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Emergence of signs of neural cells after exposure of bone marrow-derived mesenchymal stem cells to fetal brain extract

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ABSTRACT

Objective(s): Nowadays much effort is being invested in order to diagnose the mechanisms involved in neural differentiation. By clarifying this, making desired neural cells *in vitro* and applying them into diverse neurological disorders suffered from neural cell malfunctions could be a feasible choice. Thus, the present study assessed the capability of fetal brain extract (FBE) to induce rat bone marrow-derived mesenchymal stem cells (BM-MSCs) toward neural cells.
Materials and Methods: For this purpose, BM-MSCs were collected from rats and cultured and their mesenchymal properties were confirmed. After exposure of the BM-MSCs to fetal brain extract, the cells were evaluated and harvested at days 3 and 7 after treatment.
Results: The BM-MSCs that were exposed to FBE changed their appearance dramatically from spindle shape to cells with dendrite-like processes. Those neural like processes were absent in the control group. In addition, a neural specific marker, vimentin, was expressed significantly in the treatment group but not in the negative control group.
Conclusion: This study presented the FBE as a natural neural differentiation agent, which probably has required factors for making neurons. In addition, vimentin overexpression was observed in the treated group which confirms neuron-like cell differentiation of BM-MSCs after induction.

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Introduction

There is no doubt that the nervous system has a unique performance. Although it has a commander role but the point is that unlike most organs in the body, in most cases this system can't afford renewing and replacing the missed cells. It means that when a destructive occasion, endogenous or exogenous, results in cells malfunction, related disability usually remains throughout the whole life depending on the extent and damage location (1). In the majority of cases current treatments such as medical drugs play only a supportive role that prevents the damage progress and can't restore the primary function (2). Therefore, researchers seek for alternative therapies in order to retrieve the lost function. In this regard, cell therapy in which affected cells replace the new ones, becomes a promising choice in a new field of interest called regenerative medicine (3).

Mesenchymal stem cells (MSCs) are multipotent cells with self-renewal ability that can differentiate into a variety of cell types belong to endodermal and ectodermal other than mesodermal lineages. They can be easily isolated from different sources especially medullar cavity of the bones. Its autologous nature beside the immunomodulation properties can circumvent many immune-related problems particularly for *in vivo* studies. By the way, these cells can be expanded extensively and manipulated more readily *in vitro* than other stem cell types (4). Overall, MSCs' features put these cells in the frontiers of developing new strategies for struggling against a wide variety of disorders.

Potential of MSCs to differentiate into neurons and glial cells have been previously reported (5, 6) and their great capabilities for restoring nervous system operations have been declared (7).

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It is noteworthy that MSCs could migrate to neurological lesions and exert their effects at the injury site directly (8). It seems that their actions consisted of two main categories; the first, releasing a group of neurotrophines and the second, differentiation to neural cells lineage (9, 10). The present study is focused on the latter using rat bone marrow-derived MSCs (BM-MSCs), which have common surface antigens with human BM-MSCs (11). Also, it was shown that rat BM-MSCs have an intrinsic predisposition to differentiate into neural lineage (12).

Current methods for induction of neural differentiation mostly rely on utilization of compounds that may have adverse effects on cell characteristics like viability. On the other hand, these substances, mainly chemicals, are probably toxic and their use is accompanied with some considerations (13). Therefore, in the current study, the same conditions were stimulated as in the body in which the primary precursors differentiate into mature and functional neural cells. Since the completion of the brain development at the end of the pregnancy, fetal brain extract was used as a physiological environment in order to induce BM-MSCs differentiation to the neural cell lineage. In this regard expression of vimentin as a neural marker (14) was evaluated in BM-MSCs at days 3 and 7 post-treatment with fetal brain extract. In addition, this experiment has been done in order to study the differentiation process of BM-MSCs with a simple, inexpensive, and convenient protocol but in a similar approach to the one that occurred in the body.

Materials and Methods

Isolation and culture of BM-MSCs

Adult 5-week-old Sprague Dawley rats (*Rattus norvegicus*) were purchased from Center of Comparative and Experimental Medicine of Shiraz University of Medical Sciences, Shiraz, Iran. They were kept in standard conditions (temperature of 22±1°C and lighting 12 hr dark/light cycle; lighting starting at 07:00 am) and fed *ad libitum* with water and factory produced ready-meals. All procedures were done in accordance with animal guideline care of Ethical Committee of Shiraz University.

BM-MSCs of rats were isolated and characterized (15). Briefly, the rats were deeply anesthetized with ether and then killed by cervical dislocation. In sterile conditions, the abdominal portion was dissected. After removing the attached muscles and separating the remaining parts, tibias and femurs of both sides were put in a clean falcon tube filled with Dulbecco's Modified Eagles Medium (DMEM, BioWest, France) and enriched with 1% penicillin and streptomycin (BioWest, France). In the next step, under the protection of a class II biological hood, two ends of each bone were cut and the medullar cavity was flushed by a syringe equipped with a 21 gauge needle full of DMEM. In this way, cells in the bone marrow were collected on a plate. This cell suspension was subjected to centrifugation (1200 rpm, 10 min, and room temperature). The supernatant was discarded and the cell pellet was suspended in 1 ml of full culture medium including 88% DMEM, 10% fetal bovine serum (FBS, BioIdea, Iran), 1% penicillin and streptomycin, and 1% amphotericin B (BioWest, France). This volume of cells was added to a T25 culture flask prefilled with 4 ml of the same medium. The flask was transferred to a humidified atmosphere incubator with 5% CO_2. Culture medium was changed after 24 hr.

The cells in the primary passage or P0 coated the flask surface completely, spindle-shaped, which lasted about 10-12 days. After this time, the cells were passaged by removing the culture medium out of the flask and treating the cells with trypsin/EDTA (0.05%, Sigma, USA) for 3 min in the incubator atmosphere. The cell suspension was again centrifuged and suspended in the culture medium and was split into two T75 culture flasks, each containing 15 ml of full culture medium and put back into the incubator. These procedures were repeated until enough cells were obtained at passage 3.

BM-MSCs characterization

To confirm that obtained cells at passage 3 were truly MSCs, expression of MSC-specific markers was checked. Beside this, lack of expression of hematopoietic-specific markers was tested to ensure the homogeneity of the cell population. Thus the presence of CD73 and CD90 along with CD34 and CD45 was analyzed (16).

Briefly, when cells at passage 3 reached confluence, they were harvested and subjected to total RNA extraction using Column RNA Isolation Kit-III (Dena Zist Asia, Iran) according to manufacturer's instructions. RNA quality was assessed by spectrophotometry and agarose gel electrophoresis. Then RNA was considered for cDNA synthesis. This was performed by AccuPower CycleScript RT Premix (dN6) kit (Bioneer, South Korea).

At the next step, PCR was carried out with gene-specific primers (Table 1). Taq DNA Polymerase 2x Master Mix Red (Amplicon, Denmark) was applied at this stage. PCR products were analyzed for specific amplicon.

Table 1. Primer names, sequences, and amplicon length used in RT-PCR

Primer name	Primer sequence	Amplicon length (bp)
CD34-F	AGCCATGTGCTCACACATCA	257
CD34-R	CAAACACTCGGGCCTAACCT	
CD45-F	CCAAGAGTGGCTCAGAAGGG	450
CD45-R	CTGGGCTCATGGGACCATTT	
CD73-F	TGCATCGATATGGCCAGTCC	208
CD73-R	AATCCATCCCCACCGTTGAC	
CD90-F	GACCCAGGACGGAGCTATTG	177
CD90-R	TCATGCTGGATGGGCAAGTT	
GFAP-F	TTGAGTCGCTGGAGGAGGAG	130
GFAP-R	GCTGTGAGGTCTGGCTTGG	

DNA safe stain was utilized in agarose gel electrophoresis.

The BM-MSCs were differentiated to osteoblasts and adipoblasts for confirmation of their mesenchymal characters. For *in vitro* osteogenic differentiation, the rat BM-MSCs at 90% confluence were exposed to DMEM supplemented with 15% FBS, 200 µM L-ascorbic acid, 10 mM glycerolphosphate, and 100 nM dexamethasone. The medium was changed twice per week for 21 days. After 3 weeks, osteogenic differentiation was confirmed by Alizarin red staining. In brief, BM-MSCs cultures were fixed with 4% paraformaldehyde for 10 min. Then cells were incubated for 20 min at room temperature in 1% Alizarin red stain and 1% ammonium hydroxide. After incubation, cultures were washed 4 times, 5 min each time with 1 ml dH_2O replacing the water at each 5 min interval and air-dried. Alizarin red dye binds to calcium ions present in mineralized deposits resulting in a brilliant red stain. For adipogenic induction, BM-MSCs at 90% confluence were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 60 µM indomethacin, 1 µM dexamethasone, and 10 µM insulin. Induction lasted 21 days and the cells were fixed in 4% formalin at 4°C for 20 min and adipogenic induction was confirmed by Oil Red O staining (all products for differentiations were purchased from Sigma, USA)

Fetal brain extract preparation

Using the method by Razeghian Jahromi *et al* (17), a pregnant rat at the late gestation period was obtained. After euthanizing and disinfection of the abdominal portion, an incision was made to achieve the uterus. Proximal and distal end of the uterus was cut and the entire parts with its fetuses were transferred to a sterile plate. It should be noted that all of these procedures were carried out in ice-cold conditions. Every fetus was removed and put in the new plate. Their skulls were dissected separately such that the whole brains were collected. Brains were homogenized and 1 mL of DMEM culture medium was added for every 150 mg of the brain. The suspension was centrifuged at 11800 g, 4°C, and 10 min. The supernatant was considered as the fetal brain extract and kept at -70 °C until use.

Fetal brain extract exposure and vimentin expression in BM-MSCs

Two groups of cells were prepared at passage 3 in two pairs of T75 culture flasks. One pair was allocated for day 3 and the other one for day 7 cell harvesting. Negative control subgroup had only DMEM as a culture medium, while the treatment subgroup had a modification in the culture medium composition; 80% of its volume was DMEM and 20% of the remaining part was fetal brain extract (17). Flasks were put in the incubator; after 3 and 7 days, culture mediums were discarded and cells were collected using trypsin/EDTA treatment. Cells of each flask were kept separately in a microtube. Centrifugation yielded cell pellet. RNA extraction and cDNA synthesis were performed as in the former step. PCR was done using the glial fibrillary acidic protein (GFAP) specific primer (Table 1). All of the materials and procedures were the same as the previous step. To clarify the identity of amplified PCR product, PCR was repeated 5 times more to have sufficient products for gel purification. This was performed by AccuPrep Gel Purification Kit (Bioneer, South Korea) and then it was sent for sequencing.

Results

BM-MSCs characterization

The rat bone marrow cells had a round shape at the seeding day. The majority of cells were adhered after 24 hr to the flasks due to their plastic adherence, while hematopoietic counterparts were removed by changing the medium, since they were floating in the culture medium. Various morphologies could be seen at the earlier stage, including spindle-shaped, flattened, or even round. In the later, most of the BM-MSCs had fibroblastic-like morphology. Some cells gathered and formed small colonies. These colonies became much larger by proliferation and then interconnected in such a way that 10-12 days after the seeding day a confluence of around 80% was expected. Passages 2 and 3 lasted only about three days to make a T75 culture flask confluence. In order to ascertain the identity of BM-MSCs in passage 3, RT-PCR was performed (Figures 1A and 1B). This technique demonstrated that CD73 and CD90 markers were present in the cell population at passage 3. In addition, absence of CD34 and CD45 was confirmed. Also, data from Alizarin red staining of BM-MSCs in osteogenic medium revealed the osteogenic potential (Figure 1C). After adipogenic induction, the BM-MSCs differentiated into adipoblasts confirmed by Oil Red O staining (Figure 1D).

Fetal brain extract effects on BM-MSCs

As it has been shown in Figure 2A in a population of BM-MSCs in the treatment group after day 3 of exposure to fetal brain extract, cells with fibroblastic or spindle-shaped appearance were increased. In addition, some cells were observed in which three or more neurite-like processes were projected out of a cell body. Other cell types were decreased. Indeed cell density in this group was significantly more than the negative control group. On the other hand, The BM-MSCs of passage 3 in the control group at day 3 had diverse morphologies (Figure 2B).

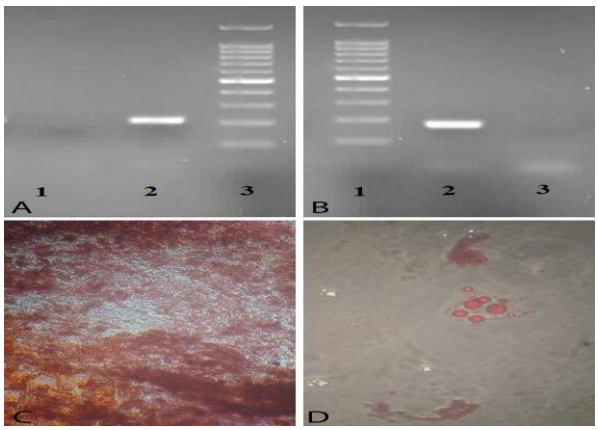

Figure 1. Presence of specific mesenchymal markers in rat bone marrow-derived mesenchymal stem cells (BM-MSCs). A, Lane 1: lack of CD34 marker (257 bp). Lane 2: presence of CD73 marker (208 bp). Lane 3: 100 bp ladder. B, Lane 1: 100 bp ladder. Lane 2: presence of CD90 marker (177 bp). Lane 3: lack of CD45 (450 bp). C, Alizarin red staining of rat BM-MSCs in osteogenic medium and D, Oil Red O staining of rat BM-MSCs in adipogenic medium

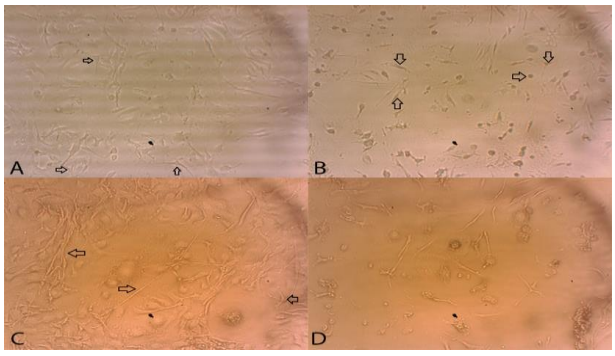

Figure 2. Differentiation of bone marrow-derived mesenchymal stem cells (BM-MSCs) exposed to fetal rat brain extract. A, BM-MSCs at day 3 in the treatment group, Branches like axons and dendrites are formed in neural-like cells in the treatment group (arrows). Transparent body, short branch, and long branch can be seen. B, BM-MSCs at day 3 in the control group kept their fibroblastic shape. Rat BM-MSCs at day 7 in the treatment group (C) and the control group (D)

Flattened, triangular, round, and some cells appeared fibroblastic or spindle-shaped.

It seems that after 7 days of exposure of BM-MSCs to fetal brain extract, longer neurite-like processes, more complicated projections, and cells with either up to three branches in different directions, were observed in the treatment group (Figure 2C).

Also, cell density was remarkably increased not only compared to the negative control group at this day but even in comparison to the treatment group at day 3. On the other hand, cells in the control group faced viability difficulties (Figure 2D).

A considerable proportion of cells became round, detached from the flask surface, and floated in the culture medium.

However some others mainly those with elongated projections were present and adhered to the flask surface.

Neural marker expression

Cells in the negative control group had only basal culture medium (DMEM). In the treatment group beside DMEM, cells faced fetal brain extract as much as one-fifth of the culture medium volume. Although GFAP specific primers were utilized in PCR, GFAP specific amplicon (130 bp) was detected neither in the treatment group nor in the negative control one. Interestingly in day 3, a band between 300 and 400 bp ladder strips was observed in the treatment group while no footprint of such a band had been detected in the negative control group. In day 7 overexpression of this marker was maintained in the treatment but not the negative control group. However, a fade expression of this size could also be observed in the negative control group in this day; but the severity of the expression in the treatment group was higher and in a continuance manner. Sequencing results showed that a 346 bp product was amplified. Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/ Blast.cgi) confirmed that this amplicon was vimentin mRNA with about 97% identity (Figure 4).

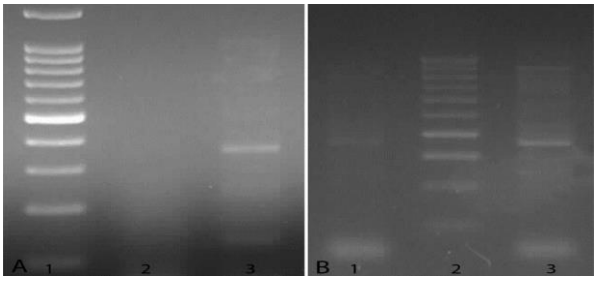

Figure 3. Vimentin overexpression detected by glial fibrillary acidic protein (GFAP) specific primers in bone marrow-derived mesenchymal stem cells treated with fetal brain extract in rat on Days 3 and 7. A, Day 3. Lane 1: 100 bp ladder. Lane 2: negative control group. Lane 3: treatment group. B, Day 7. Lane 1: negative control group. Lane 2: 100 bp ladder. Lane 3: treatment group

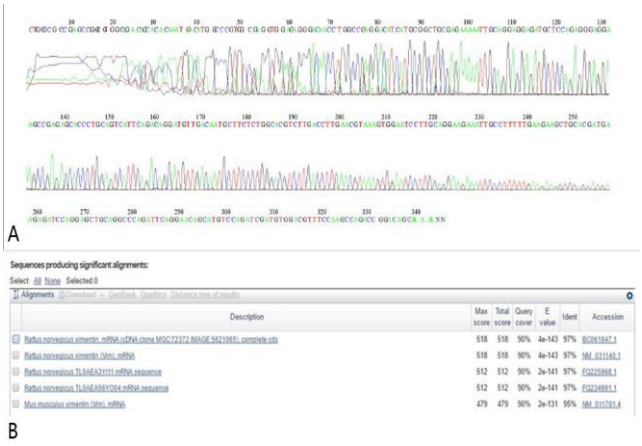

Figure 4. A, Vimentin sequencing result detected by glial fibrillary acidic protein (GFAP) specific primers in bone marrow-derived mesenchymal stem cells treated with fetal brain extract in rat. B, Results from nucleotide BLAST of our amplified product and the highly similar submitted sequences

Discussion

Findings of the present study showed the appearances of BM-MSCs that were exposed to fetal brain extract changed dramatically from spindle shape to cells with dendrite-like processes. While those neural-like processes were absent in the control group. In addition, a neural specific marker, vimentin, was expressed significantly in the treatment group but not in the negative control group. Nowadays MSCs have gained an important position in the field of regenerative medicine. Although multipotential, they can differentiate into cells belong to the three germ layers (18). Due to their adherence to the plastic surface, they can be easily separated from other groups of cells (19). High propagation capability in comparison to other cell sources like neural stem cells facilitates confronting the limitations of production of sufficient cells for research and therapeutics. It was demonstrated that the bone marrow probably has a cell population that under some circumstances could differentiate to the neural lineage (20). There are experiments that show *in vitro* differentiation of MSCs into different kinds of neural cells (neuron or glia) (21-24). In a study, MSCs expressed neural markers spontaneously without any differentiation inducing agents (25). This suggests an intrinsic predisposition of these cells for neural differentiation.

In the present study, we tried to assess the potency of brain extract at the fetal stage as a natural inducer to advance the differentiation of BM-MSCs to neural lineage. Early precursors and primary cells in the brain are nourished in this niche. It seems that fetal brain extract comprises any required elements such as chemokines and cytokines, some may be unknown, to start and terminate differentiation so that at the end neural cells are ready for specialized acts. Researchers applied different kinds of inducers for promoting neural differentiation. Some use chemical substances for MSCs differentiation but utilization of this type of stimulators has adverse side effects. For example, they may decrease cell viability and cause apoptosis (26). Others used non-chemicals such as growth factors. Woodbury and co-workers employed epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) for making neural-like cells from MSCs (5). Cell identities were judged by the elevated expression of neural markers such as nestin, beta-tubulin, Microtubule-associated protein 2 (MAP2), and NKX6 (5).

Other research such as the present study has used extracts from different parts of the nervous system for differentiation (17). Soluble extracts from rat cerebral cortex, cerebellum or hippocampus revealed bipolar and multipolar cells (27). Furthermore, treatment of embryonic stem cells with injured brain extract accelerated differentiation processes and elevated the MAP2 expression level (13). Furthermore, rat traumatized brain tissue extract was exposed to human umbilical cord MSCs. Results showed that apoptosis and proliferation were decreased and increased, respectively (28). In addition, Immunofluorescence staining confirmed that expression of GFAP and neuron positive cells were enhanced (28). Another study declared that brain extract has growth factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3/4 (NT3/4), which can promote neural cells' development. In this research omentum stem cells had more nestin and MAP2 expression than the control group (29). In another study, a specific differentiation protocol involving brain tissue extract, fibroblast growth factor, epidermal growth factor, supernatant from activated splenocytes, and electrical stimulation under physiological conditions were conducted on mouse adipose tissue-derived MSCs (30). This protocol could induce a change in cell morphology and genes expression of neuronal markers (30). Consistent with our findings, researchers assessed effects of different concentrations of striatal extract from injured or intact brain tissues on bone marrow-derived MSCs in rats (31). Cells developed bipolar or multipolar morphologies and also cells with neural specific markers like GFAP and NSE expression appeared significantly (31). Therefore, our findings in line with other studies demonstrate application of fetal brain extract can provide an appropriate media for differentiation of BM-MSCs.

On the other hand, as bone marrow was the primary source of MSCs, tibia and femur were considered for cell extraction. For making a homogenous cell population free of biological variations, bones of three rats were used in order to make a pool. Culture medium changing 24 hr after the first seeding leads to removal of a substantial proportion of non-adherent non-mesenchymal cells. The reason for the choice of passage 3 has two aspects. MSCs in the primary passages were probably not homogeneous and mixed with hematopoietic stem cells and their descendants. Furthermore, MSCs' surface antigens and consequently their properties may be changed at the later passages because of being a considerable time under *in vitro* conditions. Along with passing days, the number of cells in P0 having the fibroblastic outward, which is the typical appearance of MSCs, increased. In a similar pattern in the third passage approximately the majority of cells were MSCs with respect to morphology. Cell quantity decreased day by day in the control group; due to the lack of FBS and consequently the absence of necessary growth factors needed for cell survival and proliferation. The FBS exclusion was done because some experiments declared that this substance may act as a barrier in the neural differentiation progression (32, 33). Meanwhile, cell numbers in the treatment group was significantly higher. It shows that the fetal brain extract at least has some growth factors that stimulate cell proliferation. In addition, it means that

the extract preparation procedure was accurate enough to preserve some growth factors minimally.

It is remarkable that with the aid of GFAP specific primer (according to the BLAST results), another neural marker with a different product size has been amplified. Sequencing result confirmed that this amplified PCR product was vimentin, not GFAP. It may be due to the similarity between vimentin and GFAP mRNA sequences. Lack of existence of GFAP specific product is explained by the fact that mRNA of this neural marker was not expressed. Because GFAP is an astroglial specific marker (4), it means that fetal brain extract could not alter BM-MSCs toward this kind of neural cells but cells with vimentin overexpression emerged. Potential of fetal brain extract was sustained so that overexpression of vimentin in the treatment group was continued until day 7. The presence of cells with long processes and also vimentin footprint in the control group at day 7 probably arose from spontaneous differentiation of MSCs as mentioned in the earlier experiment (25, 34). According to microscopy observations, this extract has no negative effect on cell proliferation although it is not evaluated by a conventional proliferation test. Indeed, it seems that proliferation was stimulated in this condition. It should be mentioned that the definite identity of cells in the treatment group couldn't be judged and other neural markers and also functional properties must be evaluated.

Conclusion

Exposure of BM-MSCs of rats to fetal rat brain extract induced differentiation and formation of cells with axon- and dendrite-like projections. Furthermore, vimentin overexpression was observed in the treated groups which confirms neuron-like cell differentiation of BM-MSCs after induction.

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