

Differentiation of Mouse Bone-Marrow Mesenchymal Stem Cells into Motor Neuron Cells *in vitro*

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Abstract

Motor neuron cell is responsible for transfer neural instruct from brain to peripheral muscle through spinal cord; therefore any defect in these cells or spinal cord will affect motion. This study was designed to induce differentiation of Bone marrow mesenchymal stem cells (BM-MSCs) into neuron cells. BM-MSCs were isolated from bone stroma of femur and tibia of albino male mice and tested immunocytochemically for CD44, CD90, and CD105 expression and showed positive staining, while showed negative staining for CD34. Differentiation of BM-MSCs to motor neuron involved two main steps. First; induction of BM-MSCs by addition of 1mM mercaptoethanol (BME) in fetal bovine serum (FBS) in minimum essential medium MEM for 24 h and 2mM BME in free serum media for 2h. In the second step of induction retinoic acid, sonic hedgehog and nerve growth factor were added in free serum MEM for 4 days. Results revealed that the differentiation medium used was very efficient in directing the BM-MSCs to motor neural cell and showed positive reactivity to specific motor neural markers that used for detection of motor neuron cells like microtubule associated protein-2 antibodies and acetylcholine transferase antibody.

Keywords: Bone marrow, Mesenchymal stem cell, Motor neuron, and Differentiation.

Introduction

Motor neurons are specialized nerve cells in the brain and spinal cord that transmit the electrical signals to muscle and generate movement. There are two groups of motor neurons; the upper motor neuron at the top of the brain in motor cortex and that extended down in the spinal cord to connect the lower motor neurons, which travel out of the spinal cord and connect the muscle [1].

Neuron are polarized cells that have dendrites and axon that extend long distances from the cell body to form synapses that mediate neuronal communication. Also, the presence of cell lipid and protein are essential for control neuron cell shape and synapses [2].

Mesenchymal stem cells were used in cell therapy, regenerative medicine and tissue engineering, because such cell has the potential ability for differentiation into neural cell using differentiation reagents; and therefore its suitable as alternative source of stem cell in neurodegenerative disease [3].

Mesenchymal stem cells can be differentiated into dopamine neurons cell using cocktail of factors like sonic hedgehog,

fibroblast growth factor, and basic fibroblast growth factor. Brain derived neurotropic factor adding can increase the electrophysiological properties of these cell [4]; [5]. Microtubule associated protein 2 (MAP-2) and Acetyl choline transferase are further differentiation markers for motor neuron [6]. The aims of this study were induction the mesenchymal stem cells isolated from mouse bone marrow into motor neuron cell *in vitro*.

Materials and Methods

Mesenchymal Stem Cell Isolation

Bone marrow mesenchymal stem cells were isolated from femur and tibia albino mice by flushing method with 1ml of growth culture medium (MEM). Freshly isolated whole bone marrow cells were resuspended in 5ml MEM supplemented with 20 % fetal bovine serum (FBS) 1% Ampicillin/Streptomycin at 37°C [7]. When the cultures reached 80-100 % confluence during 5 to 7 day, the cells were suspended in culture media at density $>10^6$ cells/ml. The cell viability was assessed by using trypan blue dye. The MSCs were immunocytochemically tested for MSCs

markers by using CD 105+ CD90+, CD 44+ and haematopoietic marker CD 34⁻ [8].

Induction of BM-MSCs differentiation into motor neuron

In motor neuron differentiation the 1st passage of 2×10^4 /ml MSCs were used. The differentiation strategy involved two main steps. In the (pre induction step) the cells were cultured in 10 ml MEM medium supplemented with 20% FBS and 1 mM Betamercaptoethanol (BME). After 24 h of incubation, the media was discarded and MEM free serum media containing 2 mM BME was added and incubated for 2h, then media was discarded and cell washed with free serum media. At the second step (induction step), which lasted for four days, MEM free serum media with 1mM retinoic acid (RA), 10ng/ml nerve growth factor(NGF) (Santa Cruz biotechnology) and 0.1 ng/ml sonic hedgehog (SHH) were added and incubated at 37°C for 4 days. As a negative control, MSCs were cultured in medium without differentiation stimuli along with the differentiation experiments in the same conditions. Cells were cultured in a humidified atmosphere 5% CO₂ and 95% air at 37°C. Cultures were maintained by medium exchange every 2 days. The cell morphology was observed under inverted microscope [9].

Immunocytochemical Detection of Motor Neuron

The cell was fixed by 4% paraformaldehyde for 10 min at the end of differentiation, incubated with 1% hydrogen peroxide for 10-15 min. and washed 3 times with PBS for 5 min. Aliquot of 1.5% blocking serum was

added to cell section for 1hr, Cells sections were incubated with diluted anti acetylc transferase antibody at a ratio 1:50 vol/vol overnight as production company instruction and diluted anti microtubule associated protein antibody at a ratio of 1:100vol/vol for 1 hr then washed. Cell sections were incubated for 30 min with 1.2 ml biotinylated secondary antibody. AB enzyme reagent (avidin 50 µl and biotinylated HRP) 650 µl was added to cell section and washed two times with PBS for 2 min. Three drops of peroxidase substrate was added to cells for 10min, or until desired stain intensity develops and washed. Hematoxylin stain was added to cell section for 5-10 seconds and immediately washed with distilled water. Finally 1-2 drops of permanent mounting medium was added and examined by light microscopy at magnification powers of 20X and 40X [10].

Results and Discussion

Motor Neuron Cell Differentiation

Mesenchymal stem cell had the ability for differentiation into neuron like cell and more specialized cell like motor neuron when using simple differentiation factors in practically short period of time (not more than 5 days).

The results showed that the fibroblast like cell MSC cell membrane withdraw to the middle of the cell, extension appeared and the cell still viable and attached to the flask surface. Medium was discarded and cells were incubated for 1h in 2mM βME of free serum MEM, the cell became more radical in shape, multipolar, and extension increased in number of viable Fig.(1).

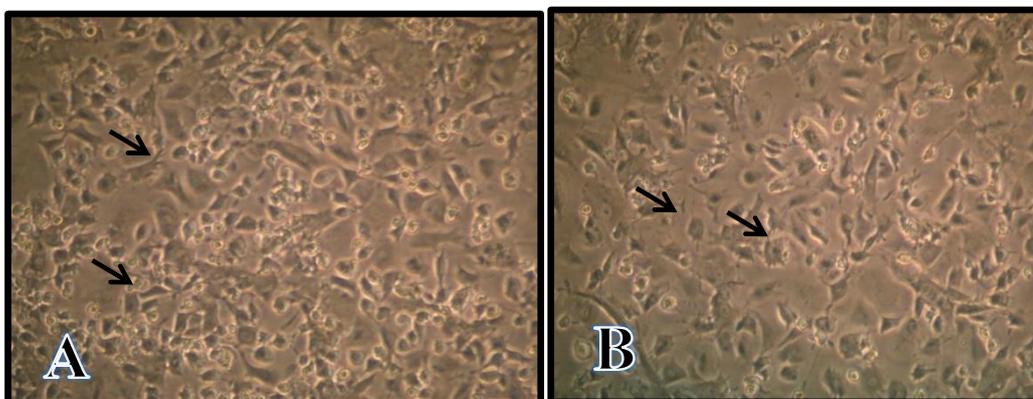


Fig.(1): BM-MSCs differentiation A. a pre induction stage after 24 h in 1M BME in MEM 20% FBS viewed by inverted microscope (20X10) showed that the fibroblast like cell MSCs cell membrane withdraw to the middle of the cell. B. a pre induction stage after 1hour in 2.mM βME of free serum MEM showed that the cell became more radical in shape multipolar (20X10).

Scientists works on bone marrow culture in 1mM BME showed that cell cytoplasm retract and when exposed to 5mM BME for 6 hours, the cell had multipolar body structure [11] and also cells incubated in BME for few hours were elongated and beard process like extensions and were immunoactive for neuron specific enolase, nestin and glial fibrillaey acidic protein [12]. The second stage of differentiation was the induction stage in which used a combination of retinoic acid and a Sonic hedgehog factors was used for just four days, cell body became typical like motor neuron cell, shiny nucleuse and with the cell soma have many dendrites and long axon like structure ended with small extensions. The MSCs under three thousand increase size using scanning microscope appeared as circular to spindle shape with no extended extension from the cell while the differentiated cell into motor

neuron showed cell body contain dendrites and long extended axon ended with small dendrite Fig.(2).

This differentiation method represent an effective method during short induction time for differentiation of mouse bone marrow MSC into motor neuron cells in compare with [13] whose cultured cells using BME as pre induction and induction in RA and forskolin for 24 h and finally 6-8 days in RA and SHH but they started the culture forming neurospher from MSC on unattached surface flask which last more time and specific culture conditions.

Other workers induce differentiation of MSCs into motor neuron cells for longer time up two weeks using large amount of growth factor and specific culture conditions [15].

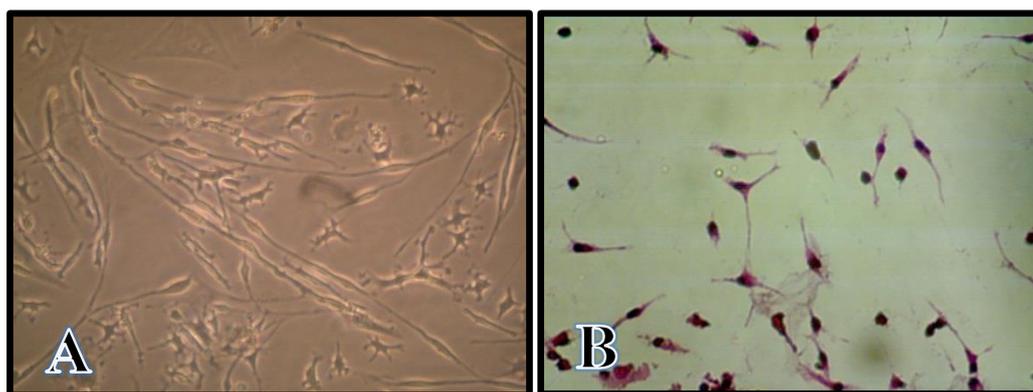


Fig.(2): Motor neuron cell A viewed (20X10) under inverted microscope at the end of induction differentiation stage, B Hematoxylin and Eosin stain of differentiated motor neuron (40X10).

Immunohistochemical Staining of Motor neuron cell

Motor neuron showed positive microtubule associated protein 2 (MAP2) and cholinacetyl transferase (CHAT) as specific marker. The results revealed that 90% of differentiated motor neuron was positive for MAP2 and about 85 % for CHAT Fig.(3).

Microtubule association protein MAP-2 was a cytoskeletal protein, present in the axon and dendrite of the cell body that reveled by mRNA in situ hybridization [14].

Bi[15] found that the cells have three neural related marker expression increased nestin, NES and MAP-2 with increasing concentration of Retinoic acid by Real time PCR, these factor were determined the differentiation into neuron.

Another marker improved the differentiation into motor neuron cells is acetyle choline transferase CHAT, The enzyme is responsible for biosynthesis of acetylcholine and present in the functional cholinergic neurons of the central and peripheral nervous system indicate motor neuron cell [16]. Other studies which was focused on embryonic stem cell differentiation into motor neuron using sequence of culturing procedures, the differentiated cells detected immunocytochemically using for HB9 and CHAT cells showed just 30% of total cell are mature motor neuron [17].

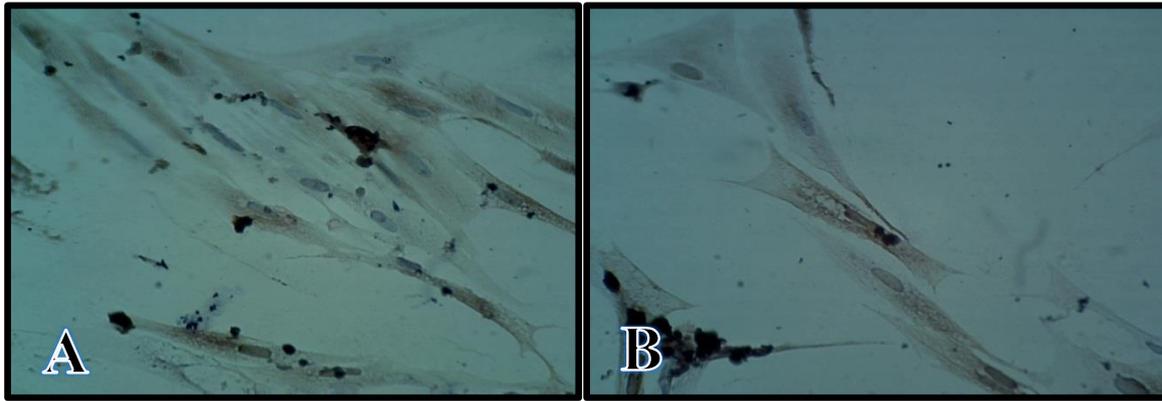


Fig.(3): A and B Immunocytochemical analysis for anti CHAT and anti MAP-2 in motor neuron after induction stage showed positive reactivity using DAP chromogenic (brown color) (10X40).

Formation of aggregated motor neuron cell structure

The formation of aggregation cell phenomena, in which motor neuron cell aggregate together forming long spindle of cell body and many other cell soma accumulated and extend their axon outside, under the scanning microscope a number of accumulated cell appeared like single long cell with edge Fig.(4).

Natural aggregates are arising from dissociated cortical neuronal cells in culture from 2-7 day *in vitro* depending on the concentration of cells. At high concentrations (> 10⁶ cells/ml) large aggregates will develop and make interconnections of grouped cellular

protrusions, which constitute bundles and the cellular knots and the bundles of protrusions interconnecting cables in the network, occupied with single cells or more forming mushroom like appearance and their cells are placed at the exterior of the head in order to get maximum exchange with the culture fluid. Phosphorylated forms of neurofilament characterize the development of immature neurons and axons to mature ones and help growing axons to accommodate the demands for plasticity and stability by modulating the structure of the axonal cytoskeleton [18].

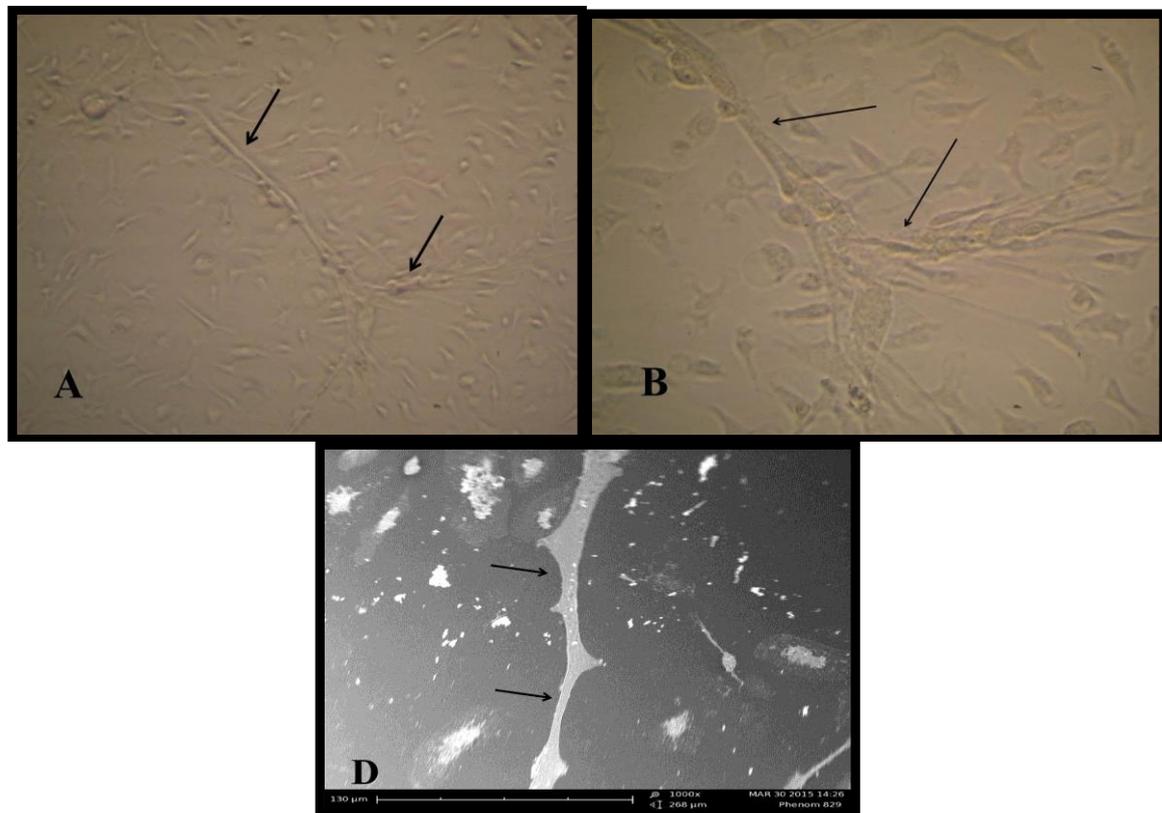


Fig.(4): A and B aggregated motor neuron cell after differentiation from MSC under the inverted microscope. C. aggregated cell under electronic scanning microscope.

Also the structure similar to all brain dissociated cells cultured for 2-5 days in free culture media with some growth factors, the cells aggregates tended to attach and grow neuritis after their transfer to poly-*l*-lysine- or Matrigel-coated dishes under stationary conditions. Early aggregates transplanted to the hippocampus of adult rats developed into identifiable grafts, with fluorescent-labeled cells show that young neural cell aggregates, maintain their ability to undergo two basic phenomena for cellular interaction, i.e., attachment and neuritic growth [19].

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الخلاصة

الخلايا العصبية الحركية مسؤولة عن نقل الإيعاز العصبيين المخ الى الاطراف العضلية من خلال الحبل الشوكي لذلك اي خلل فيها او في الحبل الشوكي يؤدي الى خلل في الحركة.

صممت الدراسة لتحفيز تمايز الخلايا الجذعية الوسيطة الى خلايا حركية عصبية. اختبرت الخلايا الجذعية الوسيطة الماخوذة من نخاع العظم والمعزولة من نسيج السترولوما لعظم الفخذ و الساق لذكر فئران الالبينو واختبرت بالتفاعل المناعي الكيميائي الخلوي للمستقبلات CD44,CD90,CD105 وسلبى لمستقبل CD34.

ان تمايز الخلايا الجذعية من نخاع العظم الى خلايا عصبية حركية تتضمن خطوتين. الاولى: خطوة قبل التمايز التي تتضمن اضافة ١ ملي مول من مادة البيتا مركبتوايثانول في سيرم جنين العجل في وسط MEM لمدة ٢٤ ساعة و في تركيز ٢ مليمول بيتامركبتوايثانول في وسك زرعي خالي من السيرم لمدة ٢ ساعة.

الخطوة الثانية تسمى خطوة التحفيز وتتضمن اضافة ١ مايكرومولاري من الرينتوك اسيد و من السونك هيدجيهوك و من نيرف كروث فاكثر في وسط MEM لمدة ٤ ايام وظهرت النتائج ان وسط التمايز المستخدم فعال جدا في تحويل الخلايا الجذعية الوسيطة الى خلايا عصبية حركية واطهرت التفاعلات المناعية الكيميائية نتائج ايجابية لمعلومات الخلايا الجذعية الحركية بواسطة استخدام CHAT و MAP-2.