Effects of Local Transplantation of Autologous Bone Marrow Mesenchymal Stem Cells in Combination with Low Level Laser Therapy in Repair of Experimental Acute Spinal Cord Injury in Rats

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Abstract
Objective- The aim of this study was to demonstrate the efficacy of MSCs transplantation in combination with low level laser irradiation (LLLI) in repair of experimental acute spinal cord injury.

Design- Experimental study.

Animals- 28 adult male Wistar Rats.

Procedures- A balloon-compression technique was used to produce an injury at the T8-T9 level of spinal cord applying Fogarty embolectomy catheter. In group-1, the autologous MSCs were transplanted to the spinal cord lesion and followed by treatment with low level laser irradiation during 15 consecutive days in group-2. The injured rats in third group were treated by LLLI alone. The functional recovery was assessed using the Basso-Beattie-Bresnahan (BBB) locomotion scoring within 5 weeks.

Results- In these three treatment groups, the scores were significantly higher than control group. The differences between group-2 and two other treatment groups were statistically significant during all five weeks after treatment. There were no significant differences in BBB score between group-1(MSCs) and group-3(LLLI) at 3rd, 4th and 5th weeks of treatment. According to the histopathological findings, the best response was observed in group-2 (MSCs+LLLI) that repair of injured parts of dorsal funiculi and less cavitation were occurred by proliferation of mesenchymal stem cells and their differentiation to glial cells especially oligodendrocytes resulting in axon regeneration and relatively spinal cord recovery.

Conclusion and Clinical Relevance- The findings of present study demonstrated that concurrent use of LLLI and local transplantation of MSCs exhibited profound effects on axon regeneration and revealed remarkable functional improvement. These results suggested that MSCs characteristics could be influenced by low level laser irradiation, so this treatment might be as a useful procedure for neural regeneration, although further detailed investigations need to be carried out particularly in clinical cases.

Keywords- Spinal cord injury, Mesenchymal stem cells, Low level laser therapy

Received: 12 May 2016; Accepted: 11 September 2017; Online: 31 October 2017
opportunity to achieve functional improvements. To date there is insufficient evidence that would support approved clinical treatments to form neurons and axons immediate protection or to increase their regeneration, but using stem cell therapy in patients with SCI gives hope for opportunity to achieve functional improvement and constitutes a target of great unmet medical demand. Among many treatments being developed for SCI, the placement of different types of grafts embedded with cell populations into areas of damage has been one of the most commonly regenerative approaches, attracting scientists and physicians for the last 15 years. Many kinds of somatic cells have been studied as transplants for the treatment of SCI, which include bone marrow stromal cells (BMSCs), Schwann cells, dental pulp-derived cells, epidermal neural crest stem cells, skin-derived precursor cells, adipose-derived stromal cells, and choroid plexus epithelial cells. MSCs have been being applied in treatment of different disorders including spinal cord injury in animal models. Oliveri et al. in a systematic review with meta-analyses suggested that recovery of locomotor function in traumatic SCI animal models can be promoted by MSCs therapy. Also, it is assumed that implantation of MSCs will have better effect than injection them into blood or cerebrospinal fluid, since it will increase amount of MSCs in damaged site. On the other hand, the use of electrotherapeutics modalities is a common practice in physiotherapy with regenerative purposes, especially using low-level laser irradiation (LLLI) holds promise for future of tissue engineering and regenerative medicine. LLLI can cause in prevention of MSCs apoptosis and improvement of MSCs proliferation, migration, and adhesion at low-powers and low-levels of red/close infrared light enlightenment, which is approved as a dose dependant procedure. Hou et al. documented that LLLI could increase growth factors secretion, stimulate proliferation and facilitate myogenic differentiation of BMSCs. Accordingly; LLLI may give a novel way to deal with the preconditioning of BMSCs in vitro before transplantation.

Thus, this study was conducted to evaluate the effect of transplantation of BMSCs associated in combination with LLLI for the treatment of the contusion-injured spinal cord of rats.

**Material and Methods**

The study was conducted with 28 male Fischer-344 Wistar rats, 8 to 12 weeks of age. In order to decrease the variability of spinal canal size, only animals with body weights between 300-350 g were included. All animals were kept in large and well-lit plastic containers. These containers were kept separately and at laboratory controlled temperature of 21 °C. Additionally, the containers were maintained with a daily photoperiod of 12 hour of light for seven days. Each animal had free access to food and water ad libitum. After 7-day adaptation period, bone marrow was extracted from femur bones of rats. MSCs isolation and propagation lasted a total of 3 weeks. At this time, spinal cord injury was induced in rats. One week after induction of SCI, injection of undifferentiated autologous MSCs was performed by using a Hamilton Syringe. One day after MSCs transplantation, laser therapy was started by a low level laser with a wavelength of 780 nm and a power of 250 mw. Two weeks later, Basso- Beattie-Bresnahan (BBB) functional scoring test was used for assessing the locomotor capacity of rats after SCI, and continued weekly for six weeks. Finally, histopathological evaluations were performed on the histopathological samples of the injured region (Fig 1).

**Isolation of bone marrow stromal cells (BMSCs)**

MSCs were harvested from the femoral bone marrows of rats and then the cells were transplanted into the same rat in order to decrease the chance of rejection. Procedures of extraction, isolation, and propagation of BMSCs were as same as Wakitan. In summary, MSCs were harvested by FNA (fine needle aspiration) technique from the femoral bone marrow. Rats were anesthetized by intramuscular injection of Ketamine HCl and Xylazine at 75 mg/kg and 10 mg/kg, respectively. Following a 5mm length skin incision a small opening (1-1.5 mm) in the femur was drilled. Then, a 2 ml syringe with a 21 G needle containing 500-750 IU heparin was used to aspirate of 0.5-1 ml of bone marrow. The samples were diluted with L-15 medium (2 ml) containing 3 ml of Ficoll. Then, samples
were centrifuged (2,000 rpm) for 15 minutes, then cells in the mononuclear layer were harvested and were re-suspended in 2 ml serum-free medium, then centrifuged (2,000 rpm) for 15 minutes and were re-suspended in 1 ml DMEM.

Spinal cord injury

Induction of SCI in rats was performed by a method described by Vanicky et al. In brief, rats were anesthetized as described above. When they were in stable situation, a midline incision of 2 cm was created over the T10-L1 spinous processes, under sterile conditions. Then the spinous processes of vertebrae T10 – T11 were removed following the dissection of the regional skin and muscles. Under magnification, vertebral arch of T10 was drilled using a micromotor bur. A groove was also drilled in the midline on the dorsal surface of T11 vertebral lamina to guide the insertion of the catheter and keep it positioned in the midline. A saline loaded 2-French Fogarty catheter was linked to an airtight 50-µl Hamilton syringe held in a precise sampling device. After insertion of the catheter into epidural space in a way that the center of the balloon was rested at T8-T9 level of spinal cord, the balloon was distended quickly with 20 µl volume of saline for 5 minutes. Then, serum was removed from the catheter and catheter was pulled out slowly. Skin and the other layers were attached together by appropriate suture placement in anatomical layers. Bladder was evacuated manually at least twice a day until reflex bladder was approved. Antibiotic therapy was performed by Enrofloxacin administration (10 mg/kg, every 24 hour) for one week. All rats were paraplegic after injury, with no signs of functional recovery. All experiments were carried out in accordance with approved guidelines of the Iran Animal Care Committee and were approved by the Faculty of Veterinary Medicine, University of Tehran Animal Care Committee.

MSCs transplantation

Seven days after induction of SCI, rats were anesthetized again as described before and vertebral arches of the T8-T9 were removed using micro-motor and a burr (Stryker Corporation, USA). Injured rats were treated with 1×10⁶ autologous undifferentiated BMSCs by insertion the tip of a 20- µl Hamilton syringe through the intact dura. Tip of the syringe were inserted into the center of the developing lesion cavity 3 mm cranial and 3 mm caudal to the cavity (penetration depth of 1.0 mm at an angle of 40–45° past perpendicular).

Low-Level Laser Irradiation

One day after MSCs transplantation, LLLI procedure has performed in group2 (MSCs+Laser) and group3 (Laser without MSCs) as described below. Briefly, groups 2 and 3 of paralyzed rats were irradiated with red or near-infrared laser via transcutaneous application. LLLI was started immediately one day after surgery and was continued daily for two weeks. A 250mW NIR laser (wavelength 780; continuous wave (CW)) was transcutaneously irradiated over the 1 cm distance between T8 and T10 for 15 min daily (spot size3 mm², laser fluence 10 J/cm²).

Behavioral Assessment

During the 6-week follow up, motor activity of hind limbs was evaluated according to Basso-Beattie-Bresnahan (BBB) open field locomotor rating scale. BBB scores include a wide range from 0-21: 0: no observable movement in hind limbs; 21: normal locomotion). These scales represent the recovery of locomotor activity and are categorized according to the sum of the animal’s joint-movements, hind limb movements, stepping, forelimb and hind limb coordination, trunk position and stability, paw placement and tail position. All of the rats movements were recorded for better analysis and more detailed assessment. Sensory recovery was evaluated by pinch test and observation of withdrawal reflex. This test gives useful information about distance and rate of regeneration of sensory neurons. In summary, rats were placed on examination desk in a way that the muscles of the hind limbs were completely relaxed (knee flexed at 130° and ankle at 90°) using anatomic supports. Then, to evaluate nociceptive withdrawal reflex, Kelly forceps were placed on the rats hind limb fingers and gradual pressure was applied until the animal showed any aversive response such as withdrawal of the limb, vocalization, and struggling.

Histopathological assessment

Five weeks after implantation of the undifferentiated BMSCs in spinal cord, the rats were deeply anesthetized by injection of 100 mg/kg pentobarbital sodium intraperitoneally, and after that perfused transcardially with 200 ml 0.1 M phosphate buffer (pH 7.4) continued by 300 ml 4% phosphate-buffered saline (pH 7.4) containing 4% paraformaldehyde and 1% glutaraldehyde. Spinal cords were sectioned transversely
from T7 to T10. The injured region of the spinal cord was fixed in formalin 10% and then tissue sections were obtained from it. After fixation, transverse sections of spinal cord at T7 to T10 were embedded, cut into 5 μm thick sections, and stained using hematoxylin and eosin. Afterwards, histopathological assessment of cells, myelinated, and dismyelinated neural fibers were performed under 40, 100, 200 magnifications by a pathologist blind to the groups. It is certified that all the animal experiments followed the applicable institutional and governmental regulations concerning the ethical use of animals.

Statistical Analysis

Statistical analyses were done by SPSS package Version 19.0. The data were described by Mean±SEM. The data were analyzed by One-Way ANOVA and Tukey Post Hoc tests. A p-value less than 0.05 were statistically considered significant.

Results

Behavioral analysis

During the 6-week follow up, the locomotor and sensory recovery of rats was weekly evaluated by two observers. Rats in all groups showed no significant locomotion in hind limbs one week after induction of SCI. Three weeks after induction of SCI (two weeks after beginning of therapies), BBB score of group-1 (MSCs) was increased to 4.88. At the same time, in the group-2 (MSCs + LLLI) and group-3 (LLLI), BBB scores were increased to 10.81 and 6.38, respectively. Six weeks after induction of SCI (after five weeks of therapy and/or at the end of behavioral assessment), BBB score of group-1 (MSCs) increased to 8.25. At the same time, BBB score increased to 17.13 and 8.31 for the group-2 (MSCs + LLLI) and group-3 (LLLI) respectively. At week-6, the differences among group-2 (MSCs + LLLI) and two other groups (MSCs and LLLI) were statistically significant (p<0.05). There were no significant differences in BBB score between group-1 (MSCs) and group-3 (LLLI) at week-3, week-4 and week-5 of therapy period (Fig 2).

Histopathological findings

In the control group, the dorsal funiculi of spinal cord were edematous with focal destruction and degeneration of myelin, swelling of axons and microcavitations was apparently seen. Proliferation of astrocytes, microglial cells and especially oligodendrocytes were present. Some of microglial cells and large foam cells were seen around of destructed areas. Gray matter especially in dorsal horns of spinal cord was atrophic with a severe hypo-cellularity of neurons. The number of these large neurons was greatly decreased from 200 neurons in intact part of spinal cord to 30 neurons in injured areas. According to histopathological evaluation, the best response was observed in the group that treated with combination of MSCs and LLLI. In this group, repair of injured parts of dorsal funiculi was occurred by proliferation of mesenchymal cells and differentiation of them to glial cells especially oligodendrocytes. It caused promotion of axon regeneration and relatively spinal cord recovery but hypocellularity of dorsal horn was apparent. There were no acute inflammatory reaction and granulomatous reaction. In laser and MSCs groups, evidences of focal destruction of dorsal funiculi and foam cells, astrocitosis and astrogliosis were still seen. In these groups, repair of injured parts of dorsal funiculi was not completed and proliferation of mesenchymal cells and differentiation of them to glial cells especially oligodendrocytes were mild to moderate. Axons degeneration and hypocellularity of dorsal horn was apparent. There were no acute inflammatory reactions and granulomatous reactions (Fig 3).

Discussion

Results of the BBB locomotors scoring test indicated that concurrent use of laser irradiation associated with undifferentiated BM-MSCs in spinal cord injured rats has better impact than use of laser therapy or BM-MSCs alone. Also it was obvious and believed that use of laser therapy or BMSCs could improve cellular structure of spinal cord and finally locomotor and sensory recovery. Although, MSCs therapy and LLLI could promote axons regeneration and recovery of injured spinal cord,
Figure 3. Histopathological findings in different groups: A, Control Group; B, BMSCs Group; C, LLLI Group; D, BMSCs + LLLI Group. A-1) A microscopic view of spinal cord in control group, dorsal funiculus is edematous with a focal destruction (a), degeneration of myelin and microcavitations (b) is apparently seen especially around of destructed area. Gray matter of dorsal horn shows severe hypocellularity (c) of neurons (H&E, × 100). A-2) A microscopic view of spinal cord in control group, dorsal funiculus is edematous with a focal destruction (a), degeneration of myelin (a), swelling of axons (d) and microcavitations (b) is apparently seen especially around of destructed area (H&E, × 200). B-1) A microscopic view of spinal cord in BMSCs group, in these groups, repair of injured parts of dorsal funiculi is not completed and proliferation of mesenchymal cells and differentiation of them to glial cells (e) is mild to moderate. Axons degeneration (d) and hypocellularity (c) of dorsal horn is apparent (H&E, × 100). B-2) A closer microscopic section of spinal cord in BMSCs group, repair of injured parts of dorsal funiculi is relatively completed and proliferation of mesenchymal cells and differentiation of them to glial cells, axon regeneration (f) and spinal cord recovery is apparent (H&E, × 200). C-1) A microscopic view of spinal cord in LLLI group, in these groups, repair of injured parts of dorsal funiculi is not completed. Axons degeneration (d) and hypocellularity (c) of dorsal horn is apparent (H&E, × 100). C-2) A closer microscopic section of spinal cord in LLLI group, repair of injured parts of dorsal funiculi is relatively completed and proliferation of glial cells (e) and axon regeneration (f) is apparent (H&E, × 200). D-1) A microscopic section of spinal cord in BMSCs + LLLI group, repair of injured parts of dorsal funiculi is completed and axon regeneration (f) and spinal cord recovery is apparent but dorsal horn is hypocellular (c) (H&E, × 100). D-2) A closer microscopic view of spinal cord in BMSCs + LLLI group, repair of injured parts of dorsal funiculi is completed and axon regeneration (f) and spinal cord recovery is apparent. In this group, repair of injured parts of dorsal funiculi is occurred by proliferation of mesenchymal cells and differentiation of them to glial cells (H&E, ×200).
It has been demonstrated that LLLI promotes differentiation and proliferation of human osteoblast cells, in vitro, proliferation of cardiac and mesenchymal stem cells in culture at 1 J/cm² and 3 J/cm². Studies on LLLI have indicated that it could play a major role in many tissue regenerating processes such as wound healing, fibroblast proliferation, nerve regeneration, and collagen synthesis and could increase migration of stem cells in vitro by changing the metabolism of stem cells and increasing the adenosine triphosphate level of MSCs. Furthermore, LLLI can improve proliferation of rat mesenchymal bone marrow and cardiac stem cells in vitro. It is remarkable that laser irradiation at 665-675 nm wave length induces the maximum effect on cell proliferation and growth factors secretion in vitro, whereas irradiation at 810 nm (or higher) inhibited cell division in vitro. LLLI can promote cell proliferation and increase, DNA, and RNA synthesis and collagen synthesis which can initiate mitosis in cultured cells. Also, mitochondrial and cell membrane receptors can be stimulated by LLLI. These receptors can convert the light energy into chemical energy (ATP) which increases cell proliferation rate and improves cellular functions.

Finally recent studies demonstrated that transcutaneous application of a 780 nm laser can improve the axonal regeneration and functional recovery. Therefore, it is assumed laser therapy can affect the grafted BMSCs and amplify their function. Based on histopathological evaluation, in this study, the best response was observed in the group treated with combination of laser-cell therapy. In this group, repair of injured parts of dorsal funiculi was occurred by proliferation of mesenchymal cells and differentiation of them to glial cells, especially oligodendrocytes. It resulted in promotion of axon regeneration and relatively spinal cord recovery.

Acknowledgments
This research was supported by grant from University of Tehran. The authors are grateful to Dr M S Pedram, Dr M S Ghodrati, Dr H Reisdanaei, Mr Isanjad and Mr Baninajar for their technical helps and advices.

Conflicts of interest
None

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ارزیابی اثرات توأم پیوند موضوعی سلول های بنیادی مرانشیمی خودی و درمان با لیزر کم توان در ابزار جراحات حاد

توجه ناخاع در رت

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هدف - هدف از این مطالعه بررسی عملکردی و هیستوپاتولوژیک اثرات توأم پیوند خودی سلول های بنیادی مرانشیمی مغز استخوان و درمان با لیزر کم توان در ترمیم ضایعات جراحات حاد

طرح - مطالعه تجربی

روش کار - آسیب حاد ناخاعی با یک تکرار نخاع بوسیله کاترون بالون در فوکاتری در محل مهره سینه ای 8، به 3 گروه در روز درگردش کردند. هر گروه به ترتیب دو، سه و چهارم صحبت ناشی بود. درگروه دوم، منجر به پاتولوژیک اثرات توأم پیوند خودی سلول های بنیادی مرانشیمی و درمان با لیزر کم توان در ترمیم ضایعات جراحات حاد

نتایج - تغییرات آنالیز رفتاری در هفته‌های اول تا پنجم بین چهار گروه مختلف دیده می‌شد. پ peanut (P<0.001). اختلاف آماری معنی‌داری بین گروه سلول بنیادی مرانشیمی و گروه سلول بنیادی مرانشیمی باعث لیزر کم توان در گروه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به پاتولوژیک اثرات توأم پیوند خودی سلول بنیادی مرانشیمی بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم توان بود. در این گروه سلول بنیادی مرانشیمی به همراه لیزر کم T

کلمات کلیدی - آسیب ناخاع، سلول بنیادی مرانشیمی، درمان با لیزر کم توان، رت

IJVS 2017; 12(1); Serial No:26