Rat bone marrow stromal cell transplantation ameliorates complete spinal cord injury induced sensorimotor dysfunctions and associated neurotransmitters

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Traumatic spinal cord injury (SCI) leads to sensorimotor dysfunction with significant impact on the patient and their family’s quality of life, social, and economic status. There is no complete restorative treatment so far. Bone marrow stromal cells (BMSCs) have anti-inflammatory and neuroprotective effects and recently emerged as a therapeutic candidate for SCI repair. Here, we examined the role of rat BMSCs transplantation on thoracic (T11) complete SCI induced dysfunctions, namely hyperalgesia, allodynia, locomotion, spinal reflexes, and spinal neurotransmitters in rats. Pre-labelled BMSCs were injected on day 9 after SCI locally. We observed that BMSCs transplantation facilitate locomotor recovery (week 2-8) and attenuated hyperalgesia and allodynia to varying sensory stimuli (week 6-8) after SCI. In addition, spinal reflexes and neurotransmitters were affected significantly by complete SCI, which were partially restored by BMSCs transplantation. Histological analyses also revealed the presence of BMSCs at the injury site and appear to fill the lesion cavities, thereby significantly reducing the lesion volume. Our data shows the beneficial effects of BMSCs transplantation on complete SCI-induced sensorimotor functional deficits in rats.

Keywords: Allodynia, Hyperalgesia, H-reflex, M-response, Nociceptive flexion reflex

Complete spinal cord injury (SCI) involves cellular, vascular, ionic, and neurotransmitter imbalance associated with structural and functional impairment of the axons, neurons, glia, and a tilt of biochemical milieu towards inhibition of regeneration and repair1-2. All these secondary processes promote inflammation and demyelination in the vicinity of the lesion site, leading to the formation of a glial scar, cavitations, and alter supra-spinal and spinal neurons sensory processing3,4. The SCI-induced hyperalgesia and allodynia are reported irrespective of injury mode, study parameters and time of evaluation, which is primarily contributed by hyperexcitability of the dorsal horn5. Activation of several cell signalling cascades, in turn, triggers microglia and excitotoxic neurotransmitters that further lead to hyperexcitability2,6. The increase in excitatory amino acids such as glutamate and serotonin contribute significantly towards hyperexcitability via receptors at microvessels and accumulation of intracellular calcium, while the descending serotonin pathway from the rostral ventromedial medulla is crucial for the genesis and maintenance of the persistent hyperalgesic state3,7-9. Besides, it also promotes secondary injury by decreasing blood flow and promoting edema8. It is apparent from the available literature that the etiology of SCI hyperalgesia and allodynia involves multiple factors constituting a secondary injury1-9.

Bone marrow stromal cells (BMSCs) have an advantage amongst stem cells as they are relatively easy to obtain, rapid expansion in vitro, non-immunogenic, capacity to migrate to the areas of inflammation, and differentiate into neurons or glia cells10,11. All these processes thereby contribute to reducing the lesion volume. However, there are only a few behavioral studies available only in the contusion and compression SCI models regarding the beneficial effects of BMSCs transplantation12-15. However, there is no available study to correlate the behavioral, electrophysiological, neurotransmitter and histological deficits in sensorimotor functions after thoracic complete SCI. In this study, we explored whether BMSCs transplantation improves the correlates mentioned above in rats.

Materials and Methods

Animals

Adult male Wistar rats (n=57) were used in the present study. They were housed individually

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(polypropylene cage) in the Experimental Animal Facility of All India Institute of Medical Sciences (AIIMS), New Delhi. They were maintained under standard conditions (24±2°C, 14:10 h light-dark cycle) and fed a standard diet. The Animal Care Committee of AIIMS approved the animal protocols in accordance with standardized Animal Care Guidelines. After SCI, rats were divided into two experimental groups as SCI rats with vehicle injection (SCI, n=17) and SCI rats with BMSCs injection (SCI+BMSC, n=15). The third group was subjected to laminectomy only (Sham, n=15) as a non-SCI control.

Exclusion

Out of above-mentioned animals, 5 rats were excluded from the study because of the bursting of the urinary bladder (n=1, SCI) and self-injurious behavior (SIB; n=3, SCI restricted to limb only; n=1, SCI+BMSC-restricted to a toe only). The rats were sacrificed after they indulged in SIB.

Spinal cord injury and post-operative care

All rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg, IM)\(^2\). The spinal cord was exposed after laminectomy and then transected (T11 vertebra) completely by a fine dura microscissors. The spinal cord ends were lifted to ensure the completeness of injury. The gap was filled with gel-foam (AbGel, India) before muscles and skin were stitched layer by layer. Body temperature was maintained (37±0.5°C) during and after SCI by thermostat pad (CMA 150, USA). All the rats have received 5 mL lactated Ringer’s (subcutaneous); systemic and local antibiotics (gentamycin, 15-20 mg/kg for 5-7 days; cefazolin, 50 mg/kg; betadine solution). Their general hygiene was maintained while urinary bladder was manually evacuated twice a day. Paraplegia was confirmed after SCI day 1 by the absence of paw pinch response and nadir BBB scores.

Isolation and characterization of BMSCs

A set of inbred rats (n=10) were used for BMSCs isolation and culture studies according to standard protocol\(^{16}\). Briefly, stroma was flushed out using a syringe (16G) with culture media (5 mL) from bone shaft under aseptic conditions and the single cell suspension was prepared by repeated pipetting. Cells were then plated onto culture flasks (T25 cm\(^2\), BD Biosciences) and cultured in Dulbecco’s modified eagle’s medium (DMEM-low glucose) including fetal bovine serum (15% FBS, Hyclone), glutamax (2 mM, Invitrogen) and Pen-Strep (100 U/mL, Invitrogen). The non-adherent cells were removed after 48 h and replenished with fresh medium. Cells were trypsinized (0.53 mM, EDTA, Invitrogen) at the 80-90% confluency (12-15 days), and re-seeded (5000 cells/cm\(^2\)) into culture flask (T25 cm\(^2\)). Cells were then sub-cultured for subsequent propagation after 5-7 days (90% confluency).

Cell surface antigens (CD44, CD45, CD90, and HLAII) were detected to characterize BMSCs using flow cytometry (LSR II, BD Biosciences). Briefly, rat BMSCs were dissociated (Tryple Express, GIBCO), re-suspended in wash buffer (Phosphate Buffered Saline (PBS) + 1% FBS + 1% sodium azide; 1×10\(^6\) cells/mL), centrifuged (1200 rpm for 5 min), and the pellet thus formed was resuspended in PBS. Subsequently, they were incubated with conjugated monoclonal antibodies of CD44-FITC, CD45-PE-Cy5, CD90-PE, and HLAII-Biotin (BD Biosciences) for 1 h at 4ºC washed once with bovine serum albumin (1% BSA) in PBS and resuspended in the same buffer (100 µL). The secondary antibody Streptavidin-FITC for HLAII (BD Biosciences) was added and incubated for 1 h in dark. Cells were then washed twice with wash buffer and re-suspended in it (300 µL) for analysis. Appropriate isotype-matched controls were used to set the instrument parameters. Cells were acquired using flow cytometer and expression of markers was assessed thrice using software (BDFACS DIVA 6.1.2).

Labeling and transplantation of BMSCs

Cells were harvested (trypsin-EDTA, Invitrogen) and counted using a hemocytometer before they were labeled with a PKH26-GL dye according to manufacturer’s protocol (PKH26-GL kit, Sigma-Aldrich). On SCI day 9, labeled BMSCs (~2.5×10\(^5\) cells in 10 µL PBS) were slowly injected (5 min) at the injury site under anesthesia. The needle was left in place for additional 5 min to prevent the backflow of cells.

Assessment of locomotor function

Rats were assessed with the Basso, Beattie, and Bresnahan locomotor rating scale (BBB score) in activity monitor (Coulbourn Instruments, USA) on day 1 and then weekly after injury\(^{17}\). The BBB is a 21-point scale designed to assess hindlimb locomotor recovery following thoracic SCI. A BBB score of 0 indicates no hindlimb movement while BBB score of 21 indicates normal locomotion. The urinary bladder was evacuated before BBB score assessment. Two unbiased observers who were blinded to animal identity assessed the BBB score (4 min session).
However, any discrepancy in the scores of observers was corrected using BBB score video.

**Assessment of urinary bladder control**

Bladder control was assessed after SCI according to the standard protocol with some modification in the assessment categories\(^{18}\). Bladder control was noted as per the following category 1=spontaneous evacuation, 2=requirement for partial assistance, 3=total assistance for evacuation, and 4=despite efforts the bladder wall ruptured.

**Sensorimotor response of tail to thermal (non-noxious/noxious) and electric stimuli**

Nociception after SCI was studied by recording tail flick latencies (TFL). It was noted after immersion of tail (4 cm from base) into an inner glass reservoir of Dale’s bath (INCO, India) filled with water of varying temperatures (5±0.5-50±0.5°C). The water of the inner bath was altered after 30 min from the non-noxious (30°C) to noxious cold (10, 5°C) and hot (45, 50°C). A cut-off time of stimulus was pre-set to circumvent tail injury (60 s for 5-45°C and 15 s for 50°C)\(^{19}\). Threshold of the tail flick (TTF) was determined by stimulating the nociceptive afferents (biphasic square wave pulses, 40 Hz, 0.2-5.0 mA) with a pair of subcutaneous needle electrodes (AMBU, Malaysia) inserted at the ankle under deep anesthesia\(^{18}\). All the 16-online responses were averaged and transferred to a personal computer for analysis (D-147, DATAQ, USA).

**H-reflex and M-response**

These responses were recorded from the interosseous muscle between 4\(^{th}\) and 5\(^{th}\) metatarsals after stimulating the tibial nerve (0.1 ms pulses, 0.16 Hz, 0.2-5.0 mA) with a pair of subcutaneous needle electrodes (AMBU, Malaysia) inserted at the ankle under deep anesthesia\(^{18}\). The strength of stimulus was increased in steps of 0.2 V until the rat flicked its tail, which was noted as TTF.

**Sensorimotor response of paws to thermal and chemical (acetone) noxious stimuli**

Hindpaw withdrawal latency (HPWL) response was noted by placing the rat on a hot plate (52±0.5°C, cut-off time-30 s; Omnitech Electronics. Inc, USA). The time between placements of the rat to the withdrawal of hindpaw(s) was frozen on the monitor and recorded as HPWL\(^{20}\). The hindpaw motor response to noxious cold was also tested using acetone (80%). Acetone (100 µL) was sprayed on the plantar surface of hindpaw and responses were noted by two observers blinded to the rat group identity. The response to acetone was categorized as: 0-no response; 1-startle; 2-withdrawal of paw; 3-withdrawal of paw/licking of paw/frowning/vocalization; 4-category 3 persisting for >10 s\(^{21}\).

**Nociceptive Flexion Reflex (NFR)**

The NFR is a withdrawal reflex involving numerous spinal cord synapses in response to activation of A\(\delta\) nociceptors\(^{22}\). It was recorded from the biceps femoris by subcutaneous electrical stimulation (0.16 Hz, 2 ms, 0.6-11 mA in incremental steps of 0.2 mA) of the sural nerve receptive field (toes 4, 5) through a set of needle electrodes (AMBU, Malaysia) under deep anesthesia. Reflex responses thus obtained were displayed on cathode ray oscilloscope (CRO; Nihon Kohden, Japan). Online, eight responses were averaged and transferred to a personal computer for analysis (D-147, DATAQ, USA).

**Neurotransmitter estimation**

Neurotransmitter concentration was estimated once after 8 weeks of SCI using liquid chromatography-mass spectrometry in different segments (cervical, thoracic, injury, lumbar, and sacral) of the spinal cord\(^{23}\). Briefly, rats were decapitated, and spinal cord segments were collected promptly, weighted and stored immediately (−80°C) until analysis. API 4000Q Trap (ABS Biosystems, USA) that operated in the positive ion mode coupled with Thermo Finnegan ultrahigh-performance liquid chromatography was used for the quantification (ABS Biosystems, USA). The inbuilt algorithm to define source and compound dependent parameters was used to reach maximum sensitivity for quantification. Singly charged Q1 mass for serotonin (5-HT), dopamine (DA), norepinephrine (NE), gamma-aminobutyric acid (GABA), glutamate (Glu), glycine (Gly), and homatropine (internal standard, IS) was determined (177.1, 154.1, 170.1, 104.2, 148.1, 76.1, 276.1, respectively). A program of linear gradient reaching 40% of acetonitrile (with 0.1% formic acid; FA) at 8 min against 0.1% FA in milliQ water (flow rate of 500 µL/min) was used to separate the neurotransmitters (sensitivity-2 ng/mL) using Supelco-C18 column. On the estimation day, pre-weighed spinal cord tissues were thawed and chopped in extracting solvent (200 µL of 70% acetonitrile in 0.1% FA+500 ng/mL IS). It was subjected to vortex (1 min) and homogenization (1 min) using Polytron (PT-2100, Switzerland). To the above homogenate, extracting solvent (300 µL) was added to make 500 µL. This was sonicated (6 min) followed by centrifugation (7840 g for
The 200 µL of supernatant was subjected to quantification.

**Histology and tracking of transplanted BMSCs**

Rats were slowly perfused intracardially with 4% paraformaldehyde and their spinal cords were separated, immersed in the same fixative (4ºC) for 24 h before being switched to 30% sucrose solution. Longitudinal cryosections (20 μm) were cut (Microm HM 550, Thermo Scientific, USA) and adhered to gelatin-pre-coated slides. These sections were examined for transplanted BMSCs under a fluorescent microscope (Nikon Eclipse 80i, Japan). Five central sections were studied per rat for determination of SCI-lesion volume (NIS Elements AR 3.0, Japan)²⁶.

**Statistical analysis**

Data are represented as mean±standard error of the mean (SEM). Neurotransmitter values were log-transferred before analysis. Differences between groups were examined for statistical significance at each time point using a one-way analysis of variance (ANOVA) with a Bonferroni posthoc test. Data within the group (week 0 vs. week 1-8) were compared by a generalized estimated equation with a Bonferroni posthoc test. The Fisher’s exact test was used to see overall changes between the groups followed by a Bonferroni posthoc test (Acetone data). The Kruskal-Wallis test was applied to examine overall change between the groups followed by the Wilcoxon Rank-Sum test with a Bonferroni posthoc test (GraphPad Prism5, USA) for multiple comparisons in urinary bladder recovery. A P value <0.05 was considered statistically significant.

**Results**

**Characterization of BMSCs**

In three different culture studies, cells were positive for CD44 and CD90 while negative for CD45 and HLAII, representing the true phenotypes of rat BMSCs in an undifferentiated state [Fig. 1A (i-iv)].

![Characteristics of rat BMSCs and their role in the functional recovery after complete SCI. Representative phase contrast image of BMSCs after passage 3 (A-i). Their characterization was done by flow cytometry (A-ii) for cell surface expressing antigen markers (A-iv). Data are presented for surface markers of BMSCs (A-iv) with their respective isotype (A-iii). Scale Bar= 100 µm (A-i); (B) Evaluation of locomotor function by BBB scores after SCI for 8 weeks. Data are presented as the mean±SD; and (C) Urinary bladder control after SCI. Bladder assessment was done after complete SCI during the period of 56 days. Data are represented as the median in day’s required for full assistance to no assistance (automatic) during micturition after SCI. [TP-transplantation; FITC-fluorescein isothiocyanate; PE-phycoerythrin; FSC-forward scatter; SSC-side scatter]
Assessment of locomotor function

The BBB scale was used to analyze the recovery pattern in these rats after complete SCI. The score was decreased to 1.0±0.0 at first week in the SCI and SCI+BMSC groups and gradually improved to 2.43±0.53 and 8.5±0.67, respectively, after 8 weeks (Fig. 1B). BBB score of the SCI and SCI+BMSC groups was lower \((P <0.001)\) compared to the Sham group (week 1-8, \(P <0.001\)), while it was improved (week 2-8, \(P <0.001\)) in the SCI+BMSC compared to SCI group (Fig. 1B).

Assessment of urinary bladder control

Rats were completely lost the bladder control after complete SCI and needed full assistance for manual micturition daily. Bladder control was attained at median day 21 (ranges 18-26 days) in the SCI rats, while it was attained significantly earlier \((P=0.0001)\) at median day 16 (ranges 12-19 days) in the BMSCs transplanted rats (Fig. 1C). Even after the spontaneous bladder evacuation, these rats needed everyday attention to check for any blockage after SCI.

Sensorimotor response of tail to thermal (non-noxious/noxious) and electric stimuli

Sensory testing was done once before and every alternate week after SCI starting at post-SCI week 2. TFL to thermal non-noxious (30°C), noxious water (5, 10, 45, 50°C), and TTF did not vary \((P >0.05)\) in the Sham group of rats during the study period (Fig. 2).

**TFL to warm (30°C) and cold (10°C) stimulus**

TFL decreased \((P <0.012)\) in the SCI (week 4 and 6 only) and SCI+BMSC (week 4 only) groups as compared to their respective week 0 TFL [Fig. 2A (i) and (ii)]. When compared to the Sham group, TFL in the SCI group was lower during week 4-8 \((P <0.001,\ ANOVA)\) while it was lower at week 4 only \((P <0.001,\ ANOVA)\) in the SCI+BMSC group. BMSC transplantation in SCI rats significantly restored TFL to warm and cold stimulus from week 6 onwards [Fig. 2A (i) and (ii)].

**TFL to cold (5°C) and hot (45°C) stimulus**

TFL decreased \((P <0.012)\) in the SCI (week 4-8 at 5°C while week 6-8 at 45°C) and SCI+BMSC (week 4-8 at both 5 and 45°C) groups as compared to their

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**Fig. 2** — Sensorimotor response of tail and paws to thermal, electric, and chemical stimuli. Withdrawal latencies of tail (TFL, A-i to A-v) to thermal (non-noxious/noxious) and electric stimuli (TTF, A-vi) of Sham (n=15), SCI (n=13) and SCI+BMSC (n=14) groups. Similar responses of hindpaw (HPWL) were assessed to thermal (B-i) and chemical stimuli (B-ii). *indicates comparison of Sham versus SCI/SCI+BMSC groups, while #indicates comparison of SCI vs. SCI+BMSC group. "*/#P<0.05, "***/###P<0.01, and "*****/####P<0.001.
respective week 0. When compared to the Sham group, TFL in the SCI group was lower ($P < 0.001$, ANOVA) during week 4-8 (5°C) and week 6-8 (45°C) while it was lower at week 4-6 ($P < 0.001$) and week 8 ($P < 0.05$) in the SCI+BMSC group. However, BMSCs transplantation improved ($P < 0.001$ versus SCI) the TFL to cold (week 6-8) and hot stimulus [week 4-8; Fig. 2A (iii) and (iv)].

**TFL to hot (50°C) stimulus**

TFL decreased ($P < 0.012$) in the SCI (week 4-8) and SCI+BMSC (week 4-6) groups as compared to their respective week 0. When compared to the Sham group, TFL in the SCI group was lower ($P < 0.001$, ANOVA) from week 4 onwards while it was lower at week 4-6 ($P < 0.001$, ANOVA) and week 8 ($P < 0.05$, ANOVA) in the SCI+BMSC group. However, BMSCs transplantation improved ($P < 0.001$, ANOVA versus SCI) TFL at week 8 [Fig. 2A (v)].

**Threshold of the tail flick (TTF)**

TTF decreased ($P < 0.012$) in the SCI (week 2-8) and SCI+BMSC (week 2-4) groups as compared to their respective week 0 [Fig. 2A (vi)]. A TTF of SCI and SCI+BMSC groups was lower during week 4-8 ($P < 0.001$) and week 2-4 ($P < 0.001$), respectively as compared to Sham group. However, TTF was significantly improved in the SCI+BMSC group from week 6 onwards ($P < 0.001$, ANOVA) as compared to SCI group.

**Sensorimotor response of paws to thermal (52°C) and chemical (acetone) noxious stimuli**

HPWL and response to acetone did not vary ($P > 0.05$) in the Sham group during the study period (Fig. 2B). HPWL decreased ($P < 0.012$) during week 4-8 in the SCI and SCI+BMSC groups as compared to their respective week 0. When compared to Sham group, HPWL was lower ($P < 0.001$, ANOVA) in the SCI (week 4-8) and SCI+BMSC (week 4-6 only) groups, respectively. However, BMSCs transplantation improved ($P < 0.001$, ANOVA) HPWL response during week 6-8 [Fig. 2B (i)].

During the acetone test, a number of rats in category 3 (withdrawal of paws) gradually increased from 42.86% (week 4, $P < 0.05$, ANOVA) to 100% (week 6-8, $P < 0.001$, ANOVA) in the SCI group while it decreased from 33.33% (week 2, $P < 0.05$, ANOVA) to 8.33% (week 8, $P > 0.05$, ANOVA) in the SCI+BMSC group. The rats in category 3 were fewer ($P < 0.01$) during week 6-8 in the SCI+BMSC group as compared to SCI group [Fig. 2B (ii)].

**Nociceptive flexion reflex (NFR)**

The NFR threshold increased ($P < 0.001$) and amplitude and duration decreased ($P < 0.01$ and $P < 0.001$, respectively) in the SCI as compared to Sham group, whereas all the NFR studied parameters were comparable in the SCI+BMSC group (Fig. 3B). However, threshold, latency, amplitude, and duration

![Fig. 3](image-url)

**Fig. 3** — Representative graphs of nociceptive flexion reflex (NFR) in the Sham (a, b), SCI (c, d) and SCI+BMSC (e, f) groups are presented. (A) Graphs (i), (iii), (v) (4.5, 10, 6.5 mA, respectively) show the stimulus artifact (arrow) while (ii), (iv), (vi) show the threshold for NFR in the Sham (5 mA), SCI (10.5 mA) and SCI+BMSC (7 mA) groups. The data show threshold, latency, duration, and amplitude of NFR (B-i-iv) in the Sham (n=15), SCI (n=13), and SCI+BMSC (n=14) groups. *indicates comparison of Sham versus SCI while #indicates comparison of SCI versus SCI+BMSC group. **P < 0.05, ***P < 0.01, and ****P < 0.001.
of NFR were improved \((P=0.0001, 0.05, 0.003, \text{and} \ 0.0001, \text{respectively})\) in the SCI+BMSC group as compared to SCI group (Fig. 3B).

**M-response**

When compared to Sham group, M-response threshold \((P=0.0001)\), amplitude \((P=0.012)\), and latency \((P=0.005)\) decreased in the SCI while all the M-response studied parameters were comparable \((P >0.05)\) in the SCI+BMSC group except the lower threshold \((P=0.0001, \text{Fig. 4B})\). However, there was a significant improvement in the M-response amplitude \((P=0.002)\) after BMSCs transplantation (Fig. 4B).

**H-reflex**

The threshold of H-reflex decreased \((P=0.0001)\) in both the groups (SCI and SCI+BMSC) as compared to the Sham group. Latency also decreased \((P=0.0001)\) in the SCI+BMSC group while it was comparable \((P >0.05)\) in the SCI group. The H-reflex amplitude decreased in the SCI (0.37 mV) and improved in the SCI+BMSC (1.15 mV) groups as compared to Sham group (0.88 mV) but it did not attain any statistical significance (Fig. 4B).

**Neurotransmitter estimation**

When compared to Sham group, 5-HT increased in the thoracic \((P=0.009)\), injured \((P=0.006)\), and lumbar \((P=0.0001)\) segments of spinal cord in the SCI group, whereas an increase was evident only in the thoracic \((P=0.003)\) and lumbar \((P=0.0001)\) segments in the SCI+BMSC group (Fig. 5A). GABA increased throughout the spinal cord in the SCI and SCI+BMSC groups, though the increase was lesser in the latter group (Fig. 5B). NE increased in the thoracic \((P=0.004)\), injured \((P=0.0001)\), and sacral \((P=0.005)\) segments of the spinal cord in the SCI group while it increased in the thoracic \((P=0.007)\) and sacral \((P=0.013)\) segments only in the SCI+BMSC group (Fig. 5C). DA increased in the thoracic \((P=0.007)\), injured \((P=0.0001)\), lumbar \((P=0.0001)\), and sacral \((P=0.0001)\) segments in both SCI and SCI+BMSC groups (Fig. 5D). Glutamate increased in both SCI \((P=0.0001)\) and SCI+BMSC \((P=0.011)\) groups in the injured segment only (Fig. 5E). Glycine increased in the thoracic \((P=0.01)\), injured \((P=0.05)\), lumbar \((P=0.01)\), and sacral \((P=0.03)\) segments in the SCI group, whereas it only increased at thoracic \((P=0.017)\) segment in the SCI+BMSC group (Fig. 5F). However, an increase in the studied neurotransmitters was attenuated in the injured segment of the spinal cord in the SCI+BMSC group except for DA.

**Histological analysis and tracking of transplanted BMSCs**

The spinal cord histology showed differences between Sham (Fig. 6A), SCI (Fig. 6B), and SCI+BMSC groups (Fig. 6C). The injured spinal cord shows the lesion cavities in the SCI group (Fig. 6B) while BMSCs were identified and tracked at lesion site (Fig. 6C and F). BMSCs were appeared to survive and filled the lesion cavities in the SCI+BMSC group (Fig. 6C, F). The lesion volume was decreased \((P=0.009)\) significantly in the SCI+BMSC group as compared to SCI group (Fig. 6D).

**Discussion**

In this study, BMSCs transplantation resulted in the attenuation of hyperalgesia and allodynia significantly in the complete SCI rats. In addition, BMSCs reduced...
lesion volume, normalized elevated neurotransmitters, improved urinary bladder control, and locomotion. Several therapeutic strategies including stem cell transplantation were attempted to facilitate SCI repair, although they remain unsatisfactory.\textsuperscript{10,11,23} BMSCs were singled out amongst all transplanted cells types, as they are easily isolated, cultured, non-immunogenic and bypass ethical issues associated with the use of stem cells.\textsuperscript{23} The outcome of sensorimotor behavior and SCI-size by transplanted BMSCs are dictated by a number of contributing factors including transplantation day, injury severity, 

Fig. 5 — Concentration of (A) 5-HT; (B) GABA; (C) NE; (D) DA; (E) glutamate; and (F) glycine in the different spinal cord segments of the Sham (☐, n=9), SCI (□, n=7), and SCI+BMSC (☐, n=7) groups. [*indicates comparison of Sham versus SCI/SCI+BMSC groups. \( P < 0.05 \), \( ** P < 0.01 \), and \( *** P < 0.001 \)]

Fig. 6 — Represented photomicrographs of the Sham (n=6, A), SCI (n=6, B), and SCI+BMSC (n=7, C) groups. Arrows indicate the lesion cavity area (B) and presence of BMSCs in the cavity (C) after 8 weeks of complete SCI. Lesion volume of the injured spinal cord compared between the SCI and SCI+BMSC groups (D). PKH26 labeled BMSCs \textit{in vitro} are shown (E) before transplantation. They were also tracked in the injured spinal cord after 8 weeks of SCI (white arrows, F). Scale Bars A-C=1000 µm. W-white matter, G-grey matter
age at SCI, and trophic factors release that enhances angiogenesis and normalization of blood flow\textsuperscript{15,23-25}. Complete SCI at thoracic level spares the central pattern generators (CPGs) in rodents which continue to generate oscillating coordinated motor patterns, permit and process peripheral inputs, albeit at the spinal level\textsuperscript{26,27}. Complete SCI in our rats sparing the CPGs as reflected by their improved BBB score after BMSCs transplantation. Nevertheless, BBB score of 9 is reported to be critical for locomotion\textsuperscript{17}. The partial functional recovery of locomotion was possibly consequent to underlying establishment of neural connectivity, the release of an optimum amount of neurotrophic factors, and normalization of neurotransmitters. BMSCs survival at the injury site further contributing towards continuous secretion neurotrophic factors and creating a conducive environment for neurogenesis since the sparing of as few as 1-10\% fibers at the injury site has profound effects on basic locomotor recovery in the absence of the more skilled forms of it\textsuperscript{28}. Moreover, trophic factors from BMSCs are reported to facilitate regeneration and exert neuroprotection\textsuperscript{10}. The 5-HT has a pivotal role in the excitability of spinal motor neurons vis-à-vis with motor performance; it increased at an injured as well as nearby segments of the spinal cord in our rats and facilitate the secondary injury via its excitotoxic effect\textsuperscript{6}. However, this increase was attenuated at the injured segment by BMSCs transplantation which has probably facilitated locomotion recovery in our SCI rats. We also observed the urinary bladder recovery after BMSCs transplantation which is also reported earlier\textsuperscript{23,29}. A recent trial (Clinical Trials.gov, NCT02165904) showed that autologous mesenchymal stromal cells improve quality of life and bladder compliance in the incomplete SCI patients (~80\%)\textsuperscript{29}.

Different types of pain (hyperalgesia and allodynia) following SCI are reported in the literature\textsuperscript{3,5,18}. We also observed hyperalgesia and allodynia below the injury in our SCI rats which are supported by both clinical and experimental studies\textsuperscript{3,5,18}. There are several factors responsible for hypersensitivity of sensory neurons to noxious and non-noxious stimuli\textsuperscript{3,5,30}. However, there is attenuation in the hyperalgesia and allodynia in our BMSCs transplanted rats. It may be due to secreted neurotrophic factors from transplanted and survived BMSCs, decreased lesion volume, scar tissue, and related normalized neurotransmitters. Hyperexcitability of the sensory neurons and ascending commissural interneurons in the spinal cord preparation reported implicating with 5-HT\textsuperscript{5,31,32} while nerve injury shifts the balance between descending 5-HT inhibitory and facilitatory influences towards the latter via enhanced activation of pro-nociceptive 5-HT\textsubscript{3} and 5-HT\textsubscript{2} receptors expressed in laminae I-II\textsuperscript{33,34}. 5-HT concentration was attenuated in the BMSCs transplanted rats, which may have contributed towards restoration of eualgesia and sensitivity to different sensory stimuli. Glutamate and GABA increase was also noticed in these rats which are supported by the existing literature; although in other SCI models\textsuperscript{35,36}. Besides 5-HT, GABA, and glutamate were also restored in these rats. It is widely accepted physiologically that GABAergic inhibitory spinal neurons counterbalance the enhanced synaptic transmission from the peripheral input, while the tone is reduced after SCI that permits enhanced synaptic transmission resulting in dorsal horn neuronal hyperexcitability. Besides, the neuroglial unit normally controls intracellular chloride ion gradient via modulation of its transporters, extracellular glutamate, and GABA concentrations via their uptake mechanisms and the intracellular "GABA-glutamate-glutamine cycle". However, it is disrupted after SCI resulting in chronic neuropathic pain\textsuperscript{35}. The excitability of dorsal horn neurons is corrected in pari passu with normalized GABA and glutamate concentration in our BMSCs transplanted rats, which significantly contributes towards the restoration of normal sensitivity. In addition, an elevated level of glycine was also normalized in the BMSCs transplanted rats. Increase in glycine plays a key role in spinal shock as well as responsible for muscle flaccidity after SCI\textsuperscript{36}. Moreover, NE also increased in our studied SCI rats which are in parallel with the immediate increase (within 3-4 h) in the injured segment of cat spinal cord\textsuperscript{37,38} and contradictory to other studies\textsuperscript{39-41}. The discrepancy is probably due to the difference in the mode of injury, evaluation technique and time after SCI. Nonetheless, it is suggested that the toxic quantities of NE are released into spinal cord post-contusion injury that leads to vasoconstriction, ischemia and ultimately, necrosis followed by vascular rupture and hemorrhage. The experimental evidence substantiated the validity of this hypothesis. BMSCs transplantation possibly reduced hemorrhagic secondary injury in our rats as indicated by reduced lesion volume and attenuation of NE. The DA concentration has also been investigated due to their contradictory role in the
pathophysiology of SCI\textsuperscript{37,39,40,42,43}. Results suggested that the trauma-related DA elevation at early time points (1-3 h) may play an important role in lesion development\textsuperscript{39,42}. DA concentration increased significantly in our SCI rats and BMSCs transplantation was ineffective in these rats especially for DA restoration. It is clear from our and other studies that neurotransmitters are the pre-dominant candidates contributing towards secondary injury in our rats\textsuperscript{39-42}. However, several factors are the determinants of concentration namely; assessment technique, neurotransmitter properties, choice of animal model, mode and type of injury, time of evaluation, and the test segment of spinal cord selected\textsuperscript{41,44,45}.

It is evident that the spinal neuronal hyperexcitability underlies the SCI-pain and allodynia\textsuperscript{46} which is reflected by a significant decrease in threshold and latency of H-reflex as well as the threshold, latency, and amplitude of M-response. An alteration in the muscle and nerve compound action potentials was reported after SCI\textsuperscript{18}. This is due to alteration in the axonal excitability and conduction properties which is mediated by a shift in the intra- and extra-cellular ion contents or Na\textsuperscript{+} and K\textsuperscript{+} channels\textsuperscript{19,47}. In addition, a marked decrease in the number of motor neurons due to injury and reduction in motor unit size due to disuse atrophy of muscles\textsuperscript{48} are implied in the decrement of M-response amplitude after SCI. Since the amplitude of M-response was restored by BMSCs transplantation in our SCI rats, it can be presumed that disuse atrophy of the muscles may have been significantly attenuated in them probably secondary to the improvement in their BBB score. We further substantiated the beneficial effect of BMSC transplantation by the recording of polysynaptic NFR in the same rats. The data clearly indicates post-SCI hypoalgesia, which is in sharp contrast to their behavioral hyperalgesia. The controversy is probably either due to our design of the experiment utilizing anesthetized rat or the insult to the reflex center since we recorded the response from biceps femoris after stimulation of the sural nerve. It is difficult to conclude; nevertheless, the polysynaptic neural circuits and the recently injured reflex center of NFR are both susceptible to anesthesia. The NFR parameters were restored by BMSCs transplantation thereby reiterating the previous conclusion of the beneficial effect of BMSCs.

Conclusion
The results have shown that BMSC transplantation facilitates locomotor recovery, attenuates hyperalgesia and allodynia and improves urinary bladder function in the complete thoracic SCI rats when transplanted on day 9 after SCI. The recovery was statistically significant from post-SCI week 6. These beneficial effects may be due to attenuation of lesion volume, normalized neurotransmitters, and survival of BMSCs at the lesion site. Limitation of the present study included host-glial cells validation of survival BMSCs and their differentiation characteristics in the injured spinal cord after transplantation. Further reproducible studies using large animal models are necessary to explore the specific mechanisms of reconstructing pathways, integration, and action of transplanted BMSCs in the SCI.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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