluorescent microscopy showing the distribution of PKH26-labeled stem cells through the sagittal sections of the spinal cord in (IL) group at 1st, 3rd, 6th weeks PCI. The transplanted BMSCs are concentrated at the site of injury of the spinal cord at 1st week PCI. However, at 3rd and 6th weeks PCI, the cells are seen to extend out the injury site rostrally and caudally. (× 100, scale bar=1 mm)

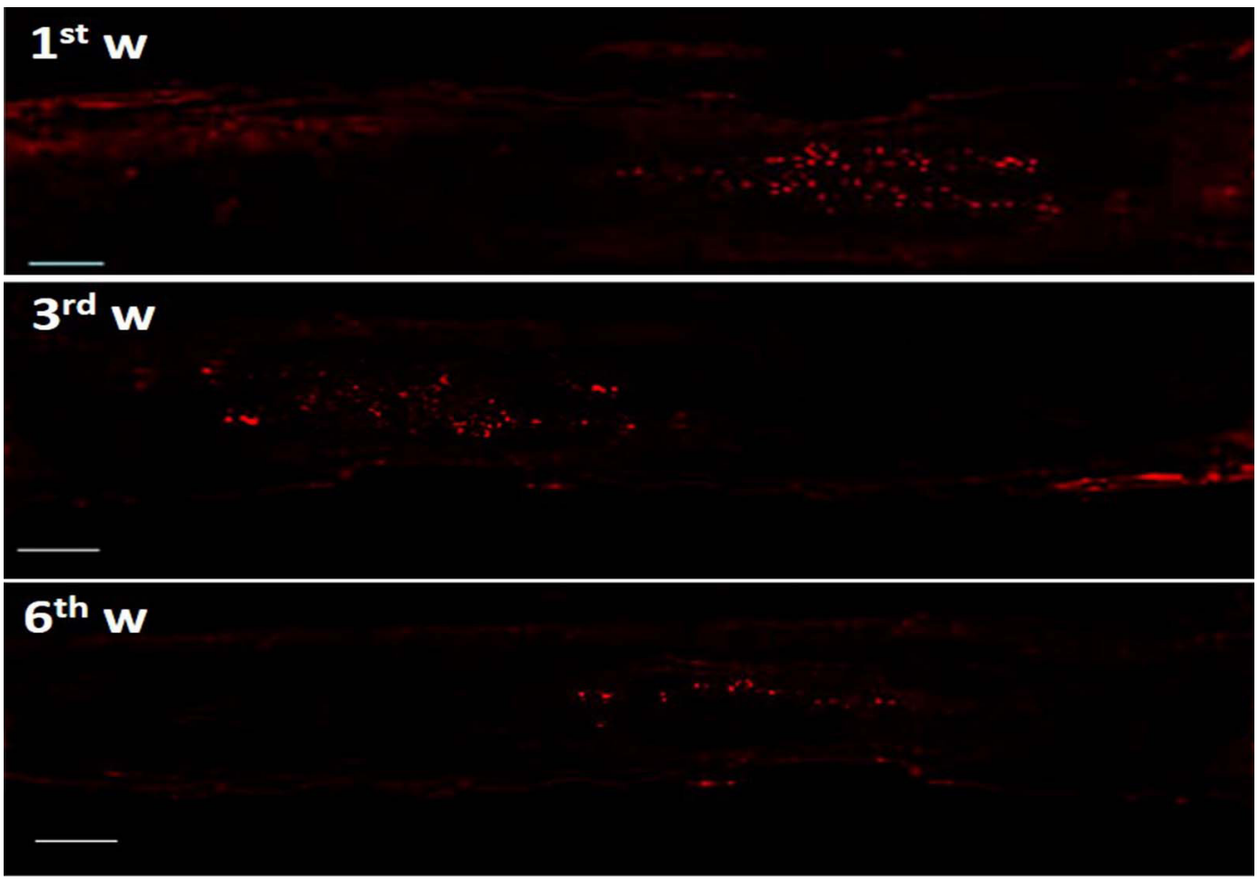


Fig. 6: Photographed Images by fluorescent microscopy showing the distribution of PKH26-labeled stem cells through the sagittal sections of the spinal cord in (IV) group at 1st, 3rd, 6th weeks post-injury. The transplanted BMSCs at 1st week PCI, are seen dispersed over the lesion site of injured spinal cord. However, at 3rd and 6th weeks PCI, the cells are dispersed over a wider area of the spinal cord including the injury site. (× 100, scale bar=1 mm)

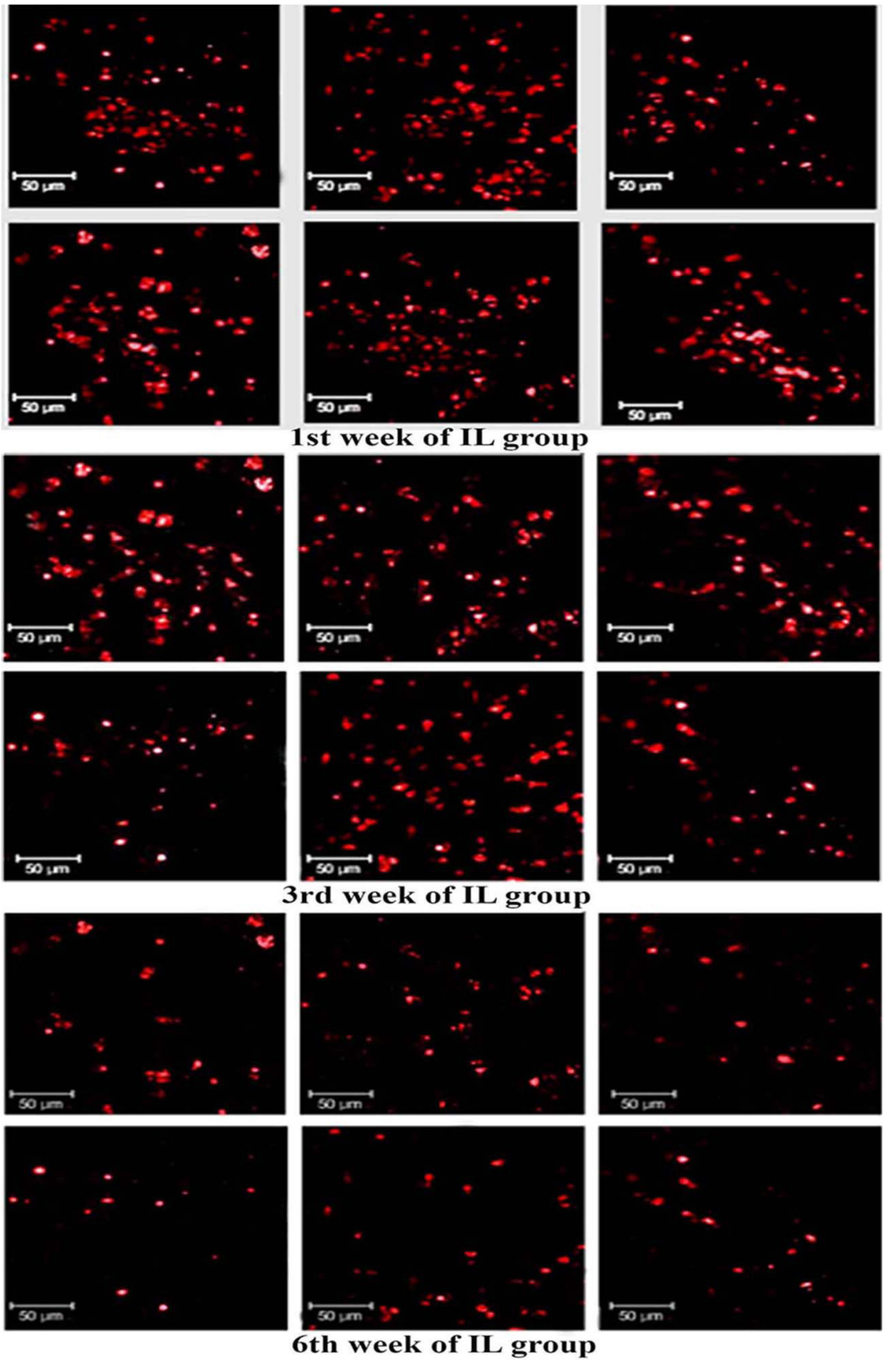


Fig. 7: Photographed Images by fluorescent microscopy showing six high magnification fields of sagittal sections of spinal cord at 1st, 3rd, 6th weeks PCI for stem cell counting in (IL) group. The numbers of PKH26-labelled MSCs engrafted in spinal cord are seen gradually decreased from 1st to 6th weeks PCI with marked reduction at the 6th week PCI. (× 200, scale bar=50µm)

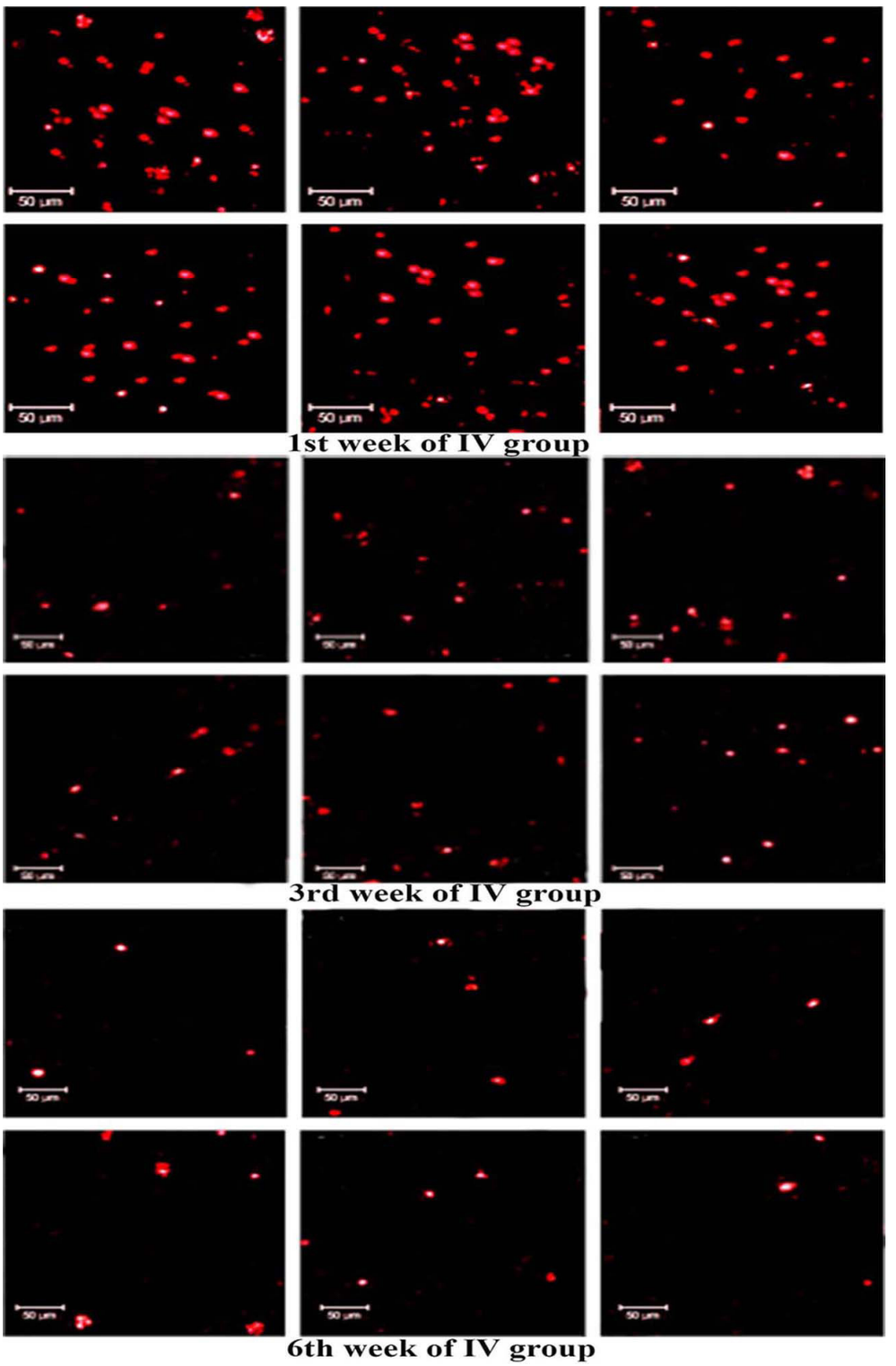


Fig. 8: Photographed Images by fluorescent microscopy showing six high magnification fields of sagittal sections the spinal cord at 1st, 3rd, 6th weeks PCI for stem cell counting in (IV) group. The numbers of PKH26-labelled MSCs engrafted in the spinal cord are seen gradually decreased from 1st to 6th weeks PCI with marked reduction at the 6th week PCI. (X 200, scale bar=50µm)

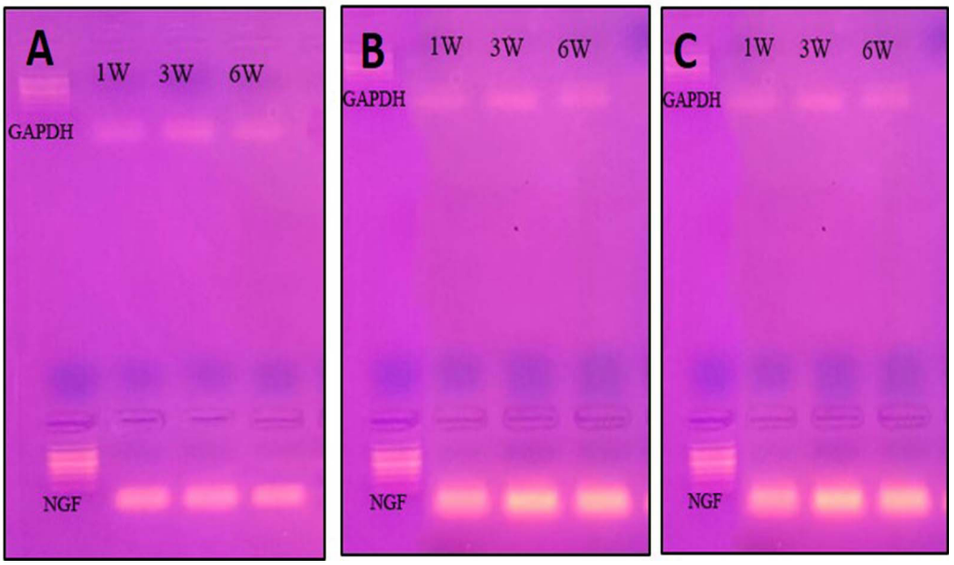


Fig. 9: Photos of agarose gel electrophoresis taken with ultraviolet trans-illumination demonstrating NGF expression levels in control (A), IL (B) and IV (C) groups at 1st, 3rd and 6th weeks PCI (1W, 3W & 6W). The NGF band intensities are higher in the 2 treated groups versus control group. GAPDH gene (496pb) is used as internal control.

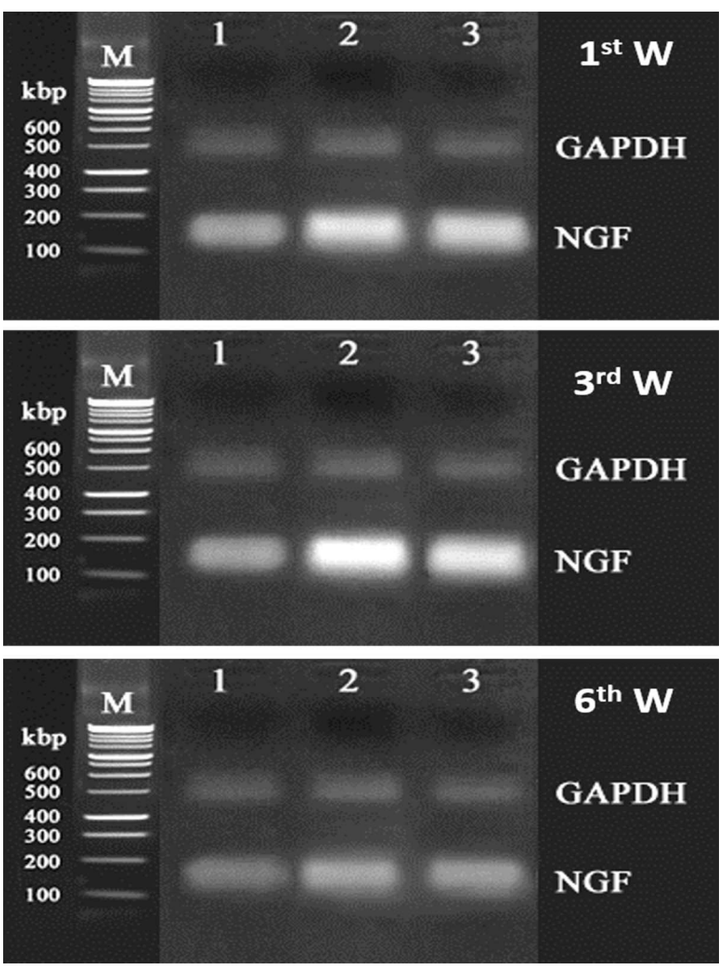


Fig. 10: Agarose gel electrophoresis for RT-PCR products for expression of NGF gene (189 bp). At 1st week PCI, M: DNA ladder, Lane 1: (+) for Control group, Lane 2: (++++) for (IL) group, Lane 3: (+++) for (IV) group. At 3rd week PCI, M: DNA ladder, Lane 1: (+) for Control group, Lane 2: (+++++) for (IL) group, Lane 3: (++++) for (IV) group. At 6th week PCI, M: DNA ladder, Lane 1: (+) for Control group, Lane 2: (+++) for (IL) group, Lane 3: (++) for (IV) group. GAPDH gene (496pb) is used as internal control.

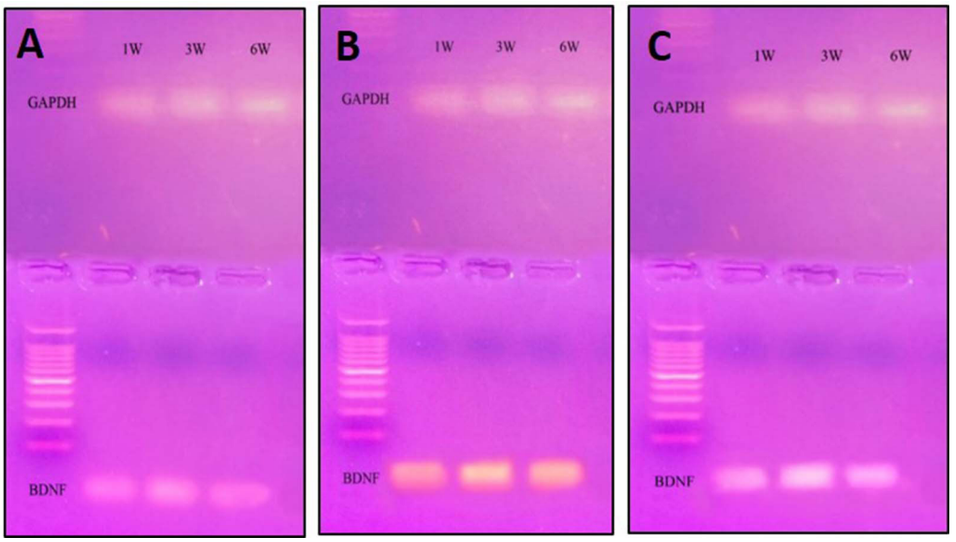


Fig. 11: Photos of agarose gel electrophoresis taken with ultraviolet trans-illumination demonstrating BDNF expression levels in control (A), IL (B) and IV (C) groups at 1st, 3rd and 6th weeks PCI (1W, 3W & 6W). As noted, BDNF band intensities are higher in the 2 treated groups in comparison to control group. GAPDH gene (496pb) is used as internal control.

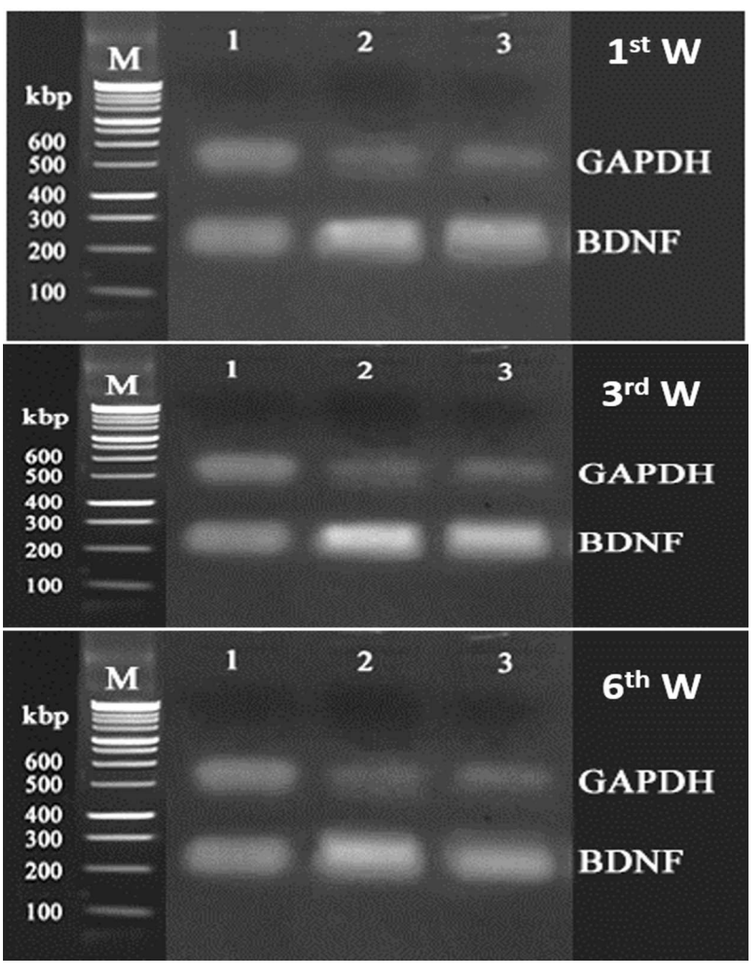


Fig. 12: Agarose gel electrophoresis for RT-PCR products for expression of BDNF gene (238 bp). At 1st week PCI, M: DNA ladder, Lane 1: (+) for Control group, Lane 2: (+++) for (IL) group, Lane 3: (++) for (IV) group. At 3rd week PCI, M: DNA ladder, Lane 1: (+) for Control group, Lane 2: (+++++) for (IL) group, Lane 3: (++++) for (IV) group. At 6th week PCI, M: DNA ladder, Lane 1: (+) for Control group, Lane 2: (++++) for (IL) group, Lane 3: (+++) for (IV) group. GAPDH gene (496pb) is used as internal control.

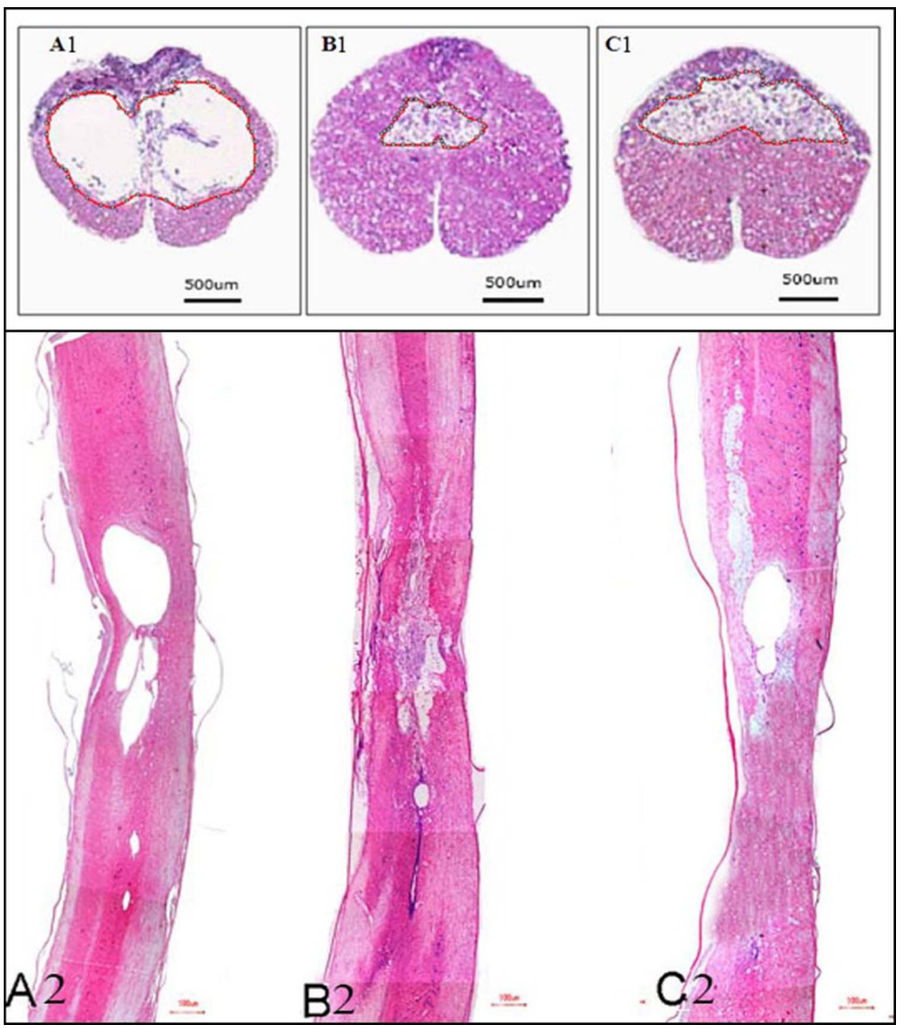


Fig. 13: Photomicrographs showing axial (A1, B1, C1) and midsagittal (A2, B2, C2) sections of lesion areas of spinal cord from rats of control (A), IL (B) and IV (C) groups. Areas of cavitation with variable degrees are apparent in the centers of site of injury in all groups. Cavitation areas are remarkably smaller in the 2 stem cells-treated groups (B & C) relative to control group (A). The (IL) group (B) has the smallest cavitation area. (H&E staining ×100, scale bar=500µm)

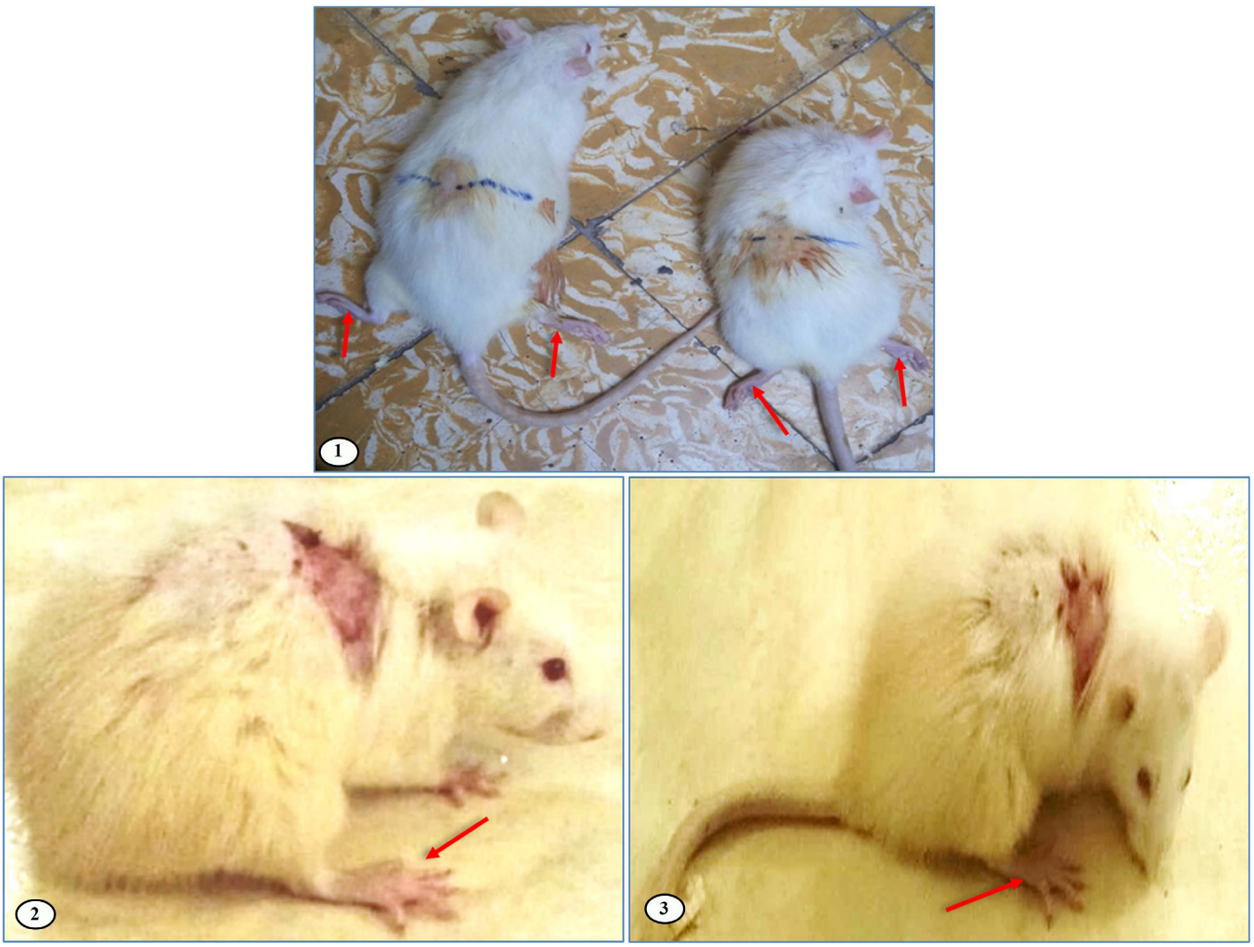


Fig. 14: [a]: photographs of rats before stem cells transplantation showing complete hindlimb paraplegia with flaccid position (arrows). [b, c]: photographs of 2 rats 6-week-old after stem cells transplantation showing improvement in hind-limb locomotor function as observed from limb positions (arrows) with weight bearing ability.

Table 2: Statistical comparison between (IL) and (IV) groups regarding numbers of transplanted stem cells in the spinal cord and spleen at 1st, 3rd and 6th PCI

Number of stem cells at specific time points (Mean score±SD) / Group		Intralesional (IL) group (n=6 rats, 2 at each time point)	Intravenous (IV) group (n=6 rats, 2 at each time point)	*p-value*
Week 1	Spinal cord	513 ± 15.6	112 ± 7.1	0.001**
	spleen	24 ±2.7	5842 ±28.6	0.001**
Week 3	Spinal cord	437 ± 18.4	81 ± 5.7	0.001**
	spleen	12 ±1.3	2372 ±17.4	0.001**
Week 6	Spinal cord	221 ± 14.1	47 ± 2.9	0.041*
	spleen	3 ±1.2	426 ±11.2	0.001**

* Significant (*P-value* <0.05), ** Highly significant (*P-value* ≤0.001)

Table 3: Showing statistical comparison between the mean relative optic densities of NGF expression in control, (IL) and (IV) groups at all studied weeks PCI using One way ANOVA and Bonferroni's post hoc analysis for comparison

1st w NGF Relative optic density / Group		Control group (n= 3)	Intralesional group (n= 3)	Intravenous group (n=3)
Mean relative optic density ± SD		1.24 ± 0.1	1.76 ± 0.3	1.63 ± 0.2
p-value	Control group versus		0.037*	0.046*
	Intralesional (IL) group versus	0.037*		0.124†
	Intravenous (IV) group versus	0.046*	0.124†	
3rd w NGF Relative optic density / Group		**Control group (n= 3)**	**Intralesional group (n= 3)**	**Intravenous group (n=3)**
Mean relative optic density ± SD		1.18 ± 0.16	1.94 ± 0.21	1.78 ± 0.17
p-value	Control group versus		0.024*	0.026*
	Intralesional (IL) group versus	0.024*		0.286†
	Intravenous (IV) group versus	0.026*	0.286†	
6th w NGF Relative optic density / Group		**Control group (n= 3)**	**Intralesional group (n= 3)**	**Intravenous group (n=3)**
Mean relative optic density ± SD		1.14 ± 0.04	1.64 ± 0.14	1.43 ± 0.12
p-value	Control group versus		0.038*	0.047*
	Intralesional (IL) group versus	0.038*		0.176†
	Intravenous (IV) group versus	0.047*	0.176†	

†Insignificant (*P-value* >0.05). * Significant (*P-value* < 0.05)

Table 4: Showing statistical comparison between the mean relative optic densities of BDNF expression in control, (IL) and (IV) groups at diferent weeks PCI using One way ANOVA and Bonferroni's post hoc analysis for comparison

1st w BDNF Relative optic density / Group		Control group (n= 3)	Intralesional group (n= 3)	Intravenous group (n=3)
Mean relative optic density ± SD		1.17± 0.08	1.62 ± 0.13	1.44 ± 0.11
p-value	Control group versus		0.039*	0.047*
	Intralesional (IL) group versus	0.041*		0.149†
	Intravenous (IV) group versus	0.047*	0.169†	
3rd w BDNF Relative optic density / Group		**Control group (n= 3)**	**Intralesional group (n= 3)**	**Intravenous group (n=3)**
Mean relative optic density ± SD		1.14 ± 0.07	1.98 ± 0.13	1.80 ± 0.17
p-value	Control group versus		0.013*	0.021*
	Intralesional (IL) group versus	0.013*		0.263†
	Intravenous (IV) group versus	0.021*	0.263†	
6th w BDNF Relative optic density / Group		**Control group (n= 3)**	**Intralesional group (n= 3)**	**Intravenous group (n=3)**
Mean relative optic density ± SD		1.13 ± 0.06	1.78 ± 0.14	1.61 ± 0.13
p-value	Control group versus		0.030*	0.045*
	Intralesional (IL) group versus	0.030*		0.128†
	Intravenous (IV) group versus	0.045*	0.128†	

†Insignificant (*P-value* >0.05). * Significant (*P-value* < 0.05)

Table 5: Showing statistical comparison between expression levels of NGF gene in the 3 studied groups at 1st, 3rd, 6th week PCI by using the Chi square (X2) test

NGF Relative expression at 1st week	Control group		Intralesional (IL) group		Intravenous (IV) group		X^2 (n=9)	*P-Value*
	No.	%	No.	%	No.	%		
(+≤ 20%) normal	2	66.7	0	0	0	0		
(++20-40%) mild	1	33.3	0	0	0	0		
+++(40-60%) moderate	0	0	0	0	2	66.7		
++++ (60-80%) high	0	0	3	100	1	33.3	13.5	0.031*
+++++ (80-100%) very high	0	0	0	0	0	0		
Total	3	100	3	100	3	100		
NGF Relative expression at 3rd week	Control group		Intralesional (IL) group		Intravenous (IV) group		X^2 (n=9)	*P-Value*
	No.	%	No.	%	No.	%		
(+≤ 20%) normal	3	100	0	0	0	0		
(++ 20-40%) mild	0	0	0	0	0	0		
+++(40-60%) moderate	0	0	0	0	0	0		
++++ (60-80%) high	0	0	1	33.3	3	100	13.5	0.020*
+++++ (80-100%) very high	0	0	2	66.7	0	0		
Total	3	100	3	100	3	100		
NGF Relative expression at 6th week	Control group		Intralesional (IL) group		Intravenous (IV) group		X^2 (n=9)	*P-Value*
	No.	%	No.	%	No.	%		
(+≤ 20%) normal	3	100	0	0	0	0		
(++20-40%) mild	0	0	0	0	2	66.7		
+++(40-60%) moderate	0	0	2	66.7	1	33.3		
++++ (60-80%) high	0	0	1	33.3	0	0	14	0.042*
+++++ (80-100%) very high	0	0	0	0	0	0		
Total	3	100	3	100	3	100		

*Significant (*P-value* < 0.05).

Table 6: Showing statistical comparison between the relative expression level of BDNF gene in 3 studied groups at 1st, 3rd, 6th weeks post-injury by using the Chi square (X2) test

BDNF Relative expression at 1st week	Control group		Intralesional (IL) group		Intravenous (IV) group		X^2 (n=9)	*P-Value*
	No.	%	No.	%	No.	%		
(+≤ 20%) normal	2	66.7	0	0	0	0	13.5	0.046*
(++20-40%) mild	1	33.3	0	0	2	66.7		
+++(40-60%) moderate	0	0	3	100	1	33.3		
++++ (60-80%) high	0	0	0	0	0	0		
+++++ (80-100%) very high	0	0	0	0	0	0		
Total	3	100	3	100	3	100		
BDNF Relative expression at 3rd week	Control group		Intralesional (IL) group		Intravenous (IV) group		X^2 (n=9)	*P-Value*
	No.	%	No.	%	No.	%		
(+≤ 20%) normal	3	100	0	0	0	0	13.5	0.015*
(++ 20-40%) mild	0	0	0	0	0	0		
+++(40-60%) moderate	0	0	0	0	0	0		
++++ (60-80%) high	0	0	0	0	2	66.7		
+++++ (80-100%) very high	0	0	3	100	1	33.3		
Total	3	100	3	100	3	100		
BDNF Relative expression at 6th week	Control group		Intralesional (IL) group		Intravenous (IV) group		X^2 (n=9)	*P-Value*
	No.	%	No.	%	No.	%		
(+≤ 20%) normal	3	100	0	0	0	0	13.5	0.030*
(++20-40%) mild	0	0	0	0	0	0		
+++(40-60%) moderate	0	0	1	33.3	3	100		
++++ (60-80%) high	0	0	2	66.7	0	0		
+++++ (80-100%) very high	0	0	0	0	0	0		
Total	3	100	3	100	3	100		

*Significant (*P-value* < 0.05).

Table 7: Showing statistical comparison between control, (IL) and (IV) groups regarding the mean cavitation areas of injured spinal cord by using one-way ANOVA and Bonferroni test of Post-hoc analysis

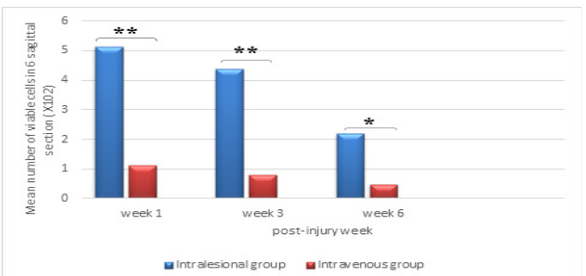
Cavitation area \ Group		Control group (n= 5)	Intralesional group (n= 5)	Intravenous group (n=5)
Mean		1.79 mm^2	1.20 mm^2	1.47 mm^2
Standard Deviation		0.08	0.27	0.28
Median		1.81 mm^2	1.43 mm^2	1.6 mm^2
Range		0.24 (1.70-1.94)	0.59 (0.93-1.52)	0.77 (0.94-1.71)
p-value	Control group versus		0.001**	0.002**
	Intralesional group versus	0.001**		0.011*
	Intravenous group versus	0.002**	0.011*	

* Significant (*P-value* < 0.05), ** Highly significant (*P-value* ≤0.001)

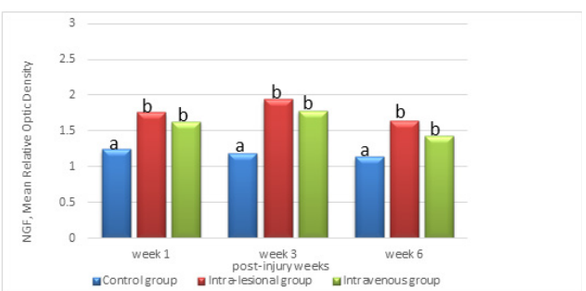
Table 8: Pairwise statistical comparisons between the mean BBB scores of the control, (IL) and (IV) groups over time from the 1st till the 6th week PCI using ANOVA and Bonferroni test of Post-hoc analysis

Comparison groups		*P- value*					
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Control group	Intra-lesion (IL) group	0.089†	0.083†	0.041*	0.041*	0.046*	0.046*
	Intravenous (IV) group	0.043*	0.035*	0.002**	0.002**	0.026*	0.024*
Intra-lesion (IL) group	Intravenous (IV) group	0.054†	0.040*	0.037*	0.035*	0.035*	0.031*

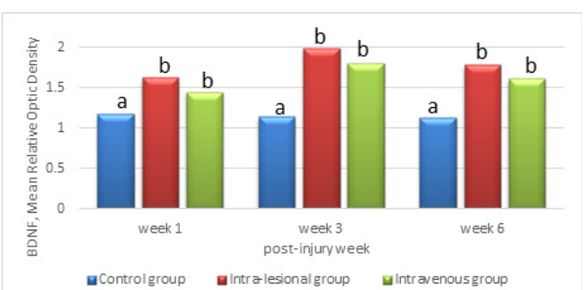
†Insignificant (*P-value* >0.05), * Significant (*P-value* < 0.05), ** Highly significant (*P-value* ≤0.001)



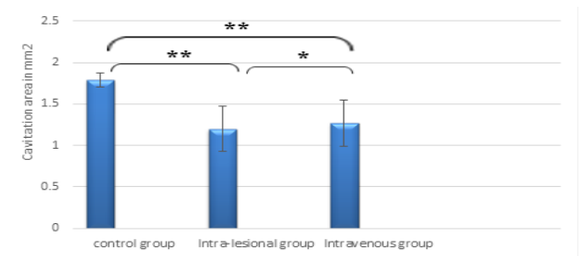
Histogram 1: showing statistical comparison between, (IL) and (IV) groups as regard engrafted cells counts in the spinal cord at 1st, 3rd and 6th weeks PCI. {N.B: *= significant (*P-value* <0.05), **= highly significant (*P-value* ≤0.001)}.



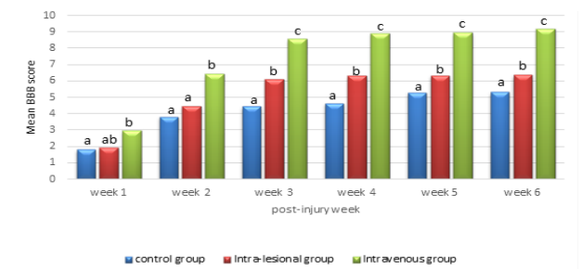
Histogram 2: Showing statistical comparison between the mean relative optic densities of NGF expression in control, (IL) and (IV) groups at 1st, 3rd and 6th weeks PCI. {N.B: the groups carrying different superscripts are significantly different (*P-value* <0.05), while the groups with similar superscripts are insignificantly different (*P-value* >0.05) at every week based on Bonferroni Post-hoc analysis



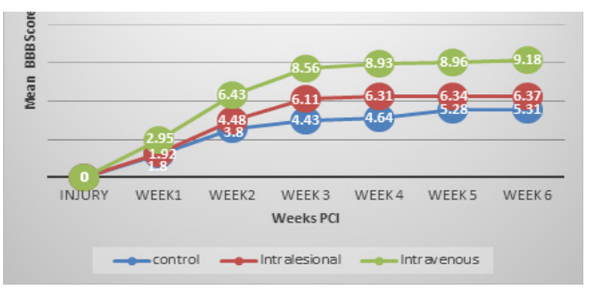
Histogram 3: Showing statistical comparison between the mean relative optic densities of BDNF in control, (IL) and (IV) groups at 1st, 3rd and 6th weeks post-injury. {N.B: at every week, the groups carrying different superscripts are significantly different (*P-value* < 0.05), while the groups with similar superscripts are insignificantly different (*P-value* >0.05) based on Bonferroni Post-hoc analysis.



Histogram 4: Showing statistical comparison between control, (IL) and (IV) groups as regard the mean cavitation areas of injured spinal cord by using one-way ANOVA and Bonferroni test of Post-hoc analysis. {N.B: * = significant (*P-value* <0.05), ** = highly significant (*P-value* ≤0.001)}.



Histogram 5: showing the mean BBB scores of control, (IL) and (IV) groups during the 6 weeks PCI duration. {N.B: at every week, groups carrying different superscripts are significantly different (*P* <0.05), while groups with similar superscripts are insignificantly different (*P-value* >0.05) based on Bonferroni Post-hoc analysis.



Line chart 1: showing the mean of BBB scores of control, (IL) and (IV) groups during the six-weeks periods of observation for locomotor assessment PCI. The BBB (0) score indicates complete hindlimb paraplegia in all injured rats immediately after the operation. Gradual recovery with variable degrees of hindlimb motor function is seen during periods of observation in all groups. BBB scores in stem cells treated (IL and IV) groups versus control group are increased and the highest scores are observed in (IV) group in all weeks.

DISCUSSION

Injured spinal cord represents a serious public health problem in the world. World Health Organization (W.H.O.) reported that 90 million people suffer from spinal cord injuries of varying degrees of riskiness in the world. The estimated annual global incidence is of 40 to 80 cases per million population (approximately 250.000 and 500.000 cases each year worldwide[34]. Since ISC is a severe obstruction that causes distress for many patients and their families, stem cell therapy for ISC, which is the subject of this study, was encouraged.

The ISC results in loss of function of sensory and motor nervous tissue. There is no treatment available that reinstates the function loss as a result of injury to the degree where an independent life is possible. Stem cells transplantation or progenitors may enhance spinal cord repair. Stem cells are characterized by their ability to differentiate to many cell types in an organism[35].

Stem cell therapy has been considered as a promising way for the treatment of vital organ damage. A lot of research were recently done to evaluate the use of stem cells transplantation therapy for spinal cord injuries[36-38]. The transplantation is based on the belief that the neurological function lost by injury might be improved by presenting new cells that can differentiate and assimilate appropriately to substitute the function of lost neurons[1,4,38].

In this study, bone marrow mesenchymal stem cells (BM-MSCs) were used because of their relatively easy accessibility, possibility for autologous transplantation and promising pre-clinical studies[36,38-40]. One of the reasons of emphasis on BM-MSCs is their liability for regeneration after trauma in resemblance to embryonic cells, but they may have less oncogenic potential than embryonic cells. Moreover, the use of BM-MSCs avoids some of the ethical issues concerning the use of stem cells of embryonic or fetal origin as described in previous literatures[11].

In addition, differentiation of MSCs to functional neurons and glia seems to be a suitable option for the replacement therapies of neuro-degenerative diseases and disorders[41]. Transplantation of MSCs is subject of many clinical trials for medication and treatment, including heart, bone and neurodegenerative diseases. Many clinical studies reported improvements of clinical symptoms[42].

In the present study, to verify that the collected cells were MSCs, their characteristic morphological appearance and adhesiveness were detected during period of culture by inverted microscope. In parallel, flow-cytometric analysis of cell surface markers in BM-MSCs expressed CD105, CD44 but did not express the hematopoietic marker CD34. The expression pattern of the surface markers of BM-MSCs was based on the guidelines of their phenotypic characterization in *vitro* as reported by International Society for Cellular Therapy[43]. The CD105, CD44, CD73 MHC (major histocompatibility complex) Class I antigens and CD90 are defined as MSC-specific cell surface markers. However, MSCs lacked the expression of some other hematopoietic markers as CD34, CD14, CD45, CD11b, CD79a and CD19[42].

By fluorescent microscopy in the current study, the transplanted PKH26-labelled MSCs were detected in the injury site of the spinal cord in the two treated groups. Statistical analysis of these numbers revealed highly significant difference between two treated groups at 1st, 3rd weeks PCI (P<0.001) but showed only significant difference at 6th week (P<0.04) with high cell counts recorded in (IL) group than (IV) group in all studied ages. These differences between the two treated groups could be explained by our results regarding the spleen because most cells transplanted via (IV) route were trapped in the spleen. Therefore, high cell counts in (IV) group of spleen were observed but few numbers in (IL) group were seen. Similar results were reported by previous literatures[11,37].

Moreover, in the current research, the MSCs counts of spinal cord were decreased from the 1st to the 6th week PCI in the two stem cells-treated groups. Percentages of cell counts detected at the 6th week in comparison to their count at the 1st week (pointed to stem cell survival rate) equal 43% and 42% for (IL) and (IV) groups respectively. One supposed explanation of such reduction is the limited survival rate of engrafted MSCs. Review of studies reported that bone marrow-derived MSC survival rates after transplantation into animal models of ISC ranged from 8% to 52% at 2 week and from 4 to 45% at 6 weeks after transplantation[44-46].

In the present work, the transplantation time was selected to enhance cell survival by performing transplantation in the early subacute stage at 3rd day after ISC. The same survival enhancing plan was used by many authors[18,35,46,47]. They reported that the survival rates at 6 weeks post-injury ranged from 38% to 45% which are nearby to the rates of this study (43% and 42% for (IL) and (IV) groups respectively).

Other different survival enhancing therapies were used by many authors including; immunosuppression[40,48,49], transplantation within a scaffold for protection against reactive oxygen species and macrophages[50,51], repeated stem cells injections[52,53], electrical stimulation[54] and culturing MSCs under microgravity conditions[55].

In this study, regarding distribution of transplanted BMSCs in the injured spinal cord of the (IL) group, at 1st week after injury, the transplanted BMSCs were concentrated around the site of injury but at 3rd and 6th weeks PCI, the cells were seen to extend out the injury site rostrally and caudally. Further studies hypothesized that the extension of stem cells in different direction to form long septae of transplanted cells that completely bridged the injury site, could be useful in guiding axons regeneration[37,56].

On the other hand, in the present study, the MSCs transplanted via IV route appeared to be dispersed from

the start over a wider area of the spinal cord including the injury site despite high numbers of cells trapped in the spleen. This finding indicated their capability to "home" towards and into the injury site. In accordance with this study, homing of MSCs into the injured spinal cord after IV injection has been confirmed in some studies[10,11,57]. Disrupted blood-spinal cord barrier after injury and stromal derived factor-1 (SDF-1)/CXCR-4 (chemokine receptor type 4) have been demonstrated to participate in the migration of MSCs from systemic circulation into the injury site[57,58].

In the current work, histologically, the cavitation areas were found to be remarkably smaller in both stem cells-treated groups versus control group with the (IL) group having the smallest mean cavitation areas. The statistical results of this study also confirmed that the mean cavitation areas in (IL) and (IV) groups versus to control group showed highly significant decrease (P=0.001) (P=0.002) respectively. However, the mean cavitation area in the (IL) group versus (IV) group showed only statistically significant decrease (P=0.011). This finding indicates that the therapy by BM-MSCs transplantation via (IL) route was associated with a better histological outcome rather than (IV) route. Similar results reported that the intralesional injection of stem cells was associated with higher engrafted stem cell counts and smaller spinal cord cavitation areas compared to intravenous injection route[37,56,58]. In addition, previous investigators found statistically significant decrease in the total cavity areas in the intralesional group at epicenter and at 4 mm rostral and caudal to epicenter compared with the control group[37]. The small reduction of the cavitation areas in (IL) group in this study may be due to increased blood supply in response to trauma which may provide good environment for the higher numbers of MSCs present at site of injury of (IL) group for repairing the damaged tissues. Recent studies added that the MSCs improve the tissue repair and functional results by promoting angiogenesis and stimulate blood vessels growth[36,59].

On the other hand, Da Silva Meirelles *et al*.[60] stated that the MSCs secreted wide-ranging of bioactive molecules like growth factors, chemokines and cytokines which instituted important biological role under injury states. In the present study, neurotrophic factors (NGF& BDNF) expression levels in the injured spinal cord were found to be significantly higher in the stem cells-treated groups versus the control group. There is a consensus in literature that following MSCs transplantation therapy, there is significant increase in neurotrophic factors expression in spinal cord tissue which plays an important role in the repair and functional recovery of spinal cord injuries[24,44,46,54,61-63].

Many studies demonstrated that BDNF prevented atrophy of neurons and reduced the extent of the lesion cavity after ISC in rats while NGF has been shown to promote or preserve neural tissue and axonal regeneration after ISC in rats[61,63,64]. On the light of the previous studies, the smallest cavitation areas were detected in this study in (IL) group versus (IV) and control groups. This could be also explained by the high expressions of BDNF and NGF detected in the (IL) group than other groups. Therefore, in this work, the transplantation of stem cells via IL route was associated with the best neuroprotective function (recording highest expressions level of BDNF and NGF) and with the best histological outcome (having smallest cavitation area) that indicating their ability in tissue repair.

As mentioned above, in this study, the NGF and BDNF expression in both treated groups versus control group were significantly higher throughout all studied weeks. However, by further analysis of the temporal changes in NGF and BDNF expression levels over the duration of study, there was an increasing pattern of their expression levels from the 1st to the 3rd week then levels decreased at the 6th week. As mentioned before at 6 weeks, the reduction in stem cell numbers could explain the decrease in neurotrophic factors expressions as well at the same time. So, it could be assumed that an additional transplantation of MSCs at 3rd week after ISC could increase the cell counts and in turn preserve the high levels of NGF, BDNF expressions. In accordance with these results, some authors reported that NGF levels reached its peak at 14 days PCI, whereas BDNF levels peaked at 21 days after ISC, then consistently decreased[65]. However, in another study, the investigators mentioned that NGF and BDNF peaked at 14 days after spinal cord transection then consistently decreased in the rat[66]. In addition, Chung *et al*.[61] demonstrated that NGF and BDNF expression reached their peak at 3rd week after SCI and decrease thereafter and they explained this decrease by the reduction of engrafted stem cells mass.

In the current study, as respect the clinical functional outcome, gradual recovery with variable degrees of hindlimb motor function was observed in rats of all groups during the six-week periods of observation. The BBB scores were higher in stem cells treated (IL and IV) groups versus control group as indicated from observation of limb positions with weight bearing ability. The (IV) group recorded the highest BBB scores of improvement comparative to other groups during all weeks of observation period. However, BBB scores of (IL) group versus (IV) group, showed significant decrease (P<0.05) from 2nd week thereafter. These findings in (IL) group may be attributable to secondary damage occurred to the injured spinal cord by needles during (IL) injection of stem cells. Moreover, as previously discussed, our observations regarding the higher number of engrafted cells after (IL) injection, the cells were concentrated around the injury site may produce mass effect on adjacent normal tissues.

On the other hand, after IV injection, BMSCs were seen dispersed over a wider area near the damaged region and therefore, no mass effect or secondary SC damage occurred like in (IL) injection. In consistence with these results, Kang *et al*.[11] evaluated the fate and efficacy of transplanted bone marrow MSCs following ISC in rats by different transplantation routes. They reported more

effective clinical improvement by IV delivery. Similar results were mentioned by numerous studies, that preferred the (IV) route for stem cell delivery because of being safe route, easy to performance, without secondary cord injury and associated with highest BBB scores of improvement more than (IL) route[4,24,39,44,46,54,63,64].

In the present work, regarding the observation of the temporal changes in BBB scores over the duration of 6th weeks PCI, revealed that BBB scores showed the best improvement rates in the (IV) group at 3rd and 4th weeks then a functional plateau was noticed which could be explained by the reduction in engrafted stem cells counts in addition to the decrease in neurotrophic factors expression from the 3rd week thereafter. So, it could be assumed that an additional transplantation of MSCs at the 3rd week after ISC could enhance the cell counts and maintain the high levels expression of neurotrophic factors resulting in high rates of BBB score improvements. These results were in accordance with previous studies which demonstrated that the functional improvement reached a functional plateau at 4 to 5 weeks post-injury[24,39,44,46,54,63,64,67,68].

Therefore, we recommend repetitive injections of MSCs instead of a single transplantation, since it would increase the cell counts, keeping the high levels of NGF, BDNF expression and the high rates of BBB score improvements.

CONCLUSION

The results of our work revealed that early transplantation of BM-MSCs in ISC resulted in remarkable improvement in the treated groups as compared to the to control group. The (IV) route therapy was associated with the best functional motor recovery proved by highest BBB scores while, the (IL) route therapy was associated with the best histological outcome manifested by the possibility of a tissue repair (smallest cavitation area) and with the best neuroprotective function verified by highest BDNF and NGF expressions.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تقييم التأثير العلاجي المبكر لزرع الخلايا الجذعية للنخاع العظمي في الحبل الشوكي المصاب في الجرذان البيضاء بطريقتين

شريهان فكرى عثمان[١]، محمد عبد المنعم محمود[١]، سمية حسن عبدالله[٢]، حنان السيد لطفى مختار[١]

[١]قسم التشريح الآدمى والأجنة، [٢]قسم الكيمياء الحيوية والبيولوجيا الجزيئية، كلية الطب البشرى، جامعة الزقازيق، مصر

المقدمة: خلقت الخلايا الجذعية بابا من الأمل في علاج الحالات المستعصية مثل إصابة الحبل الشوكي.
الهدف من الدراسة: تهدف هذه الدراسة الى تقييم التأثير العلاجي المبكر المحتمل للخلايا الجذعية للنخاع العظمي على تجديد الحبل الشوكي المصاب فى الجرذان البيضاء وأيضاً لتقييم أفضل المسارات لزرع الخلايا الجذعية في النموذج الحيواني.
المواد و الطـرق المستخدمة: تم استخدام ٦٢ من ذكور الجرذان البالغة حيث تم تخصيص إثنان من الجرذان لاستخراج الخلايا الجذعية للنخاع العظمي وتم تقسيم ٦٠ جرذً إلى ثلاث مجموعات. المجموعة الضابطة: تم تعريضهم لإصابة مستحدثة للحبل الشوكي عن طريق الجراحة، المجموعة الثانية : تلقت حقن للخلايا الجذعية عن طريق مسار الحقن المباشر في مكان الإصابة بالحبل الشوكي والمجموعة الثالثة: فقد تلقت حقن للخلايا الجذعية عن طريق مسار الحقن في الوريد الذيلي وذلك في اليوم الثالث بعد اصابة الحبل الشوكي عن طريق الجراحة. تم متابعة الأداء الوظيفي الحركي للجرذان مرة أسبوعيا ولمدة ستة اسابيع بعد الإصابة. تم استخراج عينات الحبل الشوكي المصاب وتجهيزها من أجل الدراسات النسيجية والمورفومترية والكيميائية والمناعية والإحصائية.
النتائج: بتتبع الخلايا الجذعية فقد أظهرت المجموعة الثانية المعالجة بالخلايا الجذعية بالحقن في مكان الاصابة تزايد كبير في عدد الخلايا عن المجموعة الثالثة المعالجة بالخلايا الجذعية بالحقن الوريدي وهذا في جميع أسابيع دراسة هذا البحث مع ملاحظة انخفاض عددها تدريجيا من الأسبوع الأول إلى الأسبوع السادس بعد الاصابة. وبالتحليل الإحصائي فقد لوحظ زيادة عدد الخلايا الجذعية زيادة ذو دلالة إحصائية عالية في المجموعة الثانية مقابل المجموعة الثالثة في الأسبوع الأول والثالث ولكن في الأسبوع السادس فكانت هذه الزيادة ذو دلالة إحصائية عادية. تم تركيز الخلايا الجذعية عند موقع إصابة الحبل الشوكي في المجموعة الثانية ثم تحركت عن موقع الإصابة في اتجاهين متضادين. أما في المجموعة الثالثة ، كانت الخلايا منذ البداية منتشرة على مساحة أوسع بما في ذلك موقع الإصابة مما يشير إلى قدرتها على الوصول إلى "المنشأ". كما أن متوسط قيم مساحة التجاويف الفارغة التى ظهرت في منطقة الإصابة في نسيج الحبل الشوكي في المجموعات المعالجة بالخلايا الجذعية انخفاضًا ذو دلالة احصائية عالية مقابل المجموعة الضابطة وقد أظهرت المجموعة الثانية مساحات صغيرة لهذه التجاويف مقارنة بالجموعات الأخرى. وسجلت هذه المجموعة أيضا زيادة في مستويات التعبير للتمثيل الجيني للعوامل المحفزة للنسيج العصبى (NGF& BDNF) في أنسجة الحبل الشوكي المصاب بمعدل أعلى مقارنة بالمجموعات الأخرى مع زيادة مستوى التعبيرمن الأسبوع الأول إلى الأسبوع الثالث ثم انخفاضه في الأسبوع السادس. أظهرت درجات التحسن الحركي الوظيفي في المجموعات المعالجة مقابل المجموعة الضابطة زيادة ذو دلالة إحصائية عالية و كان أعلى تحسن حركي قد سجل في المجموعة الثالثة مقابل المجموعات الأخرى.
الخلاصة: استنتج من هذا العمل إلى أن الزراعة المبكرة للخلايا الجذعية في إصابات الحبل الشوكي قد أعطت تحسنًا واضحا، كما أن العلاج بطريقة الحقن في الوريد الذيلي أدى إلى أعلى نتيجة شفاء حركي بينما أعطى العلاج بطريقة الحقن المباشر في مكان إصابة الحبل الشوكي أفضل النتائج النسيجية والوقائية العصبية.