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Comparative effectiveness of three-dimensional scaffold, differentiation media and co-culture with native cardiomyocytes to trigger in vitro cardiogenic differentiation of menstrual blood and bone marrow stem cells

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ABSTRACT

The main purpose of this study was to find effectiveness of 3D silk fibroin scaffold in comparison with co-culturing in presence of native cardiomyocytes on cardiac differentiation propensity of menstrual blood (MenSCs)-*versus* bone marrow-derived stem-cells (BMSCs). We showed that both 3D fibroin scaffold and co-culture system supported efficient cardiomyogenic differentiation of MenSCs and BMSCs, as judged by the expression of cardiac-specific genes and proteins, Connexin-43, Connexin-40, alpha Actinin (ACTN-2), Tropomyosin1 (TPM1) and Cardiac Troponin T (TNNT2). No significant difference (except for higher expression of ACTN-2 in co-cultured MenSCs) was found between differentiation potential of the cells cultured in 3D fibroin scaffold and co-culture system. Collectively, our results imply that inductive signals served by biological factors of native cardiomyocytes to trigger cardiogenic differentiation of stem-cells may be efficiently provided by natural and biocompatible 3D fibroin scaffold suggesting the usefulness of this construct for cardiac tissue engineering.

1. Introduction

Regenerative medicine and tissue engineering techniques are at the center of the most promising therapies for treatment of patients with heart failure, myocardial infarction and congenital heart diseases [1,2]. The key ingredients for tissue engineering are stem cells, inductive signals that regulate their differentiation, and an extracellular matrix that constitutes the microenvironment for their growth [3–5]. In two recent decades, stem cells have been introduced as a promising substitute for native differentiated cells due to the higher proliferation rate, trans-differentiation ability and longer survival in culture condition [6,7]. The types of stem cells that have been used widely for cardiac repair are embryonic stem cells, induced pluripotent stem cells and stem cells from various adult tissues. Recently, some favorable features of menstrual blood stem cells (MenSCs) such as easy accessibility, convenient method of sample collection and high proliferation capacity have moved scientists toward the application of these cells in the field of tissue engineering and regenerative medicine [8–11]. Adherent cells cultured from menstrual blood expand rapidly with higher propagation rate compared to bone-marrow derived stem cells (BMSCs) or even stem-cells from umbilical cord blood [12,13]. These cells have minimal risk of karyotypic abnormalities during long-term expansion [14] and trans-differentiation capacity of these cells into osteocytes [15], adipocytes [16], chondrocytes [17], hepatocytes [18,19] and neural–like cells [20]have been documented in different studies [21]. Recently, we presented convincing evidence that MenSCs have the capability to be differentiated toward cardiomyocyte under 5-azacythidin (5-Aza) stimulation and continuous treatment with basic fibroblast growth factor (bFGF) in two dimensional (2D) culture. Although expression levels of cardiac markers in differentiated MenSCs were significantly higher than

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those of BMSCs [22], providing inductive signals through co-culturing of these cells with native differentiated cells or culturing in 3D network might improve the characteristics of differentiated MenSCs [23–25]. It is well established that a suitable extra-cellular matrix (ECM) or an environment with three dimensional structure could potentially improve the functionality of cells compared to two-dimensional culture systems [26–28]. A wide variety of natural [29–34] and synthetic biomaterials [35–37] have been designed to serve as substitutes for native matrix of cardiac tissue, but challenges are remained to fabricate biologically compatible scaffolds with high cell harboring capacity, suitable porosity, and mechanical strength that mimic native cardiac microenvironment.

Silk fibroin is a natural polymer originated from both domesticated mulberry silkworm, *Bombyxmori (B.mori)*, and the non-mulberry silkworm, *Antheraeamylitta*that has unique properties such as excellent mechanical strength and high elasticity [38–41]. In a recent study, we introduced fabrication of a biocompatible *B. mori* silk fibroin scaffold with desirable mechanical and physical properties comparable with human myocardium [13]. In the present study, we evaluated the effect of 3D silk fibroin scaffold culture system and co-culturing with native cardiomyocyes on cardiac differentiation potential of MenSCs. Our main goal was to find out a more permissive condition on cardiac differentiation of MenSCs. To provide a better picture of trans-differentiation competence of MenSCs in each designed culture system, cardiomyocyte differentiation capacity of BMSCs was investigated in parallel.

2. Materials and methods

2.1. Preparation of 3-D silk fibroin based scaffolds

Silk fibroin solution was prepared as reported previously [13]. Briefly, pure silk fibroin was extracted from *Bombyxmori* cocoons (provided by Iranian Silkworm Research Center, Guilan, Iran) by degumming 5 g of fibers in 2 L of boiling sodium carbonate solution (0.02 M) for 20 min (Sigma-Aldrich, St. Louis, MO). Degummed fibers were collected and rinsed with hot and cold distilled water three times to extract the glue-like sericin proteins and then air-dried. The pure silk fibroin was then solubilized in aqueous lithium bromide (9.3 M, Sigma-Aldrich, St. Louis, MO) at 55 °C for 5 h (hr). Dried fibroin was dissolved in ultra-pure water (2%w/v), poured into 24-well polystyrene plates and lyophilized for 12 h. Porous matrices were then immersed into pure methanol, rinsed with water and lyophilized again for further 12 h.

2.2. Isolation and culture of MenSCs and BMSCs

The isolation of stem-cells from menstrual blood and bone -marrow was performed as described in our recent studies [15,17,19,20]. Menstrual blood was collected from healthy females aged 22–35 years using Diva cup (Diva International Co., Lunette, Finland) on the second day of menstrual cycle. Bone marrow aspirates (5–10 mL) were obtained from iliac crests of human donors at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran. All donors signed informed consent forms approved by medical ethics committee of Avicenna Research Institute. The isolated MenSCs and BMSCs were cultured in glucose-Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS,Gibco), penicillin G (100 U/mL) and streptomycin (100 µg/mL) (Gibco). All experiments were performed with cells at passage 2–4 from 3 to 6 donors.

2.3. Assessment of MenSCs and BMSCs attachment, migration and proliferation ability in silk scaffolds

To prepare for cell culture, silk scaffolds were sterilized by 70% ethanol immersion for 2hr. After overnight pre-conditioning in the culture medium, the scaffolds were transferred into 12-well cell culture plates and seeded with MenSCs and BMSCs at the density of 5×10^5 cells/cm^2. The plates were put in a humidified incubator at 37 °C and 5% CO_2, and the medium was refreshed next day. MenSCs attachment and growth was evaluated by scanning electron microscopy (SEM). In order to assess cell migration within silk scaffolds, the scaffolds were taken, washed 2 times with PBS, fixed in 10% buffered formalin, dehydrated, embedded in paraffin and sectioned at 5 µm thickness from the top, middle, and bottom. The sections were stained with the nuclear stain 4, 6 diamidino-2-phenylindole(DAPI) (1 µg/mL) (Sigma) for 10 min, washed three times with PBSand then observed under fluorescence microscopy.

Moreover, to assess the proliferative capability of MenSCs, the cells proliferation was analyzed at days 2, 4, 6, 8 and 10 by DNA quantification assay using propidium iodide (PI) dye according the protocol described in our previous paper [13]. The fluorescent signal was measured with a multifunction microplate reader (Synergy MicroplateReader-BioTek, Germany) at an excitation and emission wavelength of 535 and 617 nm, respectively.

2.4. Isolation of neonatal rat cardiomyocyte

Cardiomyocytes were isolated from 1 to 2 day old Wistar newborn rats according to a protocol by Iwaki et al. with some modification [41]. In brief, neonatal rats were scarified by decapitation, and their hearts were rapidly removed and placed into an ice-cold buffer (in mmol/L: NaCl 116.4, HEPES 20, NaH_2PO_4 1, glucose 5.5, KCl 5.4, $MgSO_4$ 0.8; pH 7.4). The ventricles were cut in 2–3 mm^3 pieces and digested in a buffer containing collagenase type II (0.4 mg/ml; from clostridium histolyticum) (Sigma) at 37 °C for 25 min. The digestion steps were repeated for five times and after each run of digestion, the supernatant was removed, centrifuged (600 g, 5 min) and the resulting cell pellet was resuspended in DMEM-F12 supplemented with 2% horse serum (Gibco) 10% FBS(*Gibco*), penicillin G (100 U/mL) and streptomycin (100 µg/mL) (Gibco). Cells were plated for 60 min to reduce the number of contaminating non-myocytes. Non-adherent cells were collected, centrifuged, re-suspended in DMEM/F12 medium supplemented with 5% horse serum and cultured in a 24-well plate at 500 µl/well. The cell yield was $\sim 2 \times 10^6$ per neonatal heart.

2.5. Differentiation of MenSCs and BMSCs into cardiomyocytes in differentiation media, co-culture system and 3D scaffold

MenSCs and BMSCs grown in cell culture flasks were trypsinized, counted, and plated at a density of 5×10^4 cells on 0.1% gelatin (from porcine skin)-coated cover slips (Sherkate JAM, India) in wells of a 24-well plate. After 24 h, the seeded cells were differentiated in 2D manner using differentiation media (5-azaand bFGF) or co-cultured with rat neonatal cardiomyocytes. For the first culture system, the cells were incubated in serum-free DMEM (Sigma) containing 10 mM 5-Aza (Sigma) and 10 ng/mlbFGF (Sigma). After 48 h, the medium was replaced with DMEM-low glucose (DMEM-LG) (Sigma) containing 5% fetal bovine serum and 10 ng/mL bFGF and the cells were cultured in this medium up to 21 days. For co-culture system, the cells were co-cultured by rat neonatal cardiomyocytes using 6.5mmTranswell® inserts (Sigma). Inserts were rehydrated in growth medium for 30 min, and then a cell suspension containing 2×10^4 cardiomyocytes was added to the upper membrane surface. Thereafter, cardiomyocytes-seeded Transwell® membranes were put in the wells containing mesenchymalcell-seeded coverslips and incubation in serum-free DMEM was continued for 14 days. In order to differentiate cells in 3D silk fibroin scaffolds, MenSCs or BMSCs were seeded at a density of 5×10^5 cells/cm^2 onto the surface of prepared scaffolds and differentiated using 5-Aza and b-FGF in a similar manner with the first culture system as explained above. Medium was changed every 2–3 days. Control cultures without the differentiation stimuli were made in parallel to the differentiation experiments in the same manner.