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. BM-MSCs 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 10⁴ 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% CO₂. 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-glycerophosphate and 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 1x10^6 cells per sample were analyzed with a flow cytometer.

**Effects of CSF treatment**
All experiments were carried out in triplicate. Following the second passage, 7x10^4 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 7x10^4 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 24 h, 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 fluorescence 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 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.
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**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 with 10% 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.

![Graph](image)

**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 microenvironment 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 BM-MSCs 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 line based 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.

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