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# Induction of mice adult bone marrow mesenchymal stem cells into functional motor neuron-like cells

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ABSTRACT

The differentiation of mesenchymal stem cells (MSC) into acetylcholine secreted motor neuron-like cells, followed by elongation of the cell axon, is a promising treatment for spinal cord injury and motor neuron cell dysfunction in mammals. Differentiation is induced through a pre-induction step using Beta-mercaptoethanol (BME) followed by four days of induction with retinoic acid and sonic hedgehog. This process results in a very efficient differentiation of BM-MSCs into motor neuron-like cells. Immunocytochemistry showed that these treated cells had specific motor neural markers: microtubule associated protein-2 and acetylcholine transferase. The ability of these cells to function as motor neuron cells was assessed by measuring acetylcholine levels in a culture media during differentiation. High-performance liquid chromatography (HPLC) showed that the differentiated cells were functional. Motor neuron axon elongation was then induced by adding different concentrations of a nerve growth factor (NGF) to the differentiation media. Using a collagen matrix to mimic the natural condition of neural cells in a three-dimensional model showed that the MSCs were successfully differentiated into motor neuron-like cells. This process can efficiently differentiate MSCs into functional motor neurons that can be used for autologous nervous system therapy and especially for treating spinal cord injuries.



## 1. Introduction

Spinal cord injury is a spinal lesion of a traumatic nature resulting in disruption of the nerve fiber bundles. It affects ascending and descending sensory and motor information (Julio et al., 2012). Stem cell therapy is a promising treatment method for such injuries because of a stem cell's capacity for self-renewal and differentiation to any cell in an organism, including neuron and glial cells (Tewarie et al., 2009).

Some recent experiments recommended the use of mesenchymal stem cells in treatments for spinal cord injuries based on improved functional recovery and enhanced astrologist in rats treated for balloon-induced spinal cord compression lesions, mesenchymal stem cells have a poor survival rate at the site of nervous tissue damage, limiting the possibility of using them for treatment of spinal injuries (Ritfeld and Oudega, 2014; Cui et al., 2014).

Motor neurons are specialized cells in the central nervous system that transmit electrical signals to the muscles and generate movement. Motor neurons are divided into two groups: the upper motor neurons, which are located at the top of the brain in the motor cortex and extend down the spinal cord to connect with the second group, the lower motor neurons, which extend out of the spinal cord and connect to the muscles (Talbot and Marsden, 2008). Microtubule associated protein 2 (MAP-2) and acetylcholine transferase are differentiation markers that are specific to motor neuron cells (Thonhoff et al., 2009). Acetylcholine is a neurotransmitter found at neuromuscular junctions, particularly in synapses in the ganglia of the visceral motor system but also in a variety of other locations within the central nervous system (Purves et al., 2001). MAP-2 is an important protein in neurons. It plays an important role in the growth, differentiation, and plasticity of neurons and is key in neuronal responses to growth factors, neurotransmitters, synaptic activity, and neurotoxins. This means that modification and rearrangement of MAP-2 is a necessary early step in many processes which modify neuronal function (Johnson and Jope, 1992).

Many studies focus on the differentiation of embryonic stem cells into motor neuron cells through a long process of culturing,

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which makes it difficult to use embryonic stem cells for treatments (Ebrahimi-Barough et al., 2014). In the first two-week phase of this process, pluripotent stem cells are induced to form neuroepithelial (NE) cells that, in the absence of morphogens, form neural tube-like rosettes. Over the next two weeks, the NE cells are specified to OLIG2-expressing motoneuron progenitors in the presence of retinoic acid (RA) and sonic hedgehog (SHH) or purmorphamine (Hu and Zhang, 2009). Other studies on mesenchymal stem cell differentiation into motor neuron cells also require many additives, special media and a long time (Shetty et al., 2015). This paper describes a process for mesenchymal stem cell differentiation into functionally active motor neuron cells using retinoic acid and sonic hedgehog growth factor. This study also examined the elongating capability of the differentiated cell axon and then mimicked the normal tissue structure in a collagen matrix for treatment of spinal cord injuries.

## 2. Materials and methods

### 2.1. Mesenchymal stem cell isolation

The bone marrow culture was prepared as described by Freshney (2000). Three- to six-week-old donor male Swiss albino mice were killed by cervical dislocation. The mice were provided by the ICCMGR Animal House Unite (all work related to the study was approved by the ICCMGR Animal Care and Use Committee). The mouse was killed by cervical dislocation, placed on its back on a cutting board and soaking it with 70% ethanol, followed by making a long transverse cut through the skin in the middle of the abdominal area. The skin reflected from the hindquarters and the hind legs then, the muscles were removed and the bone placed in a Petri dish containing medium MEM (US biological) free serum. Bone marrow mesenchymal stem cells were isolated from the thigh by flushing with a 1 ml syringe containing 1 ml of a growth culture medium (MEM).

The freshly isolated whole bone marrow cells were re-suspended in a 5 ml growth culture medium (MEM), supplemented with 20% FBS and 1% ampicillin/streptomycin at 37 °C and left to adhere 24 h, as for the non-adherent cells, they were removed, Mesenchymal stem cells were selected by adherence during first 24 h and maintained in growth culture media. Media changed 3 times a week and until the cultures reached 70–80% confluence.

(Soleimani and Nadri, 2009). When the cultures reached 80–100% confluence, the cells were suspended in the culture at a density of ($>10^6$ cells/ml). Immunocytochemistry was used to check for the following stem cell markers CD90 + (US biological), CD 44 + (Santa Cruz Biotechnology, USA) (Huang et al. 2015) CD 34- (Santa Cruz Biotechnology, USA) (HUSS, 2000), CD105 + (US biological, USA), (Ninagawa et al., 2011)

### 2.2. MSC passaging technique

Passaging of the cells was done in suspension culture at cell density is greater than ($>10^6$ cells/ml) as described by Freshney (2000). Typically, the first time to passage occurs between 5 and 7 days after culture. The MSCs are sub-cultured at approximately 80% confluence to prevent contact inhibition of growth and spontaneous differentiation (Solchaga et al., 2004),The culture medium was aspirated and the cells were washed three times with MEM free serum and detached by incubation with 1 ml of trypsin-versene for 5–10 min at 37 °C temperature .in order to dislodge the cells, the flask gently rocked, then 5 ml of culture media containing 20% FBS was added by using a sterile Pasteur pipette and mixed to obtain a single cell suspension. The cell suspension was centrifuged at 1000 rpm for 10 min in 18 °C then the supernatant was aspirated and the cells pellet were suspended in 1 ml of culture medium MEM containing 20% FBS, Then, the cells were counted using a hemocytometer. The cells were detached from the surface with trypsin-versene and counted.

### 2.3. Differentiation of MSCs into motor neuron cells

The first passage of ($2 \times 10^4$) MSCs were used for motor neuron differentiation. The differentiation strategy involved two steps. In the first, the pre-induction step, the cells were cultured in an MEM medium supplemented with 20% FBS and 1 mM beta-mercaptoethanol (BME) (Santa Cruz Biotechnology, USA). After 24 h of incubation, the media was discarded, and the MEM-free serum media containing 2 mM BME was added and incubated for one hour. The media was then discarded, and the cells were washed with a free serum media. The second step, the induction step, lasted for four days. During this step, the MEM free serum media with 1 mM retinoic acid (Santa Cruz biotechnology, USA), 10 ng/ml nerve growth factor (Santa Cruz biotechnology, USA) and 0.1 ng/ml sonic hedgehog (Santa Cruz biotechnology, USA) were added to the cells and incubated at 37 °C for four days. As a negative control, additional MSCs were cultured in a medium without differentiation stimuli under the same conditions as the treated cells. All of the cells were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. The cultures were maintained with a medium exchange every two days. The cell morphology was observed under an inverted microscope according to the modification method of Hu and Zhang (2009).

### 2.4. Motor neuron marker detection

At the end of the differentiation stages, the media was aspirated and the cell was fixed with 4% paraformaldehyde for 10 min, incubated with 1% hydrogen peroxide for 10–15 min and washed three times with PBS for 5 min each time. Aliquot of 1.5% blocking serum was added to the fixed cells for one hour. The cells were then incubated with diluted choline acetyl transferase antibody (1:50 v/v) (Santa Cruz, USA) overnight and with microtubule associated protein antibody (1:100 v/v) (Santa Cruz, USA) for one hour, then washed. Cell sections were incubated for 30 min with 1.2 ml biotinylated secondary antibody, according the manufacturer recommendations. AB enzyme was added to the slide and washed twice with PBS for two minutes each time. Three drops of peroxidase substrate were added for 10 min (Santa Cruz, USA). Hematoxylin stain was used as a counter stain. The slides were mounted and examined under light (Moral-Sanz et al., 2012).

### 2.5. Scanning electron microscope

For scanning electron microscopic analysis, a procedure described by Al-Shammari et al. (2015) was used. The cells were cultured on cover slips to confluency. The cells were fixed in 4% (v/v) glutaraldehyde (Santa Cruz, USA) in a 0.1 M PBS buffer (pH 7.4) for 4 h at 4 °C. The fixed cells were washed three times with a PBS buffer for 10 min each time and post fixed in 1% osmium tetroxide for 2 h at 4 °C. The samples were washed again and then dehydrated in ascending grades of ethanol (35%, 50%, 75%, 95% and 100%) and air dried for 2 h. The samples were viewed under a Phenom G2 pro desktop scanning electron microscope at an accelerating voltage of 10 kV.

### 2.6. Motor neuron function detection

Motor neuron activity was detected using high-performance liquid chromatography (HPLC), which estimations the acetylcholine concentration in the tissue culture media at different time