Differentiation of mesenchymal stem cell into motor neuron cell

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ABSTRACT
Bone marrow mesenchymal stem cells (BM-MSCs) were isolated from the bone stroma of the thigh bones of albino male mice and were tested immunocytochemically for CD44, CD90, and CD105, showing positive results and negative for CD34. Differentiation of BM-MSCs to motor neuron was induced by adding different growth factors including pre induction step using 1mM mercaptoethanol (BME) in 20% FBS in minimum essential media and then 2mM BME in free serum media for 2h and induction step using 1µM Retinoic acid and (0.1 ng/ml) sonic hedgehog in free serum media for 4 days. Results revealed that the differentiation medium used was very efficient in directing the BM-MSCs to the motor neural cell, which immunocytochemically showed positive reactivity to specific motor neural markers for detection of motor neuron cells using immunocytochemical like microtubule associated protein-2 antibodies and acetylcholine transferase antibody. Motor neuron cell activity was assessed by measuring acetylcholine level in culture media during differentiation using high performance liquid chromatography (HPLC). Results showed that the highest level was (75.0±0.57ppm (?)) in induction stage in comparison with level in the pre induction stage (15.36±0.44ppm) with significant differences P≤0.05. While BM-MCs produce acetylcholine in low quantity (15.43±0.38 ppm).

KEY WORDS: mesenchymal stem cell, motor neuron

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 group of motor neuron, the upper motor neuron at the top of the brain in motor cortex and extended down in the spinal cord to connect the lower motor neurons which travel out of the spinal cord and connect the muscle (Talbot, 2008).

Neuron are polarize cells have dendrites and axon extend long distances from the cell body to form synapses that mediate neuronal communication, cell lipid and protein essential for control cell shape and synapses (Ramirez and Couve, 2011).

Mesenchymal stem cell were found in the amniotic fluid of mammalian and readily use in cell therapy, regenerative medicine and tissue engineering. Amniotic fluid mesenchymal stem cells have the potential ability differentiation into neural cell using differentiation reagent and therefore suitable alternative source of stem cell in neurodegenerative disease (Kim et al., 2013).

Bone marrow mesenchmal stem cell differentiated into neural cells, hepatocytes and myocytes, expanding their differentiation potential. The differentiation into neural cell required either tissue plasminogen activator TPA, forskolin,3-isobuilet-1-1-methel xanthin IBMX , fibroblast growth factor FGF-1, or retinoic acid and 2-mercaptopoethanol and cytokines (Zeng et al.,2011; Scintu et al., 2006).

Mesenchymal stem cells are differentiation into dopamine
neurons cell using cocktail of factor like sonic hedgehog, fibroblast growth factor 8, and basic fibroblast growth factor. Brain derive neurotropic factor adding increase the electrophysiological properties of these cell (Trzaska and Rameshwan, 2011; Guo et al., 2005).

Microtubule associated protein 2 (MAP-2) and Acetyl choline transferase are differentiation markers for motor neuron.

Acetyl choline is a neurotransmitter at neuromuscular junctions responsible for synapses in the ganglia of the visceral motor system, and at a variety of sites within the central nervous system. Acetylcholine is synthesized in nerve terminals from acetyl coenzyme A (acetyl CoA, which is synthesized from glucose) and choline, in a reaction catalyzed by choline acetyltransferase. Choline is present in plasma at a concentration of about 10 mM and is taken up into cholinergic neurons by a high-affinity Na⁺/choline transporter (Purves et al., 2001).

High-performance liquid chromatography is a technique in analytical chemistry used to separate the components in a mixture, to identify each component, and to quantify each component (Kupiec, 2004). Define the purpose of the study in the concluding sentence

Materials and methods

Bone marrow mesenchymal stem cell isolated from thigh of Albino mice used . In using flushing method was used by flush the bone marrow cells from bones using a 1 ml syringe containing 1 ml of growth culture media (MEM) . freshly isolated whole bone marrow cells were re suspended in 5 ml growth culture medium MEM supplemented with 20 % FBS, 1 % Ampicillin/Streptomycin in 37 °C (Soleimani, M. and Nadri, 2009). when the cultures reached 80 - 100 % confluency , the cells were suspended in culture at density (>10^6 cells / ml). the cell viability studied by using trypan blue dye which allows distinguishing between healthy cells with uncompromised membrane integrity (unstained) and dead ones (stained blue). The Immunocytochemistry analysis of MSCs was done by CD 105 CD90+, CD 34−, CD 44+ (Lin et al., 2013).

Motor neuron differentiation

In motor neuron differentiation the 1st passage of (2 × 10^4) of MSCs were used. The differentiation strategy involved two main steps (pre induction step) the cells were cultured in a MEM medium supplemented with 20% FBS and 1 mM Betamercaptoethanol (Santa Cruz biotechnology, Country). After 24 h of incubation the media was discarded and MEM free serum media containing 2 mM BME was added and incubated for 1h. 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 1 mM retinoic acid (Santa Cruz biotechnology), 10 ng/ml nerve growth factor(Santa Cruz biotechnology) and 0.1 ng/ml sonic hedgehog (Santa Cruz biotechnology ) 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 of 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 (Hu and Zhang, 2009).

Motor neuron detection by immunocytochemistry

After that the media was aspirated and the cell was fixed by 4% paraformaldehyde for 10 min, incubated with 1% hydrogen peroxide for 10-15 minutes and wash with PBS for 5 min three times. Aliquot of 1.5% blocking serum was added to cell section for one. Cells sections were incubated with diluted acetyl transferase antibody at a ratio (1:50 vol/vol) overnight as production company instruction and diluted microtubule associated protein antibody at a ratio (1:100vol/vol) for 1 h then washed. Cell sections were incubated for 30 min with 1.2 ml biotinylated secondary antibody. A liquid of 650 µl of AB enzyme reagent was added to cell section and washed two times with PBS for 2 min. Three drops of peroxidase substrate was added to cells for 10 min, 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 200X and 400X (Moral-Sanz, et al. 2012).

Scanning electron microscopy solutions

4% gluteraldehyde- It was prepared by mixing 4 ml of stock gluteraldehyde with 94 ml Distilled water (Eisenbach, 2015).

Osmium tetroxide- It was prepared by dissolving 1 mg of osmium tetroxide in 10 ml Distilled water. Then stored in dark container at -20°C.
Figure 1: BM-MSCs differentiation A and B pre induction stage after 24 h in 1M BME in MEM 20% FBS viewed by inverted microscope (10X10) (10X20) showed that the fibroblast like cell MSC cell membrane withdraw to the middle of the cell. C and D a pre induction stage after 1 hour in 2mM βME of free serum MEM showed that the cell became more radical in shape multipolar.

Scanning by electron microscope
The slides were washed by PBS and fixed with gluteraldehyde 4% for 24 h at 4°C then Osmium solution was added for 2 h in dark. At the end of two hour, slides were washed with PBS two times. A serial dehydration for the sections were carried out started from (50, 70, 95, and 100) % for 30 min, dried and examined under the electron microscope (Eisenbach, 2015).

Motor neuron activity
The activity of motor neuron detected using HPLC by estimation acetylcholine concentration in media at different period of differentiation. The test was done in Ibin Sena Center /Ministry of higher education and scientific research, using shimachzy lc-2012 AHT machine.

HPLC conditions
Standard Acetylcholine was prepared by dissolving 1 mg acetylcholine chloride powder >98% purity in 1ml Distilled water. A column 250*4.6 mm column 5micron lava with Injection volume 0.7 ml/min. Column temperature was 18 °C, Detection wavelength: 210 nm and Mobile phase: Acetone, water, methanol 80%, 14%, 6% (Landgraf et al., 2010). Acetylcholine concentration was calculated according to this equation (Chen and Yun, 2009).

Concentration of compound (ppm) = area of sample × conc. of standard/ area of standard

Results and Discussion
Mesenchymal stem cell have the ability to differentiated into neuron like cell and more specialized cell like motor neuron when using differentiation factors in two stages of differentiation using monolayer of passage one MSCs. The first stage of differentiation, a pre induction stage, the cell cultured in β-mercaptoethanol in two concentration, 1mM β-mercaptoethanol contained MEM 20 % Fetal bovine serum for 24 h showed that the fibroblast like cell MSC cell membrane withdraw to the middle of the cell, extension.
became appear and the cell still viable with account 1.9 X10^6 and attached to the flask surface Figure 1. The media discarded and incubated for 1hour in 2mM βME of free serum MEM, the cell became more radical in shape, multipolar, and extension increased in number of viable count to about 1.40 X10^6 Unit.

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 (liu, et al., 2011) and also cells incubated in BME for few hours were elongated and beard process like extensions and were immunoactive for neuron specific enolase, nestin, glial fibrillaey acidic protein (Mareschi et al, 2006).

The second stage of differentiation was the induction stage in which used a combination of Retinoic acid and a Sonic hedgehog factors for just four days, cell body became typical like motor neuron cell, shiny nuclease 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 under two thousand increase in size Fig 2 and 3.

The first and second stages of induction represent the most effective, efficient and shortest induction time method for differentiation of mouse bone marrow MSC into motor neuron cells in compare with Shetty and his workers (2015) whose cultured cells in BME as pre induction, induction in RA and forskolin for 24 h and finally 6-8 days in RA and SHH but they started culturing forming neurospher from MSC on unattached surface flask.

Other workers induce differentiation of MSCs into MNCs for longer time up two weeks using large amount of growth factor and specific culture conditions (Ebrahimin- Barough, et al.,2014).
Figure 3: A MSC using scanning electron microscope 3000 time increase in size showing dipolar structure of cell. B. Differentiated motor neuron under scanning electron microscope 2000 time increase in size cell soma appeared less in size with many dendrites and extended of cell axon.

Motor neuron cell detection using immunocytochemistry
Bone marrow mesenchymal stem cell differentiation into motor neuron cell was detected by immunocytochemistry study. Motor neuron cell are mature differentiated cell have microtubule associated protein 2 (MAP2) and cholinacetyl transferase (CHAT) as specific marker. Results revealed that 90% of differentiated motor neuron was positive for MAP2 and about 85 % for CHAT Figure (4).

Differentiation indication markers MAP-2 used in this study and the results proved that mature spinal cord neuron, MAP-2 a cytoskeletal protein, present in the axon and dendrite of the cell body reveled by mRNA in situ hybridization (Hirokawa et al., 1996). Bi, et al. (2010). 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 determined the differentiation into neuron.

The other marker improved the differentiation into motor neuron cells is acetylle choline transferase (CHAT), the enzyme responsible for biosynthesis of acetylcholine and present in the functional cholinergic neurons of the central and peripheral nervous system indicate motor neuron cell (Oda ,1999) and also other studies on embryonic stem cell differentiation into motor neuron using sequence of culturing procedures detected immunocytochemistry using Quantification of HB9 and CHAT cells showed just 30%of total cell are mature motor neuron (Hester, 2011).

Figure 4: A and B Immunocytochemical analysis for MAP-2 and CHAT in motor neuron after induction stage showed positive reactivity (brown color) (10X40).

Level of Acetyl choline by HPLC
The activity of motor neuron by measuring the level of acetylcholine produced by differentiated cells in culture media. The increase level of neurotransmitter acetyl choline produced in culture media estimated functionality of motor neuron.
Results revealed that a significant increase (75.0±0.57pmm) in Acetyl CoA produced by motor neuron in differentiation medium at 4th day after induction stage in
comparison with zero day and after 24h (15.43±0.38 and 15.36±0.44pmm) Figure (5).

Motorneuron cell produce acetyl choline as cholinergic compound from choline and acetyl co A metabolized by enzyme Acetyl choline transferase which expressed strongly in differentiated cell immunocytochemicaly by CHAT primary antibody as shown in figure (5), therefore acetylcholine present in media indicate that motoer neuron functionally active cell.

Figure 5: Level of Acetyl CoA produced by motor neuron after different differentiation period

Liu, et al. (2013) showed the production and secretion of acetylcholine from motor neuron cell derived umbilical cord mesenchymal stem cell, positive for acetylcholine transfarase, increased during the period of differentiation from very little in the 9th day of 15 differentiation day, about 2.8 ng/ml to 5.7 ng/ml at the end of differentiation using heparin end fibroblast growth factor comparing with mesenchymal stem cell control which not produce any acetylcholine.

Murine and human embryonic stem cell synthesize acetyl choline and express acetyl choline transferase in regulation of embryonic stem cell as calcium dynamics, cell survival and proliferation, it’s increased the viability, but decreased the proliferation of embryonic stem cells this indicate the presence of low concentration of acetylcholine in the culture of MSCs and pre induction stage of differentiation (Landgraf et al.,2010; Takahashi, 2015)

References


