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Dental pulp stem cells differentiation into retinal ganglion-like cells in a three dimensional network

Reza Roozafzoon [a,b], Alireza lashay [b], Mohammad Vasei [a,*], Jafar Ai [a], Ahad Khoshzaban [b,c], Saeed Heydari Keshel [b], Hoda Bahrami [b]

[a] Department of Tissue Engineering, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[b] Stem Cell Preparation Unit, Eye Research Center, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran
[c] BioDental Materials Department, Dental Faculty of Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

The loss of retinal ganglion cells (RGCs) in majority of retinal degenerative diseases is the first seen pathological event. A lot of studies aim to discover suitable cell sources to replace lost and damaged RGCs. Among them dental pulp stem cells (DPSCs) have a great potential of differentiating into neuronal lineages as well as RGCs. Moreover, three-dimensional (3D) networks and its distribution for growing and differentiation of stem cells as much as possible mimic to native tissue holds great potential in retinal tissue engineering. In this study, we isolate DPSCs from rat incisors and validate them with flow cytometry. Briefly, we differentiated cells using DMEM/F12 containing FGF2, Shh and 0.5% FBS into retinal ganglion-like cells (RGLCs) in two conditions; 3D state in biocompatible fibrin hydrogel and two-dimensional (2D) or conventional culture in polystyrene plates. Immuncytochemical and gene expression analysis revealed the expression of Pax6, Atoh7 and BRN3B increased in 3D fibrin culture compared to 2D conventional culture. In combination, these data demonstrate that using 3D networks can resemble near natural tissue properties for effective generating RGCs which used to treat neurodegenerative diseases such as glaucoma.

1. Introduction

Retinal degeneration which terminates in neuronal cells of retina is the leading cause of blindness in this century. The loss of neurons in the retina is routinely referred to be the inevitable cause of blindness. Age-related macular degeneration (AMD), glaucoma, and diabetic retinopathy are three most common figures of visual impairment and blindness in progressive loss of the neural cells of the eye. Glaucoma, the second leading cause of blindness worldwide, is a chronic degenerative disease that affects only RGCs [1,2]. Enhancing viability and function of RGCs remains a major goal through basic and translational researches in ophthalmology.

Stem cells have been suggested as an alternative therapeutic approach for treating a large spectrum of disorders. There are currently several trials in progress to treat neuronal related diseases of the retina using stem cells [3,4]. The results of these trials have been shown stem cell therapy for retinal degeneration diseases is a viable approach. Partially differentiated stem cells or even stem cells themselves which have the ability to produce trophic factors to rescue the dying cells of the retina can eventually restore their functions [5,6]. In this regard, stem cells derived from umbilical tissue, autologous bone marrow-derived stem cells and fetal stem cells are being transplanted to the eye in an attempt to maintain a healthy retina [7,8].

A major barrier to studying and treating RGCs related diseases is the amount and accessing of RGCs sources. Thus, the generation of RGCs in vitro is crucial to advance research and therapy in optic neuropathies. Dental pulp is a specific tissue originating from the neural crest [9] and it contains dental pulp stem cells (DPSCs) [9]. DPSCs are a heterogeneous population of cells including mesenchymal and ectodermic cells. This population of cells has been shown to possess properties similar to neural stem cells and mesenchymal stem cells. [10]. By isolation and expansion of these stem cells usually for long-term culture in media containing higher concentrations of serum followed by the induction of neuronal lineage with appropriate neurogenic conditions, neuronal differentiation of DPSCs is possible.[11,12]

Thanks to the tissue engineering approach three main components have become available in regenerative medicine; biomaterial

* Corresponding author at: Department of Tissue Engineering, School of Advanced Technologies in Medicine, Italia St., Keshavarz Blvd., Tehran 1417755469, Iran. Fax: +98 88995517.
E-mail address: mvasei@tums.ac.ir (M. Vasei).

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which act as a scaffold, stem cells and growth factors. This would be very effective to restore cell functions that remain no longer capable to carry out their function or even in damaged tissues. Fibrin is a suitable natural polymer for tissue engineering applications [13–15]. In the formation of the fibrin network, fibrinogen is converted into fibrin through the reaction of thrombin in the presence of a large amount of calcium ions [16]. These reactions make 3-dimensionally organized clot resemble to classic wound healing which accrue in the body [17]. Beside of its biocompatibility, elasticity, it provides suitable mechanical support for cell migration and cell entrapment [15,18].

In this study we use the fibrin network as a three dimensional environment to mimic mechanical properties of the native retina in developing the retina. In this regard, we were used rheological properties of the fibrin and differentiate DPSCs into retinal ganglion-like cells in an aforementioned environment in comparison to two dimensional conventional cell culture.

2. Materials and methods

2.1. Isolation of dental pulp stem cells

Three adult male Sprague–Dawley rats weighing 170–200 g were housed under the ethical principles of Tehran University of Medical Sciences (TUMS) guidelines for animal researches. The dental pulp was removed under sterile conditions and locate in transferring medium containing 1× PBS (TAKARA BIO INC., Japan) and 1% penicillin (100 μ/ml) streptomycin (100 μg/ml) in DMEM (Gibco®, USA) supplemented with 1% amphotericin-B (200 μg/ml) (Gibco®, USA) and then sliced into 1 mm^3 fragments and incubated in 4 ml of 0.25% trypsin–EDTA [21] for 120 min at 37 °C. Trypsin was inactivated by adding an equal volume of DMEM containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Gibco®, USA). A single cell population was obtained by passing the cell suspension through a 70 μm cell strainer (BD Biosciences, Oxford, UK), which was centrifuged at 150g for 5 min. Cell pellets were resuspended in DMEM containing 1% P/S and 10% FBS and seeded into T25 flasks (Corning, Amsterdam, NL) in a total volume of 5 ml. Cultures were maintained at 37 °C in 5% CO_2. The medium was changed 72 h after seeding and every 4 days thereafter for up to 4 weeks. Moreover, when cells reached 80% confluent they passaged using 0.05% Trypsin/EDTA Solution (Gibco®, USA).

2.2. Identification of cell phenotypic markers by flow cytometry analyses

Primary antibodies as well as CD44, CD73, CD90, CD31, CD34, CD45 and CD105 and secondary antibodies with fluorescent conjugates were used for assay according to abcam protocol (abcam, Cambridge, UK). At the end, the cells were checked using flow cytometry and data analysis was performed by flowing software (BD Biosciences Inc.).

2.3. Osteogenic differentiation and mineralization assay

Isolated DPSCs were seeded at a concentration of 1×10^4 cells/ml in a 6-well plate (SPL Life Sciences, Korea) with 0.7 ml media per well. The medium was changed to the osteogenic induction media which was contain 50 μg/ml ascorbic acid, 10 μM β-glycerophosphate, 0.1 μM dexamethasone and 10% FBS in low glucose DMEM (Sigma, Germany) for 21 days. Then the cells were stained with alizarin red S (Sigma–Aldrich Co., Germany) for 15 min [19]. The cells were visualized under an inverted phase contrast microscope (LabPro CETI, OXFORD).

2.4. Differentiation to retinal ganglion-like cell

Differentiation of DPSCs into retinal ganglion-like cells was done according the protocol of Jagatha et al. with some modifications in 2D/3D culture systems [20]. Briefly, isolated DPSCs were differentiated on 150 μg/ml poly-D-Lysine and 1 μg/ml Laminin substrate for 11 days in a differentiation medium which was contained DMEM/F12 supplemented with 1% N2 supplement (Invitrogen), 0.5% FBS, 2 μg/ml Heparin and 10 ng/ml FGF2 (Sigma–Aldrich Co., Germany). Afterward, the medium was altered to the growth factor containing media consisted of DMEM/F12 and combinations of 500 ng/ml Shh, 8 ng/ml FGF2, in 0.5% FBS for 16 h with the aim of effective differentiation. N2 supplemented DMEM/F12 with 0.5% FBS was used as control differentiation medium.

2.5. Cell Immobilization in fibrin gel

To prepare fibrin gel, 1.5 mg of fibrinogen (Sigma) was dissolved in 0.5 ml M199 medium (Sigma) and 2×10^5 cells/ml of DPSC were loaded to 500 μl of the prepared fibrinogen solution then added to 24-well culture plate. Then, 15 μl of a thrombin solution (120 U/ml in 1 M $Cacl_2$, Nacl, pH: 7.4 Sigma) and 50 μl of fetal bovine serum (FBS) were added to fibrinogen solution (3 mg/ml). After gelatin occurred at 25 °C, the dish was placed in an incubator at 37 °C and 98% relative humidity for 1 h to form its final three dimensional network structure [21].

2.6. Rheological analysis

Fibrin gel rheometry was done using a Physica MCR 300 rheometer (Anton-Paar, Ashland, VA). Fibrin gels were placed directly onto the parallel plate with 25 mm diameter, and 0.05 mm was a gap between the plates. Plate temperature was set 37 °C at the start of the experiment. A constant stress mode applied to the sample. The tests were done by dynamic method at strain (1%). The storage (G′) and loss moduli (G″) were measured by small-amplitude oscillatory shear measurement at the frequencies whiten 0.1–10 Hz.

2.7. Biocompatibility

500 μl of 5 mg/ml fresh MTT (3-[4,5-dimethyl-2-thia-zolyl]-2,5-diphenyl-2*H*-tetrazolium bromide) (Sigma–Aldrich, Germany) was added to each well and they were incubated in a humidified 5% CO_2 incubator at 37 °C for four hours. Afterward, the formazan salts were dissolved with 200 μl of DMSO and finally the optical density was measured at 575 nm by using a microplate reader (PHOmo, Autobio-labtec, China).

2.8. Immunofluorescent analysis

All antibodies were purchased from abcam (abcam, Cambridge, UK). According to abcam procedure the cells prepared. Primary antibodies as well as Rabbit polyclonal Anti-GFAP antibody, Mouse monoclonal anti-MAP2 antibody and Rabbit polyclonal Anti-BRN3B/POU4F2 antibody was added to the cells and then the secondary antibodies were added to the cells at recommended dilution. Moreover, DAPI (Sigma–Aldrich, Germany) was used to counterstaining the nuclei. Samples were visualized using a fluorescent inverted microscope (Trinocular Epi Flu Inverted microscope, Ceti Microscopes, UK).

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