Original Article

Cultivation and Neural Differentiation of Embryonic Cerebrospinal Fluid Treated Adipose Stem Cells on the Scaffold of Amniotic Membrane

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Article Information
Received: 2020-05-10
Revised: 2020-06-14
Accepted: 2019-07-13

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Cite this article as:
Keshavarzi R, Nabiuni M, Jalali H. Cultivation and Neural Differentiation of Embryonic Cerebrospinal Fluid Treated Adipose Stem Cells on the Scaffold of Amniotic Membrane. Archives of Advances in Biosciences : 2020:11(2)

Abstract

Introduction: Adipose stem cells (ASCs) are ideal candidates for cell therapy of neurological disorders. *In vitro* methods require the use of a variety of growth factors and multi-step protocols to induce neuronal differentiation. This study was aimed to assess the neural differentiation of adipose stem cells in a co-culture system.

Material and Methods: ASCs were obtained from male Wistar rats and were characterized, using flow cytometry. Harvested ASCs were cultured on a scaffold prepared from amniotic membrane (AM). Cerebrospinal fluid (CSF) was collected from rat embryos and was added to culture medium for 7 days. Structure of scaffold and cell attachment was assessed through scanning electron microscopy (SEM). Neural differentiation of ASCs in the co-culture system was confirmed with immunofluorescence (IF) staining for β-tubulin III and MAP-2 markers.

Results: SEM results confirmed the decellularization of AM and attachment of ASCs on the AM derived scaffold. MTT assay revealed that ASCs proliferated on AM significantly during the 7 days of culture. IF data confirmed that the CSF treated cells were expressed by β-tubulin III and MAP-2 but untreated cells were negative for the expression of neural markers.

Conclusion: Cultivation of ASCs on the scaffold and their treatment with CSF induced them into the neural lineage fate in the absence of any chemical inducing factor. This method of co-culture may represent a new method to improve *in vitro* neural differentiation of ASCs.

Keywords: Cell therapy, Scaffold, Neurodegeneration, Brain, Growth factor

1. Introduction

Adipose tissue stem cells (ASCs) are mesenchymal cells (MSCs) with the capacity for self-renewal and multi-potential differentiation. The ease of access without ethical concerns, high rate of proliferation, long term survival in host tissue and modulation of immune system are among their advantages which make ASCs ideal candidates for cell therapy and regenerative medicine purposes [1]. ASCs express a marker for neural progenitor cells, Nestin, and have a differentiation potential into neuronal and glial cells [2].

At present, the use of growth factors, chemical inducers, gene transfection, conditioned growth medium, and combined methods are main procedures which are used to induce neural differentiation in stem cells [3]; however, inducers that are close to the microenvironment of the human body are under consideration. Cerebrospinal fluid

(CSF) is an important component of the central nervous system (CNS) that has been shown to be critical in neuronal development [4]. CSF contains large quantities of proteins and trophic factors such as the brain-derived and fibroblast growth factors [5] which affect the function of stem cells and acts as the regulator for stem cell differentiation into nerve cells [6,7]. Comparison between embryonic and adult CSF showed that embryonic CSF had high rate of protein content [8].

The biochemical signals and environment, in which cell-cell and cell-environment connections are established, are two factors which conduct stem cell differentiation toward a specific lineage [9]. It has been proven that the conventional two-dimensional culture systems do not mimic the actual tissue structure and the cellular interactions which occur in vivo [10]. An alternative way to resolve this problem is using three-dimensional (3D) scaffolds [11]. 3D scaffolds may allow the control of proliferation and differentiation of stem cells and also their derivatives [12]. Scaffolds derived from decellularized tissues act as excellent three dimensional media for stem cells [13]. They provide a natural microenvironment for cell migration and differentiation and are a proper alternative to synthetic polymers [14]. Amniotic membrane (AM) is a fetal-derived tissue with significant properties such anti-inflammatory, immunomodulary and antimicrobial effects. In recent years, it has been considered as a fascinating biomaterial in the field of tissue engineering and regenerative medicine [15]. The extracellular matrix of AM contains collagen IV, collagen VII, Laminin 5, and Perlecan, which provide an exhaustive environment for cell adhesion and proliferation [16] .

This study aims to assess the ability of embryonic CSF (e-CSF) to stimulate neural differentiation of ASCs in a natural 3D culture medium. The main goal was to demonstrate that a biological three-dimensional medium provides a good environment for the proliferation and differentiation of ASCs into neural cells in the absence of additional inducing factors. This is the first study that evaluates the effect of e-CSF on ASCs in the 3D environment.

2. Materials and Methods

2.1 Isolation and Characterization of ASCs

ASCs were obtained from two-month-old male Wistar rats and characterized according to expression of CD90 and CD45 markers [17]. Briefly, rats were anaesthetized and the skin was pulled off from the inguinal region. The fats were removed from the animal body and placed in a falcon containing cold phosphate buffered saline (PBS; Gibco, UK). Under the laminar hood, fatty fragments were converted into small pieces and washed with PBS several times. Then, fine fragments were transferred to another falcon containing Trypsin-EDTA (Gibco, UK) of 0.5%, and the falcon was placed into an incubator at 37 ° C and 50 rpm for 55 minutes. After this step, the Dulbecco Modified Eagle Medium (DMEM; Sigma-Aldrich, UK) was added to the sample and the falcon was centrifuged for 15 minutes at 1500 rpm. The settled cells were transferred to DMEM, supplemented with 15% fetal bovine serum (FBS; Gibco, UK), 50 U/mL penicillin, and 50 mg/mL streptomycin (both from Gibco, UK). The suspension was cultivated in 25 cm^2 flasks and incubated at 37 ° C in 5% CO_2. After 48 hours, incubated cells were replaced with fresh medium and the non-adherent cells were removed [18]. For flowcytometry analysis of specific surface antigens, adherent cells at passage 3 were treated with 0.25% trypsin and washed twice with PBS. Cells were incubated with conjugated antibodies (Biolegend, USA) for 30 minutes at 4 ° C and suspended in 100 µl of PBS. Unbounded antibodies were removed by washing with PBS and the cells were

suspended in PBS for FACS analysis. At least 1×10^6 cells per sample were analyzed with flow cytometer.

2.2 CSF Collection

The cerebrospinal fluid was collected from Wistar rat embryos at embryonic day 17 (E17) according to previous results showing protein concentration of embryonic CSF [19,20]. The CSF was collected from the cisterna magna using glass micropipette and to remove the remaining cells and debris, samples were centrifuged at 1500 rpm. The supernatants were transferred to sterile micro-tubes and were immediately frozen at – 80 ° C. To prevent protein degeneration, all stages were carried out on the ice.

2.3 Scaffold Preparation

Human placenta was obtained from healthy donors during caesarian sections in the sterile condition and the amniotic membrane was separated from other associated membranes of the placenta. Washing was performed with normal saline containing penicillin and streptomycin and tissue was placed on cellulose filter paper and maintained in the vial containing equal ratios of DMEM/glycerol and stored at -80 ° C. To prepare the scaffold, frozen amnion membrane samples were thawed at 37 °C, and then incubated in 0.25% trypsin- EDTA at 37 ° C for 20 minutes. To complete the cell elimination process, membrane was gently scraped with cell scraper. The decellularized amniotic membrane (dAM) was transferred into DMEM and incubated for 24 hours at 37 ° C in 5% CO_2.

2.4 Scanning Electron Microscopy

The scaffolds were fixed with glutaraldehyde 2.5 M in PBS 0.1% at 4 ° C for 1 hour and then fixed in the osmium tetroxide 1% for 2 hours. Following fixation, specimens were dehydrated in ascending grades of ethanol, sputtered with gold and viewed with a scanning electron microscope (Tescan, Czech Republic).

2.5 Cell cultivation on dAM scaffold and e-CSF treatment

The present study consisted of two experimental groups: group 1: ASCs cultured on dAM without treatment with e-CSF (control) and group 2: ASCs cultured on dAM with treatment with e-CSF (test). Briefly, the dAM pieces were spread on culture plates and 2×10^5 cells of ASCs were transferred to them in DMEM, containing 1% Pen-Strep and 15% FBS. Culture medium was replaced 48 hours later and non-adhered cells were removed using washing.

To study the effect of e-CSF on ASCs seeded on dAM derived scaffold, 48 hours after cell cultivation, treatment with a concentration of 7% v / v of E17 CSF was started and continued for one week. On the seventh day of treatment, cells were examined for neuronal differentiation.

2.6 MTT Assay

To investigate the proliferation rate of ASCs seeded on dAM, the 3-(4, 5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay was performed in control group. Briefly, 2×10^5 cells were seeded on AM derived scaffold and after cell attachment, MTT assay was carried out at fifth and seventh day after cell cultivation. A total volume of 10 µL of 5 mg/mL MTT (Sigma-Aldrich, UK) solution was added to each well and incubated for 4 hours, and then the medium was discarded. Subsequently, 100 µL dimethyl sulfoxide (DMSO; Merck, Germany) was added to dissolve formazan salts, and the absorbance value for each well was measured at 450 nm. The viability percentage was determined using the ratio of absorbance in the first day to the absorbance of 5th and 7th days.

2.7 Immunofluorescent Analysis

The expression of neural related proteins MAP-2 and β-tubulin III in e-CSF treated ASCs was analyzed using immunostaining.

Briefly, cultured cells on scaffolds were fixed in 4% paraformaldehyde in PBS for 45 minutes, permeabilized with 0.4% Triton X-100 for 30 minutes at room temperature and subsequently blocked with 1% BSA in TPBS (Tween 20 in PBS) for 1 hour at room temperature. Specimens were incubated at 4 °C overnight in the presence of either anti-MAP-2 monoclonal antibody (1:500 dilution; Sigma-Aldrich, UK) or anti-β tubulin III monoclonal antibody (1:500 dilution; Sigma-Aldrich, UK). The following day, after three washes with TPBS, FITC conjugated goat anti-rat IgG as secondary antibody (1:1000 dilution; Sigma-Aldrich, UK) was added at room temperature for 1 hour. Scaffolds were then washed and photomicrographs were taken with a fluorescence microscope (Olympus, Tokyo, Japan).

2.8 Ethical Statement

All procedures performed in the current study were in accordance with the ethical standards of the 1964 Helsinki declaration and its ethical standards.

2.9 Statistical Analysis

Data were analyzed using SPSS statistical software and statistical test, ANOVA (one-way). A difference between groups was considered statistically significant if $p<0.05$.

3. Results

ASC Culture and Characterization

Adipose stem cells were harvested from the inguinal region of Wistar rats and were transferred to culture plates. Observation under inverted microscopy confirmed their spindle shape morphology (Figure 1). Surface antigen expression analysis revealed that the isolated ASCs were positive for mesenchymal stem cell marker CD90 and were negative for hematopoietic marker CD45 (Figure 2).

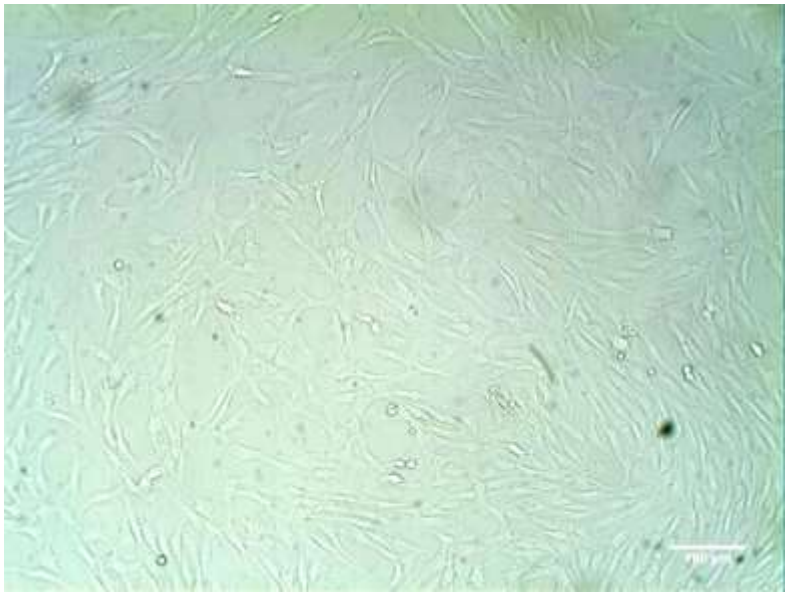

Figure 1. Photomicrograph of spindle shaped adipose stem cells under inverted microscopy (magnification: 400 x).

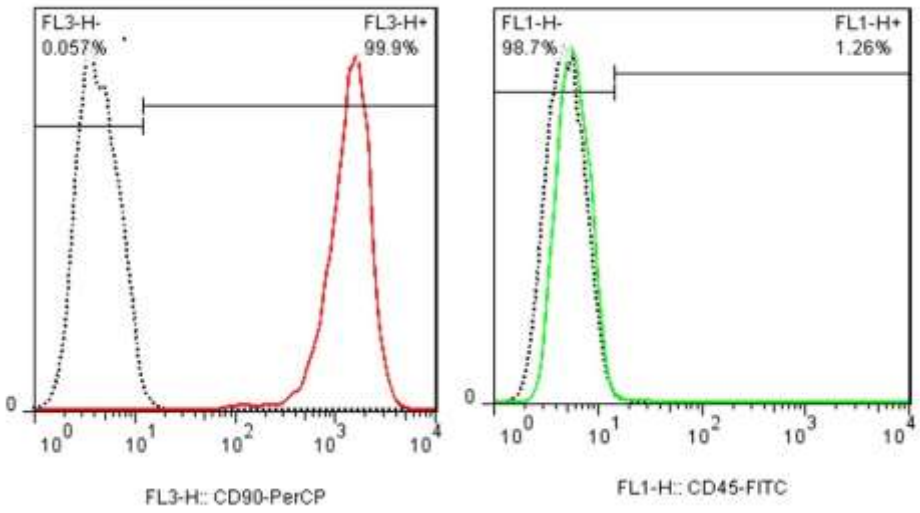

Figure 2. Flow cytometric analysis of mesenchymal stem cell marker CD90 and hematopoietic marker CD45. As shown the isolated adipose stem cells expressed high rate of mesenchymal marker (99.9%) and less amount of hematopoietic marker (1.26%).

Attachment of ASCs on dAM

To confirm the decellularization process of amnion membrane, electron microscopy scanning was performed and the structure of contact amnion membrane (Figure 3a) was compared with amnion membrane without epithelial layer (Figure 3b). The results revealed that there were no cell fragments or epithelial cells on dAM and dAM had a non-cellular structure. Twenty-four hours after AM scaffold preparation, ASCs were seeded on dAM; SEM images detected the fibroblast (like ASCs) attached to dAM (Figure 3c).

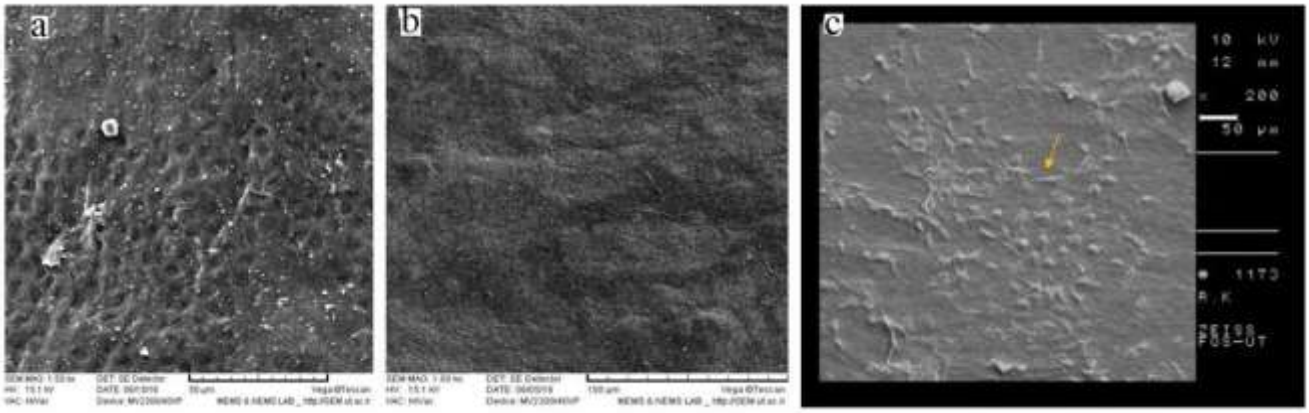

Figure 3. Electron microscopy images of a) contact amnion membrane with an epithelial layer; b) decellularized structure of amnion membrane; c) attachment of adipose stem cells to matrix of amnion membrane. Arrow is showing ASCs on dAM.

Proliferation of ASCs on dAM

Evaluation of the rate of cell proliferation in non-treated cells showed that ASCs were divided on dAM significantly during the 7 day of experiment, so that the rate of viable cells on the first day of cultivation was 15%, reaching to 32.87% on the fifth day and 44.39% on the 7th day. Differences were significant at $p < 0.05$ (Figure 4).

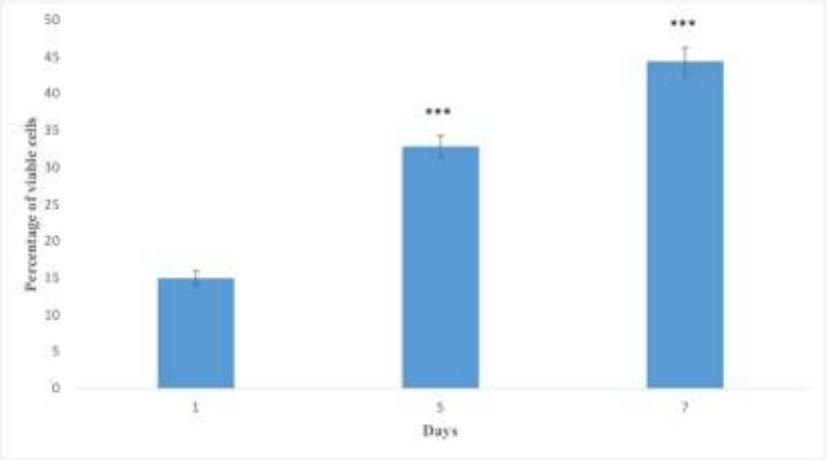

Figure 4. Evaluation the proliferation of ASCs on dAM during 7 days of culture. Differences are shown compared to the first day of cell cultivation (***: $p < 0.001$)

Neural Differentiation of ASCs Treated with e-CSF

The ASCs were cultured on AM derived scaffold in the presence of e-CSF for 7 days and their neural differentiation was evaluated with IF method. Immunofluorescence staining showed that ASCs differentiated into neurons in the presence of e-CSF and expressed two neural-related markers β- tubulin III and MAP-2. No expression of neural markers was observed in the ASCs which were cultivated on AM without e-CSF treatment (Figure 5).

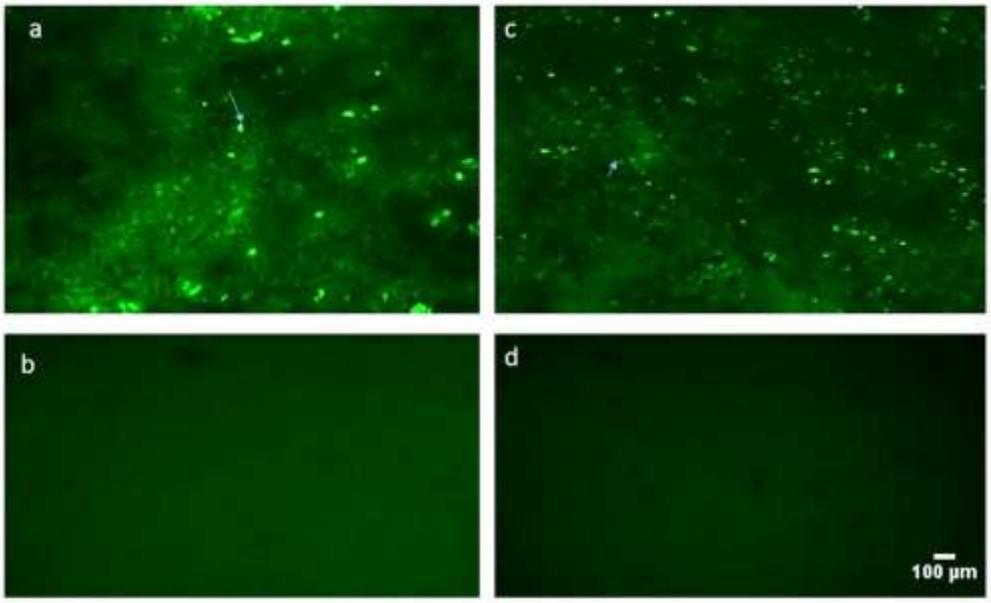

Figure 5. Immunonoflurescent of β-tubulin III (a and b) and MAP-2 (c and d) in adipose stem cells (ASCs). a and c: e-CSF treated ASCs; b and d: non-treated ASCs. Arrows are showing protein expressing cells. Observation under fluorescent microscopy (Magnify: 100 ×).

4. Discussion

Cell-based therapies have been taken into consideration as a promising method for the treatment of neurological disorders [21]. MSCs from a variety of tissue sources possess several qualities which make them appropriate choices for treating the diseases of the nervous system [22]. MSCs have the ability to promote neuronal repair, protect damaged neuronal tissues and down-regulate the immune responses [23]. Among the different kinds of MSCs, the adipose stem cells have attracted a lot of attentions in cell therapy of neurological diseases and considerable results have been achieved in pre-clinical studies [24,25]. Nevertheless, ASCs have shown variability in their ability to differentiate toward neural lineage [26] where improving *in vitro* conditions are important as to increase the rate of their differentiation.

The use of scaffolds can enhance the rate of neural differentiation of ASCs [12] by controlling the mechanical, chemical, and other properties of microenvironment [27]. In the current study, ASCs were cultured on amnion membrane derived matrix and their proliferation and neural differentiation were investigated. The MTT assay revealed the evidence that the cells replicated during their cultivation on amnion membrane and their number increased significantly throughout the culture period. Our results were consistent with Salah and colleagues, revealing that matrix of decellularized AM provides a proper three dimensional bioscaffold for culturing of human mesenchymal stem cells [28]; the findings were also in line with Gomes and colleagues concluding that HAM is an ideal substrate to support growth of limbal stem cells [29]. In terms of neuronal differentiation, ADSCs-seeded onto amnion-based scaffold developed into neuron-like cells when treated by embryonic CSF and expressed MAP-2 and beta-III tubulin neural markers. Promotion of neural fate in ADSs in the presence of e-CSF showed that the e-CSF was contained sufficient factors to induce neuronal differentiation in ASCs. In agreement with previous studies, the results of our research showed the importance of CSF for stem cell differentiation [3,30,31]. Several studies reported that embryonic CSF plays a role as a neural stem cell niche which provides a microenvironment for supporting viability, proliferation and differentiation of cortical cells and neural stem cells [32]. The mechanism of CSF-induced differentiation of stem cells into nerve cells has been studied rarely; however, it is theoretically proposed that CSF contains growth factors and cytokines which affect the proliferation or differentiation of stem cells [5]. Lehtinen showed that CSF plays an essential role in distributing signals in the central nervous system and promotes the proliferation of neural progenitor cells especially through insulin growth factor-2 (IGF-2) pathway [19]. Following that, Zhu and colleagues revealed that CSF protects proliferation and inhibits apoptosis of stem cells mainly through IGF-1 [33]. Chen and colleagues suggested the idea that CSF obtained from patients with subarachnoid hemorrhage (SAH) supports neural differentiation of neural stem cells due to the presence of trophic factors such as BDNF (brain derived neurotrophic factor), VEGF (vascular endothelial growth factor), FGF-2 (fibroblast growth factor-2) and IGF-2 (Insulin like growth factor-2) [7].

5. Conclusion

In conclusion, the present study revealed that embryonic CSF is a biologically active compound which induces neural differentiation in ASCs. The use of 3-D culture system can be a suitable way to increase the stability and differentiation of ASCs and the results revealed that amnion membrane derived scaffold provides a proper extracellular matrix for implantation, growth and neural development of ASCs. Results of the current study can be

advantageous as of improving *in vitro* culture methods to achieve effective outcomes for ASCs neural differentiation, using natural compounds.

Acknowledgement

This article was based on the results of MSc thesis of Raziyeh Keshavarzi with ethical committee number of 13099, supported by faculty of biological sciences of Kharazmi University.

Conflict of interest

The authors declare no conflict of interest.

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