Fetal Cerebrospinal Fluid Promotes Proliferation and Neural Differentiation of Stromal Mesenchymal Stem Cells Derived from Bone Marrow

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ABSTRACT

Embryonic cerebrospinal fluid (E-CSF) contains many neurotrophic and growth factors, acts as a growth medium for cortical progenitors, and can modulate proliferation and differentiation of neural stem cells. Mesenchymal stem cells (MSCs) are multipotential stem cells that can differentiate into several types of mesenchymal cells as well as nonmesenchymal cells, such as neural cells. In the present study, the effect of E-CSF on proliferation and neural differentiation of bone marrow mesenchymal stem cells (BM-MSCs) was investigated to test whether E-CSF is capable of driving these cells down the neuronal line. To verify the multipotential characteristics of BM-MSCs, the cells were analyzed for their osteogenic and adipogenic potential. Expression of the neural markers, MAP-2 and β-III tubulin, was determined by Immunocytochemistry. BM-MSCs differentiate into neuronal cell types when exposed to b-FGF. BMMSCs cells cultured in medium supplemented with CSF showed significantly elevated proliferation relative to control cells in media alone. E-CSF (E17-E19) supports viability and stimulates proliferation and, significantly, neurogenic differentiation of BM-MSCs. The data presented support an important role for CSF components, specifically neurotrophic factors, in stem cell survival, proliferation and neuronal differentiation. It is crucial to understand this control by CSF to ensure success in neural stem cell therapies.

Key words: Cerebrospinal fluid, Neural differentiation, Proliferation

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INTRODUCTION

After closure of the anterior neuropore, the cranial neural tube enlarges and generates the cephalic vesicles.
These are delineated by the neuroepithelium, composed of neural stem cells/radial glial cells and Cajal-Retzius cells forming the cortical preplate. These will eventually generate all the neurons and glial cells of the cerebral cortex together with the median and lateral eminence that provide post-natal neurogenesis and cortical interneurons (reviewed in(1, 2). The neural tube and cephalic vesicles are filled with embryonic cerebrospinal fluid (E-CSF) which plays important roles in neural development at both embryonic and fetal stages, regulating the survival, proliferation, and neural differentiation of the neuroepithelial progenitor cells (3-5).At the start of the major phase of cortical development, high volume CSF is secreted by the ventricular choroid plexus (6). The generation of neurons and glia from proliferating neural progenitor/stem cells is a complex process (7-9). During this development CSF is rich in proteins, in contrast to very low protein content in normal adults(10, 11). CSF contains growth factors that change during different stages of embryonic development. Several evidence suggest that the E-CSF contains diffusible factors such as transforming growth factor-s (TGF-s), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), insulin-like growth factor (IGF) and hepatocyte growth factor (HGF), basic fibroblastic growth factor (b-FGF) that regulating the survival, proliferation, and differentiation of primary cortical progenitor cells and neuroepithelium (7, 12). In chick embryos at early stages of CNS development, E-CSF contains FGF2, that this FGF2 is involved in regulating the behavior of neuroectodermal cells, including cell proliferation and neurogenesis(3). NGF, BDNF, NT-3 and NT-4 are involved in many more aspects of neural development and function(11). It also contains small molecules, salts, peptides, proteins and enzymes that play critical roles in a number of physiological processes. Changes in, concentration, composition and modifications of CSF proteins and peptides can accurately reflect pathological processes in the CNS, and offers a unique window to study CNS disorders (13-15). We have already demonstrated how CSF acts as a growth medium for cortical progenitor cells and how the age of CSF and the age of stem cells interacts to ensure that different parts of the CNS develop at the correct times (16).
Stem cells are characterized by their capacity for long-term self-renewal, in addition to their ability to differentiate into multiple cell types in response to instructive cues (17, 18). In adult bone marrow, there are two distinct populations of stem cells, hematopoietic stem cells (HSCs) that renew circulating blood, including red cells, monocytes, platelets, granulocytes and lymphocytes (19, 20), and non-hematopoietic mesenchymal (stromal) stem cells (BM-MSCs) are able to differentiate along number of different mesenchymal cell lineages (19, 21-27). The existence of nonhematopoietic stem cells in bone marrow was first suggested by Owen (21) ,who referred to these cells as stromal stem cells and whose work was based, at least in part, on the efforts of Friedenstein and his colleagues, who described bone marrow stromal cells that have the potential to differentiate into bone, cartilage, fat, and myelosupportive stroma (22, 28). MSCs are defined by their ability to adhere to plastic, and express surface markers(CD29+, CD44+, CD73+, CD90+, CD105+, CD146+, PDGFR+, CD31-, CD34–, CD45-, and Stro-1-)(29, 30) . This current investigation examined the differentiation of BM-MSCs into neural cells (evaluating two neural marker expression: MAP- 2 and β-III tubulin using E-CSF from various gestational ages, with the aim of testing whether fetal rodent CSF could drive proliferation and neural differentiation of BM-MSCs towards use for cell therapy.

MATERIAL and METHODS

Animal
All experiments were performed following ethical review by the animal use committee of The University of Kharazmi. Wistar rats were bred in house in the research facility of the Department of Biology, Kharazmi University and were kept in large rat boxes at constant temperature on a 12 hour light/dark cycle starting at 8am and with free access to food and water. For timed mating, individual male and female rats were paired in mating cages and checked regularly for the presence of a vaginal plug which was taken as an indication of successful mating and the day noted as embryonic day 0 (E0). At specific gestational time points pregnant dams were euthanized by cervical dislocation, the uterus rapidly removed onto ice, and fetuses dissected out onto ice. Each pregnant dam usually produced between 10–15 fetuses.

Collection of CSF samples
CSF was collected from the cisterna magna of rat fetuses at E17, E18, E19 using glass micropipettes and capillary action without aspiration. Aspiration invariably resulted in bleeding and contamination of the samples. Fetuses were positioned with heads flexed down on to the chest to allow penetration into the cisternal cavity through the skin and underlying muscle. Samples containing undesirable blood contamination, visualized as a pink color in the fluid, caused by damaging a blood vessel within the cisternal cavity, were discarded. All samples were collected into sterile microtubes and centrifuged at 4,000 rpm to remove cells or debris from the fluid, and the supernatant was transferred into another sterile tube. These samples were stored at $-40°C$ until use. The volume of CSF collected from each fetus by this method was between 5 and 50 µl and samples were pooled for each experiment.

Total protein analysis
Samples were pooled for specific fetal ages and total protein concentration measured using the Bradford protein assay with absorbance measured at 595 nm wavelength.

Preparation and culture of BM-MSCs
Adult NMRI mice (6–8 weeks) were sacrificed before the femur and tibia were removed from both hind legs of four mice per age group. After cleaning, the ends of the bones were cut and the bone marrow flushed out with Dulbecco's modified Eagle's medium (DMEM) using a syringe needle. Cells were disaggregated by gentle pipetting through decreasing needle bore size. The suspension of cells obtained was centrifuged at
1500 rpm at 25 °C for 5 min before resuspension in 1ml of medium. Cells were seeded at a density of 10_4 cells/cm2 in a 25 cm2 plastic flask in DMEM, 10% FBS (fetal bovine serum), 100 U/ml penicillin, and 100 mg/ml streptomycin, and incubated at 37°C with 5% CO_2. After 48h, nonadherent cells were removed by replacing the medium and addition of fresh medium which was repeated every 3 or 4 days. When confluent, cells were harvested with a scraper. Cells were used in experiments from the second passage.

Characterization of BM-MSCs
To verify the multipotential mesenchymal characteristics of the generated cell lines, cells were analyzed for their osteogenic and adipogenic potential (25, 31, 32).

Osteogenic differentiation

Osteogenic differentiation was assessed by incubating the cells with DMEM-LG and 10% FBS supplemented with 0.1 μM dexamethasone, 10 μM β-glycerophosphateand ,50 μM ascorbate, for 2 weeks. To assess mineralization, cultures were stained with 2% Alizarin Red (Sigma).

A dipogenic differentiation

Adipogenic differentiation was induced by culturing 90% confluent cultures in DMEM-LG supplemented with 10% FBS, 0.5 mM IBMX (isobutylmethylxanthine), 10 mg/ml insulin,1 mM dexamethasone and 100 mM indomethacin, for 2-4 weeks. The medium was changed every third day. To detect adipocytes, cells were stained with Oil Red (Sigma).

Surface antigen analysis of BM-MSCs by flow cytometry

BM-MSCs were characterized by flow-cytometric analysis of specific surface antigens of cluster of differentiation (CD) CD45, CD44, and CD29. Adherent cells at passage 3 were treated with 0.25% trypsin and washed twice with PBS. Cells were incubated with antibodies (mouse anti-CD44, mouse anti-CD29, mouse anti-CD45 (Abcam, UK) for 30 min at 4 ∘C and resuspended in 100μl of PBS. Unbound antibodies were removed by washing with PBS. After washing, cells were incubated for 40 min at room temperature in

the dark using FITC conjugated secondary antibody and resuspended in PBS for FACS analysis. At least $1 \times 10_6$ cells per sample were analyzed with a flow cytometer.

Effects of CSF treatment

All experiments were carried out in triplicate. Following the second passage, $7 \times 10_4$ cells were seeded in 24- well plates and the media changed every 2-3 days. Following attachment, cells were exposed to CSF (E17, E18 and E19) at 0, 3, 7 and 10% v/v concentrations in DMEM, 100 U/ml penicillin, and 100 mg/ml streptomycin, without FBS. Cell morphology was examined for neurite outgrowths after one week. Neuronlike

cells with processes were present in those cultures treated with CSF compared to control groups with no observable processes. Cells in individual wells were photographed and further analyzed using Image-J software (NIH)(10). A neurite was counted when a cellular process was longer than the diameter of the cell body. The average length of neurites was calculated from measurements of 10 cells in each of 6 wells for

each age of CSF tested. B-FGF is potent regulator of neurogenesis that promote differentiation of cortical

progenitors. CSF has been reported to contain b-FGF and other neurotrophic factors (7, 33), because of this

fact we utilized the b-FGF (10 ng/ml) to compare its effects on differentiation with those of E-CSF treatment.

MTT assay

Cell viability and/or proliferation were quantitatively determined by the MTT method. MTT (3-(4, 5- Dimethyl 2 thiazolyl)-2, 5- diphenyl 2 tetrazolium bromide) is a yellow tetrazolium dye that responds to metabolic activity. Reductases in living cells reduce MTT from a pale yellow color to dark blue formazan crystals. In 24- well plates, cells were seeded at $7 \times 10_4$ cells/well in 500 μL DMEM without FBS. Following

attachment, cells were exposed to CSF (E17-E19) with concentrations of zero, 3, 7 and 10% (V/V). After 24h, 100 μl of MTT (5 mg/mL in PBS) was added to each well and the cells were incubated for 4h at 37°C.

Finally, the supernatant was removed and 2 ml dimethylsulfoxide (DMSO) was added to each well to dissolve the blue substance. Absorbance was read at 570 nm in disposable cuvettes. All experiments were carried out in triplicate.

Immunocytochemistry
For immunocytochemistry cells were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilised with 0.1% Triton X-100 for 30 min at room temperature, blocked with 5% BSA in TPBS (Tween 20 in PBS) for 1 h at room temperature and then incubated at 4°C overnight in the presence of either anti-β-III tubulin (Abcam, UK, 1:100 dilution) or anti-MAP-2 (Abcam, UK, 1:100 dilution).). Negative controls, to verify the
specificity of the antibodies were obtained by omitting primary antibodies and incubating only with secondary antibodies. After three washes with TPBS, FITC conjugated goat anti-mouse IgG (1: 50 dilution Abcam, UK) was added at room temperature for 1 hr. Cells were washed and photomicrographs were taken
with a florescence microscope (Olympus, Tokyo, Japan).

Statistical analysis
All values are expressed as mean • } standard error of the mean (SEM). Statistical analysis was performed using the one-way ANOVA and tukey test, and significance was accepted for p values of <0.01.

RESULTS and DISCUSSION

After two or three passages, BM-MSCs became homogeneous in appearance with two distinct populations of large flattened cells and relatively elongated or spindle-shaped cells (Fig. 1 C).
Cultures were exposed to osteogenic differentiation medium as described in Methods. (Fig. 1 A) shows representative plates of cells stained with Alizarin Red after differentiation treatments. Microscopic visualization identified >95% of cells as positive for Alizarin Red. BM-MSCs were exposed to adipogenic differentiation medium for 21 days, stained with Oil-Red and examined microscopically for cytoplasmic lipid
droplets (Fig. 1 B).
Surface antigens of passage 3 cells were analyzed using flow cytometry and revealed that the BM-MSCs were positive for CD44 and CD29 and were negative for CD45 (Fig. 2). CSF of rat fetuses aged E17 had a mean total protein concentration of 2 • } 0.1 mg/ml, which was higher than E18 and E19 that were 1.7 and 1.6 mg/ml, respectively (Fig. 3). 3 days following CSF treatment, spindle shape BM-MSCs turned elongated. The
cell-processes became longer and more evident after 7 days. Inverse microscopic examination of BM-MSCs revealed neuron like cells in cell cultures treated with E17-E19 CSF and b-FGF (positive control) compared to the control group (without CSF) (Fig. 4).
Furthermore, the average neurite outgrowth of cells was significantly greater than controls when cultured in the presence of b-FGF (p<0.001), or CSF from E17 (p<0.001) or E19 (p<0.001) for 7 days (Fig. 5) while it was only slightly increased compared to the controls in CSF from E18, which was non-significant. In addition, as shown in Fig. 5, the length of neuritis was much greater in b-FGF treated cells than in CSF (E17 and E19) treated cells.
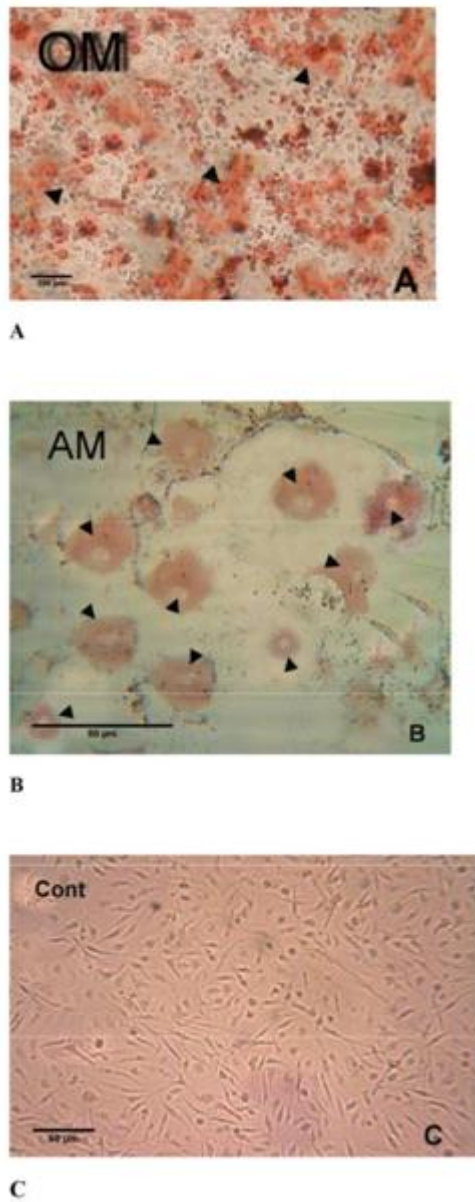
Fig. 1: Characterization of the BM-MSCs and tested for multipotential characteristics: (A) Cells grown in osteogenic medium and stained with Alizarin red, (B) Cells grown in adipogenic medium stained with Oil red O, (C) Cells grown in control medium with no defined cytokines to promote differentiation.
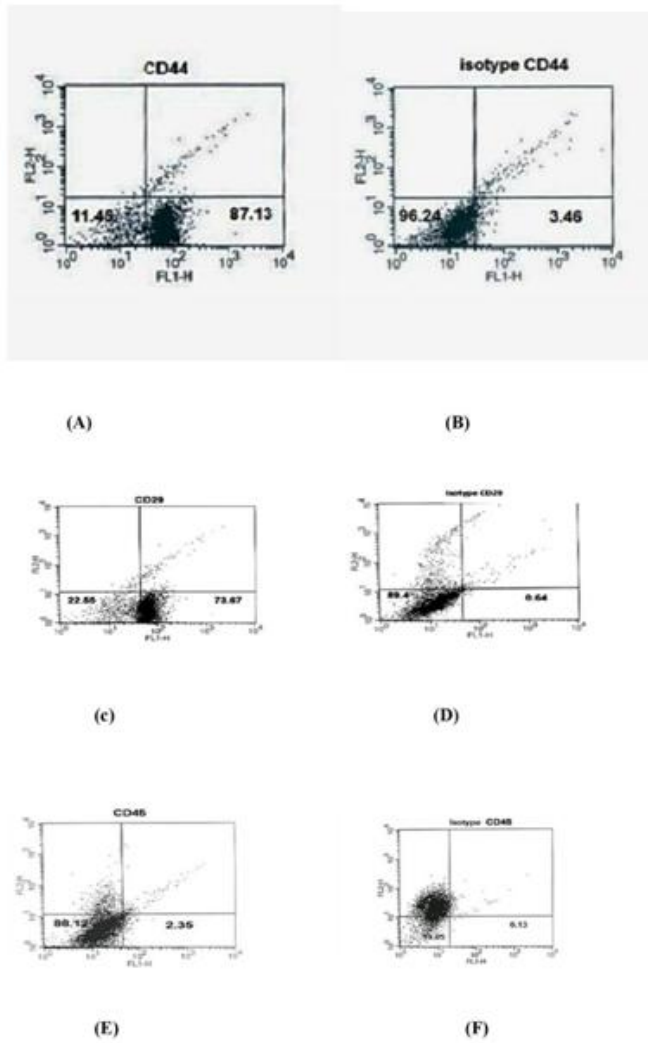
(A) (B)

(c) (D)

(E) (F)

Fig. 2: Flow cytometry for surface markers of BM-MSCs .The BM-MSCs suspension was immune stained for CD29, CD44, and CD45. FACS analysis revealed that BM-MSCs were positive for CD29 and CD44 but negative for CD45 .Each analysis is shown with its isotype control stain for comparison.
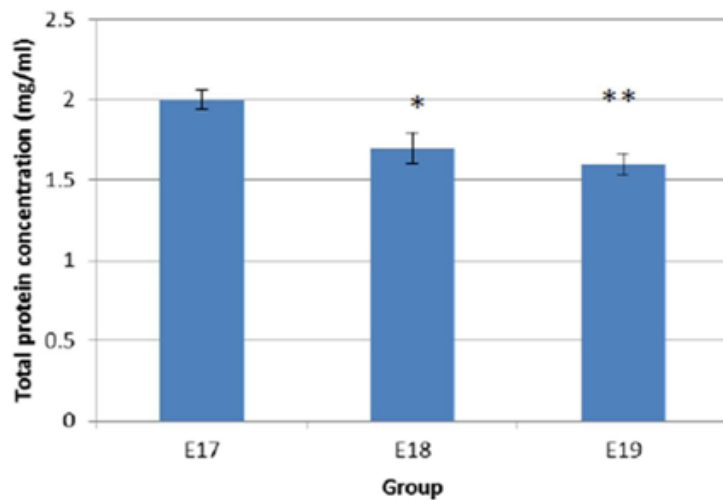

Fig. 3: Total protein content of fetal rat CSF. Histogram of total protein concentration in pooled samples of cerebrospinal fluid (CSF) from rat fetuses at embryonic days E17 to E19. There are the significant reduction in total protein through this short developmental period (* $p < 0.05$, ** $p < 0.01$).
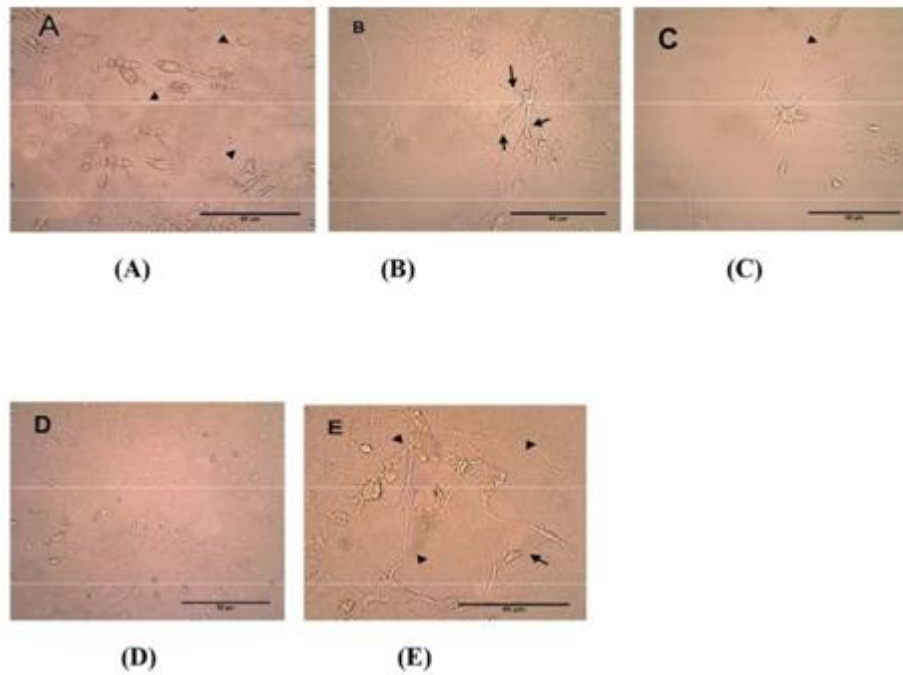
Fig. 4: In vitro growth of BM-MSCs. Cells were photographed with phase-contrast optics after 7 days (A) cultured with 10% E17 CSF, (B) cultured with 10% E18 CSF, (C) cultured with10% E19 CSF, (D) negative control cells growing in media without CSF addition and, (E) positive control cells cultured in b-FGF(10 ng/ml).
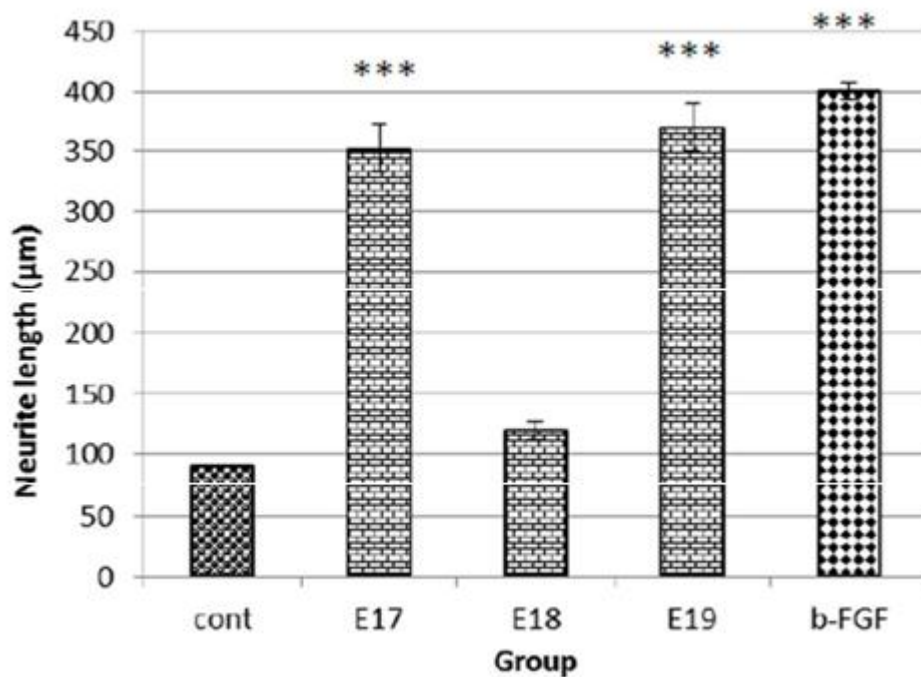

Fig. 5: In vitro neurite growth from BM-MSCs. Cells was cultured for 7 days in media alone (control) or media supplemented with 10% CSF from E17 to E19 CSF and 10ng/ml b-FGF. Neurite length was measured for at least 5 representative cells from each of 3 wells for each age of CSF tested. *** $p < 0.001$ compared to control culture.

Viability and cell proliferation measures of BM-MSCs cultured in E17 or E18 CSF-supplemented medium were greater than those of other treated groups (E19) as well as showing a concentration effect (Fig. 6) with a significant increase in 10% CSF compared to 3% and 7%. E18 CSF gave a significant increase in viability over that seen in media alone which fits with our previous data from primary cortical cells where E18 gave increased proliferation of cells (34). Thus it was concluded that the effective concentration of CSF for proliferation of BM-MSCs was 10% (Fig. 6). Moreover, we found β-III tubulin and MAP-2 expression in BM-MSCs grown in CSF supplemented media as well as b-FGF supplemented media indicating neuronal differentiation (Fig. 7). s-III tubulin and MAP-2 have been used as sensitive and specific markers for neural differentiation.
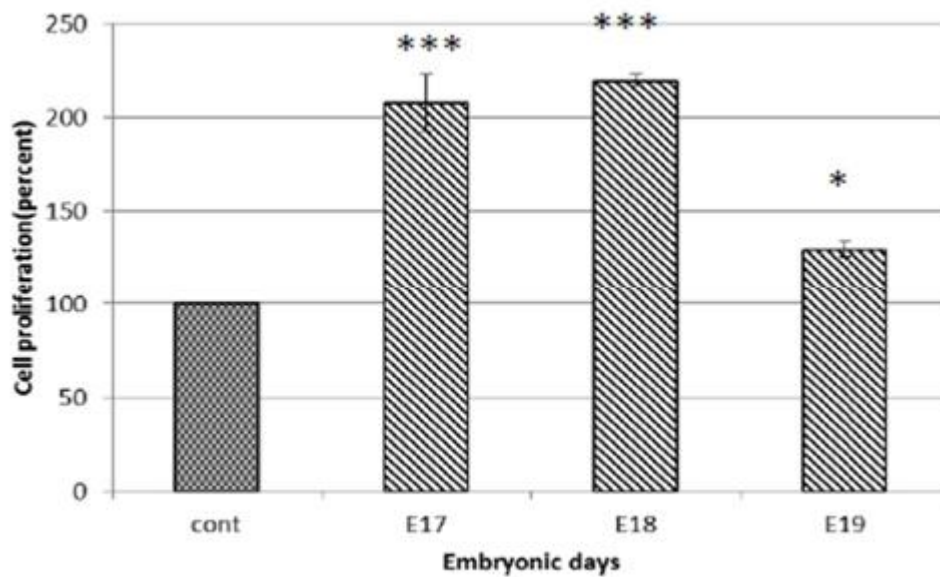

Fig. 6: Survival and proliferation of BM-MSCs cultured with fetal rat CSF. Reduction of MTT measured color metrically by the absorbance of formazan product. Cells were treated with 10% CSF from gestational ages E17 to E19 and analyzed after 24h in culture. Results are expressed as a percentage of control levels (cultures without added CSF). All cultures with added CSF had higher viability and proliferation and this was significant with E18 and E17 CSF compared to controls (*p < 0.05‹**p < 0.01, ***p < 0.001).
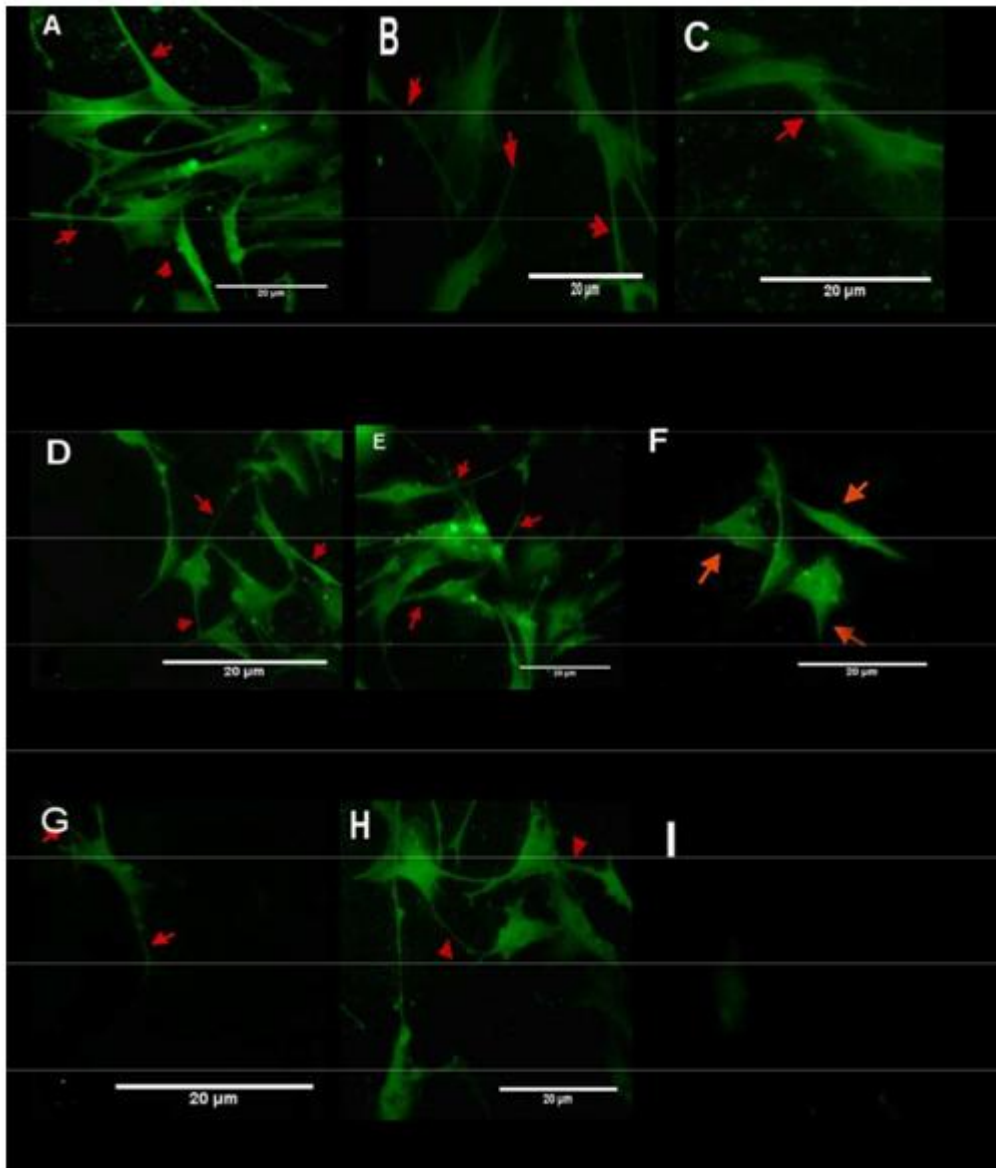
Fig. 7: β-III tubulin expression: in BM-MSCs cultured with 10ng/ml b-FGF (A), 10% E17 CSF (B), E18 CSF (C) and E19 CSF (D). MAP-2 expression: in BM-MSCs cultured with 10ng/ml b-FGF (E), 10% E17 CSF (F), E18 CSF (G) and E19 CSF (H). (I) shows example control (cultures without added CSF) cells showing no stain when the primary antibody was omitted. E17 and E19 give similar staining intensity to b-FGF treated for β-III tubulin while E18 CSF gave less intense staining.

It is broadly agreed that stem cells are going to be an important part of cell-based therapies in which a relatively high number of healthy cells are needed[21]. Among the stem cells that have been isolated and studied, BM-MSCs are proving to be of great interest for potential use in brain repair[35]. In this study, we were able to isolate BM-MSCs and expanded them in vitro. It was observed from this present study that MSCs appeared as population of plastic adherent, highly proliferative cells and able to form colonies according to theory of Friedenstein et al (1987)[36]. These cells were similar to those reported by others (Azizi et al., 1998) based on morphology and expression of cell-surface markers. In our FACS experiments we found no evidence of hematopoietic precursors and the isolated BM-MSCs were negative for the lymphohematopoietic marker CD45. BM-MSCs are shown to have

an inherent potential to differentiate along the mesodermal lineage such as adipogenic, chondrogenic and osteogenic cells that most commonly used to identify cell populations with multilineage differentiation capabilities (37, 38). Our results demonstrated that BM-MSCs are also capable of multilineage differentiation.

The most critical constituents of CSF are its protein components, the quality and quantity of which change during CNS development (39). Critical growth factors are important for development of the cerebral cortex, including FGF, TGF-β, NGF, BDNF, NT-3, IGFs and others(40) which are found in active concentration of fetal CSF. Proteomic studies have also revealed the presence of mitogenic factors in CSF (41).Growing evidence suggests that CSF plays an important role as a neural stem cell niche and provides the microenvironment regulating neuroepithelial cells (42), and indeed has been shown to be capable of supporting viability, proliferation and differentiation of primary cortical progenitor cells(10) as well as PC12 cell (10). Proteomic composition of fetal CSF suggested that it has all the secretory factors, growth factors, cytokines, extracellular matrixproteins, adhesion molecules and many other materials and nutrients. These components sufficient to maintain neural stem cells survival and regulate proliferation and differentiation of the progenitor cells in to mature cells (43). So we hypnotized that add E- CSF (E17, E18 and E19) with different concentration to the culture media provides a better micro-environment for inducing the neural differentiation of BM-MSCs.

We have demonstrated that fetal CSF has a noteworthy potential to induce differentiation in vitro culture. In this study expression of β-tubulin and MAP-2 neural markers was significantly increased compare with the control group. So, we could say CSF indeed promotes neuronal differentiation and proliferation of BMMSCs

in an age dependent manner. This study adds to the body of evidence supporting a vital role for CSF as a growth medium for the developing brain and as the "missing" factor in thinking about neurological conditions, both through developmental abnormalities and later in life as neurodegeneration. The evidence from these studies indicates that understanding the detailed role of CSF in development, function and pathophysiology of the brain will be one key area to promote normal development and to develop strategies and treatments to prevent abnormal development and neuropathological conditions . Moreover, understanding CSF and the possibility of manipulating its composition through action on the choroid plexus, may prove to be vital in the successful use of neuronal stem cells and BM-MSCs in the treatment of brain damage and neurodevelopmental and neurodegenerative conditions (44). The current study provides a parallel cell linebased analysis system to that of primary brain cells to investigate the role of CSF.

CONCLUSIONS

In conclusion we have described evidence that CSF can induce proliferation and neural differentiation of BM-MSCs in an age dependent manner confirming that CSF is a powerful growth medium promoting brain development. CSF provided an essential niche for promoting the differentiation of BM-MSCs in vitro. Thus, further studies seem necessary to investigate CSF components and synergistic effects of them.

ACKNOWLEDGEMENTS

REFERENCES

1. Paridaen JT, Huttner WB. Neurogenesis during development of the vertebrate central nervous system. EMBO reports. 2014:e201438447.
2. Urbán N, Guillemot F. Neurogenesis in the embryonic and adult brain: same regulators, different roles. Frontiers in cellular neuroscience. 2014;8.
3. Alonso M, Martín C, Carnicero E, Bueno D, Gato A. Cerebrospinal fluid control of neurogenesis induced by retinoic acid during early brain development. Developmental Dynamics. 2011;240(7):1650-9.
4. Gato A, Desmond ME. Why the embryo still matters: CSF and the neuroepithelium as interdependent regulators of embryonic brain growth, morphogenesis and histiogenesis. Developmental biology. 2009;327(2):263-72.
5. Vera A, Stanic K, Montecinos H, Torrejón M, Marcellini S, Caprile T. SCO-spondin from embryoniccerebrospinal fluid is required for neurogenesis during early brain development. Frontiers in cellular neuroscience. 2013;7.

6. Miyan JA, Nabiyouni M, Zendah M. Development of the brain: a vital role for cerebrospinal fluid. Canadian journal of physiology and pharmacology. 2003;81(4):317-28.
7. Mashayekhi F, Azari M, Moghadam LM, Yazdankhah M, Naji M, Salehi Z. Changes in cerebrospinal fluid nerve growth factor levels during chick embryonic development. Journal of Clinical Neuroscience. 2009;16(10):1334-7.
8. Stevens HE, Smith KM, Rash BG, Vaccarino FM. Neural stem cell regulation, fibroblast growth factors, and the developmental origins of neuropsychiatric disorders. Frontiers in neuroscience. 2010;4.
9. Yari S, Parivar K, Nabiuni M, Keramatipour M. Effect of embryonic cerebrospinal fluid on proliferation and differentiation of neuroprogenitor cells. Cell Journal (Yakhteh). 2013;15(1):29.
10. Nabiuni M, Rasouli J, Parivar K, Kochesfehani HM, Irian S, Miyan JA. In vitro effects of fetal rat cerebrospinal fluid on viability and neuronal differentiation of PC12 cells. Fluids Barriers CNS. 2012;9(8).
11. Orešković D, Klarica M. The formation of cerebrospinal fluid: nearly a hundred years of interpretations and misinterpretations. Brain research reviews. 2010;64(2):241-62.
12. Salehi Z, Mashayekhi F. The role of cerebrospinal fluid on neural cell survival in the developing chick cerebral cortex: an in vivo study. European journal of neurology. 2006;13(7):760-4.
13. Mashayekhi F. The importance of cerebrospinal fluid in cerebral cortical development. IJST. 2012;4:493-9.
14. Naureen I, Waheed Kh A, Rathore AW, Victor S, Mallucci C, Goodden JR, et al. Fingerprint changes in CSF composition associated with different aetiologies in human neonatal hydrocephalus: inflammatory cytokines. Childs Nerv Syst. 2014;30(7):1155-64.
15. Naureen I, Waheed KA, Rathore AW, Victor S, Mallucci C, Goodden JR, et al. Fingerprint changes in CSF composition associated with different aetiologies in human neonatal hydrocephalus: glial proteins associated with cell damage and loss. Fluids Barriers CNS. 2013;10(1):34.
16. Miyan JA, Zendah M, Mashayekhi F, Owen-Lynch PJ. Cerebrospinal fluid supports viability and proliferation of cortical cells in vitro, mirroring in vivo development. Cerebrospinal Fluid Res. 2006;3:2.
17. Lairson LL, Lyssiotis CA, Zhu S, Schultz PG. Small Molecule–Based Approaches to Adult Stem Cell Therapies. Pharmacology and Toxicology. 2013;53.
18. Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. Blood. 2003;102(10):3483-93.
19. Lei Z, Yongda L, Jun M, Yingyu S, Shaoju Z, Xinwen Z, et al. Culture and neural differentiation of rat bone marrow mesenchymal stem cells< i> in vitro</i>. Cell biology international. 2007;31(9):916-23.

20. Morikawa S, Mabuchi Y, Kubota Y, Nagai Y, Niibe K, Hiratsu E, et al. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. The Journal of experimental medicine. 2009;206(11):2483-96.

21. Jamous M, Al-Zoubi A, Khabaz MN, Khaledi R, Al Khateeb M, Al-Zoubi Z. Purification of mouse bone marrow-derived stem cells promotes ex vivo neuronal differentiation. Cell transplantation. 2010;19(2):193-202.

22. Bae KS, Park JB, Kim HS, Kim DS, Park DJ, Kang SJ. Neuron-like differentiation of bone marrow-derived mesenchymal stem cells. Yonsei medical journal. 2011;52(3):401-12.

23. Kassem M, Kristiansen M, Abdallah BM. Mesenchymal stem cells: cell biology and potential use in therapy. Basic & clinical pharmacology & toxicology. 2004;95(5):209-14.

24. Ma K, Fox L, Shi G, Shen J, Liu Q, Pappas J, et al. Generation of neural stem cell-like cells from bone marrow-derived human mesenchymal stem cells. Neurological research. 2011;33(10):1083-93.

25. Rostovskaya M, Anastassiadis K. Differential expression of surface markers in mouse bone marrow mesenchymal stromal cell subpopulations with distinct lineage commitment. PloS one. 2012;7(12):e51221.

26. Foudah D, Redondo J, Caldara C, Carini F, Tredici G, Miloso M. Expression of neural markers by undifferentiated rat mesenchymal stem cells. BioMed Research International. 2012;2012.

27. Sun S, Guo Z, Xiao X, Liu B, Liu X, Tang PH, et al. Isolation of mouse marrow mesenchymal progenitors by a novel and reliable method. Stem cells. 2003;21(5):527-35.

28. Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem cells. 2001;19(3):180-92.

29. Karaoz E, Aksoy A, Ayhan S, Sarıboyacı AE, Kaymaz F, Kasap M. Characterization of mesenchymal stem cells from rat bone marrow: ultrastructural properties, differentiation potential and immunophenotypic markers. Histochemistry and cell biology. 2009;132(5):533-46.

30. Rostovskaya M, Anastassiadis K. Differential expression of surface markers in mouse bone marrow mesenchymal stromal cell subpopulations with distinct lineage commitment. 2012.

31. Rombouts W, Ploemacher R. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. Leukemia. 2003;17(1):160-70.

32. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. science. 1999;284(5411):143-7.

33. Martin C, Alonso M, Santiago C, Moro J, De la Mano A, Carretero R, et al. Early embryonic brain development in rats requires the trophic influence of cerebrospinal fluid. International Journal of Developmental Neuroscience. 2009;27(7):733-40.

34. Owen-Lynch PJ, Draper CE, Mashayekhi F, Bannister CM, Miyan JA. Defective cell cycle control underlies abnormal cortical development in the hydrocephalic Texas rat. Brain. 2003;126(3):623-31.

35. Taran R, Mamidi MK, Singh G, Dutta S, Parhar IS, John JP, et al. In vitro and in vivo neurogenic potential of mesenchymal stem cells isolated from different sources. Journal of biosciences. 2014;39(1):157-69.

36. Friedenstein A, Chailakhyan R, Gerasimov U. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell proliferation. 1987;20(3):263-72.

37. Zheng YH, Xiong W, Su K, Kuang SJ, Zhang ZG. Multilineage differentiation of human bone marrow mesenchymal stem cells in vitro and in vivo. Experimental and therapeutic medicine. 2013;5(6):1576-80.

38. Sudo K, Kanno M, Miharada K, Ogawa S, Hiroyama T, Saijo K, et al. Mesenchymal progenitors able to differentiate into osteogenic, chondrogenic, and/or adipogenic cells in

vitro are present in most primary fibroblast-like cell populations. Stem cells. 2007;25(7):1610-7.

39. Yuan X, Desiderio DM. Proteomics analysis of human cerebrospinal fluid. Journal of Chromatography B. 2005;815(1):179-89.

40. Mashayekhi F. The importance of cerebrospinal fluid in cerebral cortical development. Iranian Journal of Science and Technology. 2012;36(A4):493.

41. Zappaterra MD, Lisgo SN, Lindsay S, Gygi SP, Walsh CA, Ballif BA. A comparative proteomic analysis of human and rat embryonic cerebrospinal fluid. Journal of proteome research. 2007;6(9):3537-48.

42. Johansson PA, Cappello S, Götz M. Stem cells niches during development—lessons from the cerebral cortex. Current opinion in neurobiology. 2010;20(4):400-7.

43. Lehtinen MK, Bjornsson CS, Dymecki SM, Gilbertson RJ, Holtzman DM, Monuki ES. The choroid plexus and cerebrospinal fluid: emerging roles in development, disease, and therapy. The Journal of Neuroscience. 2013;33(45):17553-9.

44. De Meyer G, Shapiro F, Vanderstichele H, Vanmechelen E, Engelborghs S, De Deyn PP, et al. Diagnosis-independent Alzheimer disease biomarker signature in cognitively normal elderly people. Archives of neurology. 2010;67(8):949-56.