HISTOPATHOLOGICAL ASSESSMENTS OF AUTOLOGOUS BONE MARROW DERIVED MESENCHYMAL STEM CELLS ON THE REGENERATION OFSPINAL CORD INJURY IN LABORATORY WHITE RATS (*RATTUS NORVEGICUS*)

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ABSTRACT

A spinal cord injury (SCI) prompting the perpetual loss of sensation and deliberate development underneath the sore level is one of the most challenging clinical problems. Until now, treatments for (SCI) are,to a great extent, ineffectual. Subsequently, this investigation was intended for the purpose of evaluating the effects of bone marrow-derived Mesenchymal Stem Cells (MSCs) on useful recuperation in rodents after spinal line injury. There are fourteen adult rats partitioned into three groups, group 1 (laminectomy andwithout SCI), group 2 (laminectomy with SCI), and group 3 (laminectomy with SCI + implanted BMSCs). Thorough investigations were including the clinical signs and histopathological assessment. Afterthe sixteenth week's post-surgical operation,Tarlov scores were utilized to clinically assess the restorative impacts. The mean average functional recovery scores in the third group have been demonstrated considerably (P< 0.05) high compared to the second group's score.A while later, the lesion site of the spinal cordhas been directlyassessed by conventional histology. Group III showeddecreasedtissue loss from secondary processes and decreased glial scarring tissue, conspicuous remyelination regenerative nerve fibers, reduction of cavitation, and significant angiogenesis at 16th week's post-surgical operation compared to group II. In conclusion,thisexamination demonstrated the clinical adequacy of BMSCs as one of the tools related to the enlistment of neuro-regeneration following traumatic SCI.

Key words: Rats, bone marrow, spinal cord injuries, regeneration, functional recovery

Introduction

Recuperation from SCI is considered extremely difficult due to the fact that the central nervous system (CNS) shows a limited capacity for regenerating the injured cells, replacing damaged myelin sheath, and storing the functional associations (Urdzikova *et al*., 2006). In humans, somewhere in the range of 3 and 5 individuals for each every 100,000 are influenced by SCI (Coutts and Keirstead, 2008). Traumatic SCI frequently results in the mortality of various neurons that might not be recovered. Also, the process of SCI was indicated via neural apoptosis, demyelination, as well as posttraumatic inflammatory reactions (Coutts and Keirstead, 2008). Concerning SCI, the functional and structural damage regarding the spinal cord happens via secondary and primary injuries. These results in loss of development, sensation, and autonomic nerve brokenness beneath harmed level, such effect might be harming patients' emotional wellness and spot an immense weight on society from a general well-being viewpoint (Joyce *et al*., 2010). SCI treatment strategies were focused on impacting secondary damage. Surgical stabilization and decompression are the major traumatic SCI treatments. Neurotrophic rehabilitation is reducing the damage, saving damaged area, relieving spinal cord ischemia and hypoxia, rescuing the necrotic neurons, and promoting a neuronal function (Nishio *et al*., 2006). MSCs that have been separated from the bone marrow showneuro-protective characteristics after implantation in the lesion of thespinal cord (Joyce *et al*., 2010). Besides, such impact was anticipated in their paracrine characteristics,which are mediated viathe release of growth factors, antiinflammatory cytokines and antiapoptotic molecules,generating a promising environment for neuronregeneration, remyelination (Hardy*et al*., 2008; Nishio *et al*., 2006) and immuno-modulation (English, 2013). A few MSC preferences were evident in the case when compared with other stem cell types utilized for SCI; for instance, they might be obtained easily from the adult donors, expanded, and autologously transplanted insignificant malignant transformation risks. Besides, the immunosuppressive and immunomodulatory properties related to MSC were lately assessed in many clinical and experimental investigations Nishio *et al*., 2006; Stagg and Galipeau, 2013). Up until this point, using MSC was verified to be safe in various clinical investigations (Jalil and Karevskiy, 2020; Karussis *et al*., 2010), nonetheless, more data in terms of their underlying mechanism was anticipated for enhancing their applications in clinical practice. Furthermore, this examination's major aimis elucidating the effects of BMSCs embedded into the hem section on the second lumber spinal cord segment on the conduct execution of rodents after SCI.

Materials and Methods

Animals and groups

This investigation was conducted on fourteen white laboratory rats (*Rattus norvegicus*) with a weight of (200±50)gm. Rats were independently housed in wire cages, given commercial pellets as food, and provided with water *ad libitum.* The rodents were kept in their particular confines for 15 days for acclimatization before experimentation. All strategies utilized in this examination have beenaffirmed by the College of Veterinary Medicine's Ethics Committee, Univ. of Qadisiya. The rodents have beenpartitioned into the next 3 groups;a control group (group 1), which includes 4 rodents subjected to the

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sham operation. The animals experienced dorsal laminectomy just at the 2nd lumbar vertebra of the spinal cord. After that, the animals have been sacrificed with corresponding experimental groups. The trauma control group (group 2) includes 5 rodents subjected to dorsallaminectomy as well as longitudinal incision related to Dura matter then, left lateral hemisection of the spinal cord at 2nd the 2ed lumbar vertebra of the spinal cord,the hemisection defect has been treated with phosphate buffer saline (1 ml). Finally, a BMSC group (group 3), it includes 5 rodents subjected to dorsal laminectomy and excision related to spinal cord comparable to group 2, and afterward implanted BMSC 50 µl ($5x10^6$) in a hole between proximal and distal ends regarding the spinal cord. Then Dura matter has been repaired for preventing CSF spillage.

***In vitro* study**

BMSCs isolation and culture

Isolation and expansion of mouse BMSCs under sterile conditions, the femurs were removed from anesthetized mice, and bone marrow cells were isolated by flushing method for the purpose of flushing the bone marrow from bones with the use of a syringe and a media based on the establishedprotocols (Chi *et al*., 2015). Bone marrow cells were suspended with Dublecco modified Eagle medium (DMEM) of 3 ml. High-glucose DMEM has been supplemented with 30% (FBS), pencillin G of 10 U/ml, streptomycin of 10 U/ml, amphotericin B of 25 mg/ml, 1% non-essential amino acid as well as sodium pyruvate (100 ng/ml), all of them provided via (GIBCO, USA). FBS of 3 ml has been seeded in 75 cm^2 flask for 3 min and then the blended media with BM is added to flask. After that, subject to incubation at temperature of 37°C in CO_2 of 5% in air for a total of 3 days, and following 72 hours, non-disciple cells have been collected, while the medium was supplanted. In addition, the culture reached a conjunction stage onthe twelfth day, after that, the monolayer cells have been washed two times with PBS (PH 7.2) of 2 ml. Then, the cultures have been digested with 2 ml of 0.20% trypsin 0.02%, ethylenediaminetetra acetic acid (EDTA Sigma, U.S.), also appropriated on the layer's surface for 2 min. Furthermore, checking the cells under a microscope till recapturing their rounded shape and disposing of the trypsine. DMEM, which contains FBS of 10%, has been added to the medium and,after that, trapped (tenderly) for separating cells for the next three subcultures. Furthermore, the cells are reaped *via* tapping the medium from the flask, PBS is washed; Trypsine bathing and substituting with the DMEM of 10 ml; after that, the cells and medium have been collected in sterile test tubes, subjected to centrifuging for 10 min at 2000 rpm. DMEM of 1 ml has been added to isolated precipitated pellets as well as blended. Also, thecells are counted with the use of hematocytometer for ensuring a minimal count of ($1x10^6$) MSCs in a culture medium of 10 µl.

Immunohistochemistry analysis of MSCs

Following MSCs have been dispersed with a trypsinPBS, also suspended in a DMEM growth media, the cell has been recultured in a multiwell tissue culture slides, a total of 8 wells in the DMEM supplemented with FBS of 15%, while the plates are subjected to incubation at a temperature of 37ºC for allowing the cells for development ofa monolayer related to adherent cells in a period between 1 and 3 days, then the media has been aspirated, while the cellsare fixed for 10 mins for paraformaldehyde (4%). In addition, the sections in all the steps are put in a humid chamber between a temperature of 20-25ºC. For the purpose of inhibiting endogenous peroxidase, the cells have been subjected to incubation with hydrogen peroxide (1%) between (10 and 15) minutes and washed three times by the PBS for 5 min. Furthermore, an aliquot of 1.5% blocking serum has been added to the cell section for 1 hr and after that washed with 3 changes of (PBS) for a total of 5 mins, to decrease background staining. Cells sections were subjected to incubation with diluted primary antibody (125 µl) a t (1:50) ratio for 1hr at room temperature or overnight at a temperature of 4ºC, after that with 3 PBS changes for 5 mins. Also, cell sections have been subjected to incubation for 30 min with biotinylated secondary antibody (1.2 ml) as well as washed with 3 PBS changes for 5mins. Analiquot of 650 µl of AB enzyme reagent has been added to the cell section and washed 2 times with PBS for 2mins. A total of 3 peroxidase substratedrops have been added to the cells for 10 min, or till the required stain intensity is developed and washed for 5 min with distilled water. Hematoxylin stains have been added to the cell section between (5 and 10) and washed immediately with distilled water. Lastly, between (1 and 2) permanent mounting medium drops are added and analyzed *via* light microscopy at (40x and 100x) powers of magnification (Caddick *et al*., 2006).

***In vivo* study**

Modified surgical protocol

Before anesthesia, the rats were left without food for a period of two hours. A mixture of 10 mg/kg of Xylazine hydrochloride (ILIUM XYLAZIL-20, Australia) and 75 mg/kg Ketamine hydrochloride (Bioketan, Vetoquinol Biowet, Sp. Zo.O, France) was used for intraperitoneal injection so as to attain induction and maintenance anesthesia (Flecknell, 2003). Spinal semi-cordectomy is illustrated in Fig. 3. The rats have been put in a sternal recumbence; also, a surgical incision has been done over a dorsal midline between L1-L3 (Fig.1A). This study sub-periosteallyincreased the epaxial muscles from dorsal spinous processes, articular facets, laminae, and pedicles of L2 vertebrae. In addition, the Gelpi retractors have been utilized for separating the epaxial muscles throughout dissection. The interarcuate ligament has been dissected with a No-11 scalpel blade for removing the exposed dorsal spinous processes to the dorsal lamina level with large, single-action duckbill ronguors. As soon as the inner cortical layers regarding the two laminae are evaluated, the soft periosteal has been palpated with a dental spatula, and the periosteum was gently penetrated for removing the fat tissue which surrounds the spinal cord. Furthermore, a longitudinal incision related to meninges left lateral sectioned is accomplished with a surgical blade No-10 under a magnifying lens. In the BMSC group, it was treated by implantation BMSC 50 µl ($5x10^6$) (Fig. 1B). After that, a dura matter is closed via 4/0 Vicryl with subcutaneous fat, put over the laminectomy for avoiding adhesions (Fig. 3C). The fascia, as well as epaxial muscles,have been closed with 3-0 Polydioxanone simple continuous sutures. Close subcutaneous tissue and skin.

Behavioral evaluation

Animals' neurologic deficits have been estimated daily prior to and after surgery, from day 1 till the study ended on sixteen weeksvia utilizing the next scoring (Grade 0: paraplegia without low extremity motor function; Grade 1: poor lower extremity motor function; Grade 2: excellent hind limbmovement, yet unable to stand; Grade 3: the ability to stand, yet with no ability to walk in a normal way; Grade 4: total recovery (Tarlov *et al*., 1953).

Sample harvest and observation of BMSCs in spinal cord samples

Sixteen weeks after surgery, they were deeply anesthetized with intraperitoneal injection related to the mixture of 75 mg/kg ketamine hydrochloride as well as 10 mg/kg of Xylazine hydrochloride. They were euthanized by an overdose of 10% formaldehyde by direct intracardial injection and exsanguination. A 1cm-long segment regarding the spinal cord that contains the lesion site was fixated, dehydrated, and embedded in paraffin.Furthermore, the tissue blocks have been cut into sections with a thickness of 5 µm. Hematoxylin-eosin staining to evaluate the regeneration of the injured spinal cord.

Statistical analysis

Statistical analysis was carried by expressing and analyzing the data as means as well as standard deviations (M±SD). Then the results from each group were compared against each other through the Kruskal Wallis and Mann-Witney tests by Statistical Package for Social Sciences (SPSS) v. 16.0 software (non-parametric tests). Here, the significant level has been set at $P \leq 0.05$.

Results

Morphology of bone marrow-derived mesenchymal stem cells

In the experimental work, following (48-72 hrs), small heterogeneous cell colonies have been indicated, the cells are flattened and a round shape and started to attach at the coated surface related to plastic culture flask with primary colonies' formation (Fig. 2A). Following 10 days, the cells and colonies expanded markedly, and colonies were progressively expanded in size and interface together to form a monolayer of adherent cells, and flasks were confluent (Fig. 3B). These cells displayed small rounded, spindle-shaped, and large flatted morphology after 12 days of culture at passage 1 (P1) (Fig. 2C).

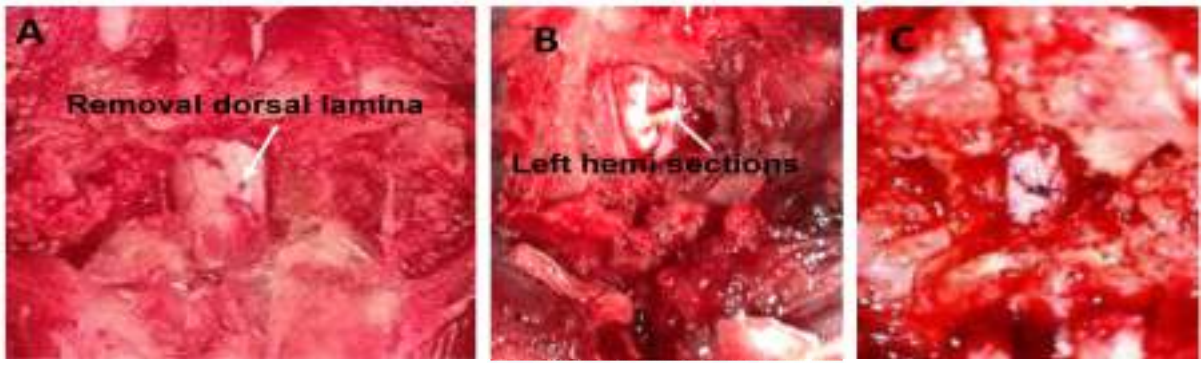

Fig.1: Photograph showing the initial steps of hemicordectomy (A) Surgical incision over dorsal midline andremoval of the dorsal lamina (B)Hemi sections on the left side (arrow) and implanted bone marrow stem cells (D) Dura matter sealing.

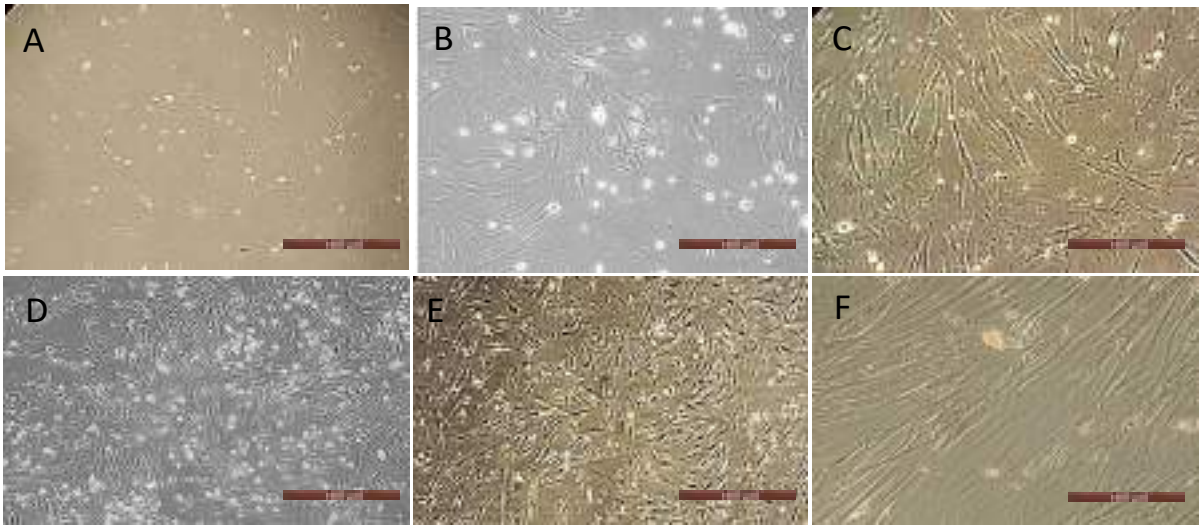

Fig. 2: Light micrograph related to bone stromal stem cells (A) following 48-72 hrs, small heterogeneous cell colonies have been evident, the cells are flattened and round shape. (B) After 10 days, the colonies and cells showed a significant increase, also the colonies were gradually expanded in size to form a monolayer of adherent cells. (C) after 12 days on passage 0 showed spindle polymorphic cells, round and polygonal cells (D) after 2 days of p 2, (E) after 5 days of p 3, (F) after 5 days of p 4, fibroblast-like cells; Scale bar = 100 µm

On (P2), after two days post culture, BMSCs indicated high confluence of spindle-shaped cells, and the cells aligned themselves along their longitudinal axis (Fig.2D). Following 5 days, the BMSCs at the P3 subculture demonstrated a mixture of large, flat, spindle-shaped, round, and polygonal-shaped cells (Fig. 2E). Following P4, indicated fibroblastic morphology spindle-shaped with multiple nucleoli that were common MSC morphology. The cells have kept up their morphology in the case when sub-cultured beyond passage 4 (Fig. 2F).

Immunophenotypic analysis of MSCs

The immunocytochemical staining method was used to a

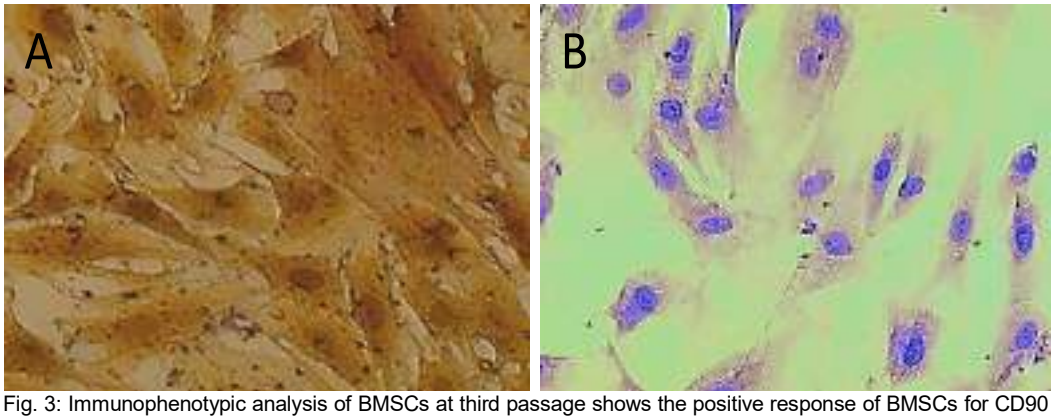

Fig. 3: Immunophenotypic analysis of BMSCs at third passage shows the positive response of BMSCs for CD90 markers and stained with brown color stain (A) and the cells reveal negative response for CD34 markers and stained with blue color with Harris Hematoxylin stain (B) X400

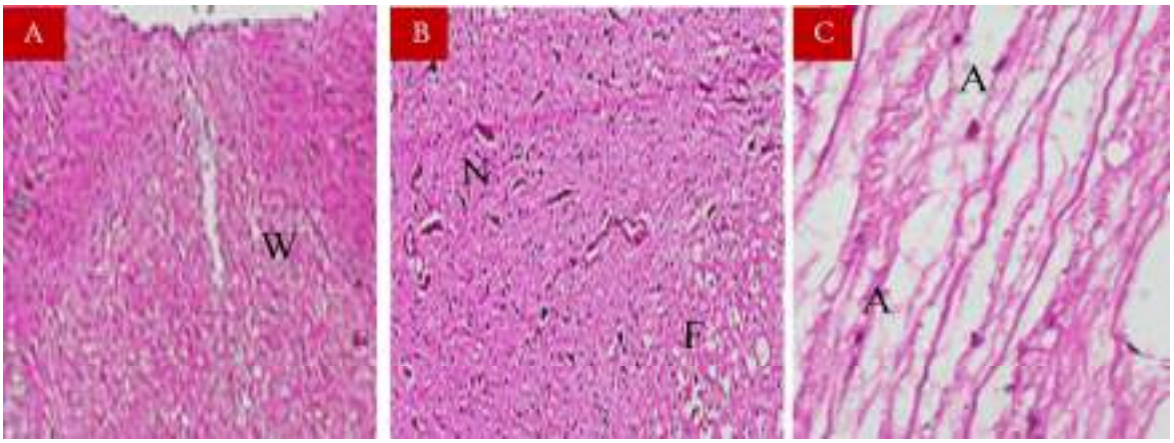

Fig. 4: Light micrographs of the rat spinal cord sections showed in Group1 (A): central canal (C) grey matter (G) and white matter (W) all showing normal structures. (B): multiple neurons (N) in grey matter and nerve fibers each containing an axon (F) in the white matter (100) (C): Multiple axons in the white matter (A) (H&E, X400).

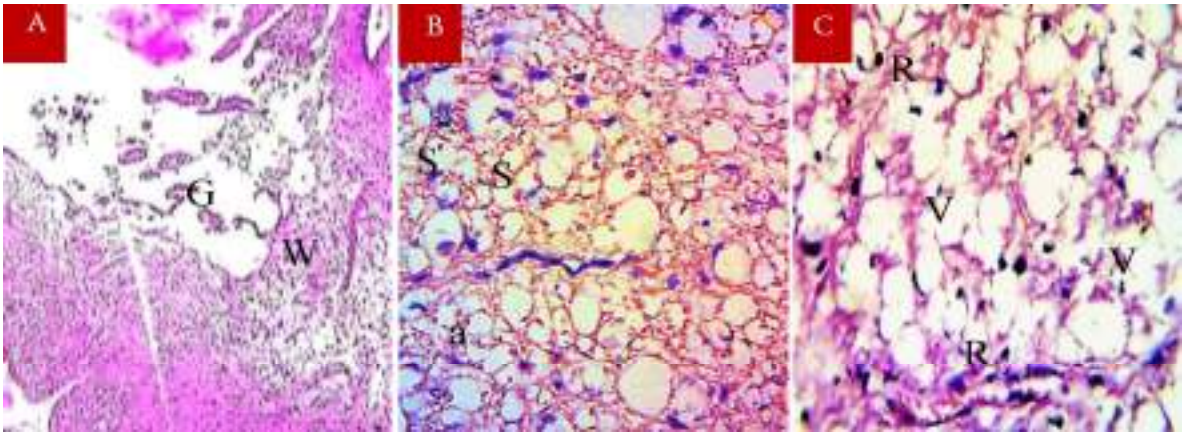

Fig. 5: Light micrographs of the rat spinal cord sections showing in In Group II (A): vacuolations in the grey (G) and white (W) matter (100) (B): swelling of axon fiber (a) with spheroid bodies in spaces of the axon (S) (100) (C): vacuolations in white matter with wallerien degeneration of axon fiber (V) and wide reactive gliosis (R) (H&E, ×) 400).

quick separation of MSCs from a mixed cell suspension. This analysis revealed that most of the adherent MSCs were positive for $CD90^{+}$ markers and were strongly stained with brown color (Fig.3A). Interestingly, these cells were negative for $CD34^{-}$ which was strongly stained with blue color by utilizing Harris Haematoxylin stain (Fig. 3B).

***In vivo* effects of BMSC transplantation on the spinal cord injury**

The neurological evaluations areprovided in Table 1. With regard to group 2, neurological scores have been low. Whereas, in group 3, the Tarlov scale increases and showed a considerable difference ($P \leq 0.05$) in comparison to group 2.

Histological findings

After 16 weeks, rats were evaluated histologically is illustrated in Fig. 5 & 6 below. With regard to group ², it was indicated that the central canal, grey matter, and white matter all with normal structure (Fig. 4A). Multiple neurons have been indicated in the grey matter as well as nerve fibers with axons are identified in cross-sections regarding the white matter (Fig. 4B). In addition, the longitudinal sections related to white matter displayed multiple continuous axons (Fig. 4C). Yet, in group 2, the vacuolation is identified in white and grey matter (Fig. 5A). The swelling of axon fiber with spheroid bodies in spaces of the axon (Fig. 5B), Disrupted axons are identified in white matter with wallerien degeneration of axon fiber and wide reactive gliosis (Fig. 5C). While, in group III showednarrow cavitation surrounded by dense regenerative nerve fiber (Fig. 6A), Injury site apparently normal regenerative neuron cells in gray matter and very prominent axonal regeneration associated with the proliferation of glial cells, in white matter regenerate axons arranged in transverse and longitudinal directions (Fig. 6B). Injury site filled with a high density of regenerative nerve fiber with a proliferation of glial cells and angiogenesis (Fig. 6C).

Table 1: Statistical analysis of motor clinical observation in every group

Groups	Group II (Control)	Group III (Treatment)
14 day	C 0.00 ± 0.00b	D 1.00 ± 0.00 a
28 day	C 0.00 ± 0.00c	C 2.00 ± 0.00 a
56 day	B 0.50±0.28 b	B 4.50± 0.28a
112 day	A 1.25 ± 0.25b	A 6.00± 0.00a
LSD	0.50*	1.105 *

Discussion

The current examination indicated that BMSC transplantation improved the motor function as estimated by the modified Tarlov Scale, locomotor rating scale. These outcomes proposed that improved the functional recovery maybe because of the trophic support or paracrine effect neurotrophic factors, cytokines, along with neuro-protective factors that have been secreted vianeurally-differentiated cells could be further conspicuous therapeutic impact compared to the integration related to grafted BM cells as well as establishing the functional associations (Chi *et al*., 2015). Many reports (Horky *et al*., 2006; Corti, *et al*., 2002) explained that neurotrophic factors including GDNF, BDNF, NGF, NT-3, or the ones secreted from the transplanted stem cells. Our finding demonstrated that neuro histopathological examination in group II (control group) showedexhibited vacuolation in the gray matter because of the necrotized and degenerated neurons, neuronal cell necrosis resulting from direct trauma that happens following spinal cord secondary injury. In addition, the ischemia resulting from insufficient supply of blood to the tissues results in hypoxia as well as a decrease in perivascular pH from the accumulation related to acid metabolites including lactate, such tissue perfusion might be increasing the cellular damage via promoting an influx regarding free radicals as well as other toxic byproducts, that have been in concurrence with the investigation directed via Anwar *et al*. (2016). What's more, swelling of axon fiber with spheroid bodies in spaces of the axon; this attributed to Wallerian degeneration is the rapid degeneration of the distal stump of a transected axon after injured spinal cord. These results correspond with Wu *et al.*

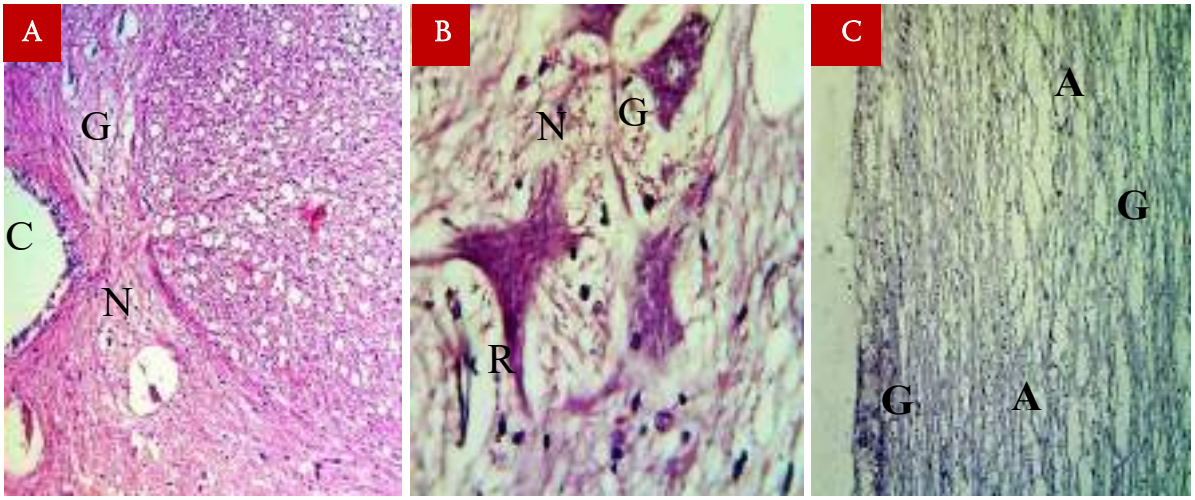

Fig. 6: Light micrographs of the rat spinal cord sections showing in Group III (A): central canal (C) narrow cavity (G) surrounded by dense regenerative nerve fiber (N) (100) (B): injury site apparently normal regenerative neuron cells in gray matter (N) and very prominent axonal regeneration associated with proliferation of glial cells (G), in white matter regenerate axons arranged in transverse and longitudinal directions (R) (400) (C): injury site filled with high density of regenerative nerve fiber with proliferation of glial cells (G) and angiogenesis (A) (H & E, × 400).

(2003).

In group III, (treated group) demonstrated the BMSCs have low migration of the inflammatory cells. In addition, a likely explanation regarding such phenomenon was that BMSCs shows low expression levels related to histocompatibility complex antigens (class 2) as well as releasing soluble factors and cytokines for the purpose of regulating the inflammation reaction in response to the SCI. This outcome concurs with an investigation got by Boido *et al. (*2014) suggesting that the capability of transplanted BMSC for the penetration into a glial cyst and reducing its volume might improve the axonal regeneration as well as degrading the nerveinhibitory molecules at the site of theinjury.

Therefore, implant BMSCs revealed acceleration and enhancement in myelin sheath formation and the neovascularization regarding the injured spinal cord. BMSC's might be reducing the lesion cavity size through diminishing the glial cyst and increases the sparing related tothe white matter, as stated via Forostyak *et al*. (2013). Our findings recommend that BM-MSC reduced cystic cavity, offered orientation in terms of regenerative nerve fibers in the white matter, more regenerative cells of theneuronsin the gray matter, increase in angiogenesis along witha formation of a limited scar tissue at the site of the sectioned spinal cord. Zhou *et al*. (2016) detailed that after BMSCs were utilized *in-vivo*, BMSCs were quickly attacked and degraded via macrophages and other immune cells. Throughout release growth factors, degradation, and cytokines TGF,VEGF, bFGF, and PDGF are contributing to healing. BMSCs apparently promote axonal regeneration through a paracrine effect by delivering NGF, BDNF, FGF -2, VEGF, IGF-1, and TGF-β (Mahmood *et al*., 2004).

In conclusion,the transplantation of autologous bone marrow-derived mesenchymal stem cells might be one of the significant cell-basedtreatmentsin terms of SCI, conceivably extend to using such cells to treat other neurological diseases.

Acknowledgments

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