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RESEARCH ARTICLE

Differentiation of Wharton's jelly mesenchymal stem cells into neurons in alginate scaffold

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Abstract

Alginate scaffold has been considered as an appropriate biomaterial for promoting the differentiation of embryonic stem cells toward neuronal cell lineage. We hypothesized that alginate scaffold is suitable for culturing Wharton's jelly mesenchymal stem cells (WJMSCs) and can promote the differentiation of WJMSCs into neuron-like cells. In this study, we cultured WJMSCs in a three-dimensional scaffold fabricated by 0.25% alginate and 50 mM $CaCl_2$ in the presence of neurogenic medium containing 10 μM retinoic acid and 20 ng/mL basic fibroblast growth factor. These cells were also cultured in conventional two-dimensional culture condition in the presence of neurogenic medium as controls. After 10 days, immunofluorescence staining was performed for detecting β-tubulin (marker for WJMSCs-differentiated neuron) and CD271 (motor neuron marker). β-Tubulin and CD271 expression levels were significantly greater in the WJMSCs cultured in the three-dimensional alginate scaffold than in the conventional two-dimensional culture condition. These findings suggest that three-dimensional alginate scaffold cell culture system can induce neuronal differentiation of WJMSCs effectively.

Key Words: *nerve regeneration; Wharton's jelly mesenchymal stem cells; mesenchymal stem cells; neurons; motor neurons; alginate; 3D scaffold; neural regeneration*

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Introduction

Human Wharton's jelly can be considered as a rich source of mesenchymal stem cells (MSC). Isolation of MSCs from the umbilical cord is easy, non-invasive and less expensive. Wharton's jelly mesenchymal stem cells (WJMSCs) meet the stemness criteria, such as self-renewal and the ability to differentiate into various cell lineages (Anzalone et al., 2010). Their greater expansion (Weiss et al., 2008), pluripotency potential (Nagamura-Inoue and He, 2014), and the lack of HLA-class II marker expression (La Rocca et al., 2009) make them an appropriate cell source for cell therapy without any ethic concern. WJMSCs have shown to express a gene profile that is more similar to the embryonic stem cells than MSCs derived from the adult tissues (Fong et al., 2011). They have also shown the ability to differentiate into the derivatives of all three germ layers (Nagamura-Inoue and He, 2014) and express both embryonic and mesenchymal stem cell markers (Fong et al., 2011). WJMSCs also secrete the factors involved in neurogenesis and have been demonstrated to act as neuroprotective cells because their secretory growth factors stimulate neuronal growth and reduce cell apoptosis in primary cortical cells (Hsieh et al., 2013).

A subset of the naïve human WJMSCs has been shown to express nestin, a neuroblast marker (Montanucci et al., 2011), glial cell line-derived neurotrophic factor, and glial cell marker (Weiss et al., 2006). They also express pluripotency markers, such as Oct4, Nanog, and Sox2. WJMSCs showed a very high potential to differentiate into the neuronal cell lineage (Montanucci et al., 2011; Tantrawatpan et al., 2013). Neurogenic induction of WJMSCs led to an increase in the frequency of the nestin-positive cells in two-dimensional (2D) conventional culture system (Messerli et al., 2013).

The neurogenic induction of bone marrow-derived MSCs in three-dimensional (3D) fibrin (Shakhbazau et al., 2011) and 3-hydroxybutyrate scaffolds (Wang et al., 2010) was demonstrated previously. Culturing MSCs in 3D environment promoted neuron formation more intensively compared with 2D conventional monolayer culture condition (Wang et al., 2010). The stiffness of 3D scaffolds controls the neurogenesis even in the absence of the biochemical signals (Wang et al., 2010). The method of loading MSCs into the biomaterials such as chitosan-silicate hybrid, collagen has been confirmed to improve the clinical outcome of sciatic nerve lesions (Ribeiro et al., 2013).

Alginate as a popular biomaterial is a non-toxic linear polysaccharide. Alginate cross-links into a 3D scaffold by divalent cations, such as Ca^{2+}. 3D alginate scaffold can embed cells and the cells can be harvested by administration of Ca^{2+} chelating agents. 3D alginate scaffold supports cell proliferation and influences the differentiation fate of stem cells (Lee and Mooney, 2012). Alginate is considered as a carrier for neuron transplantation (Novikova et al., 2006). A previous study showed that after culturing in the alginate scaffold, adult neurons from various origins present neurite formation as time progressed (Frampton et al., 2011). Alginate scaffold has been suggested as an appropriate biomaterial for promoting the differentiation of embryonic stem cells toward neuronal cell lineage (Frampton et al., 2011). With regards to these considerations, the objectives of the current study were to differentiate WJMSCs into neurons in 3D alginate scaffolds and compare the differentiation fate of WJMSCs grown in 3D alginate scaffolds with that of WJMSCs grown in conventional 2D monolayer system.

Materials and Methods

Isolation of WJMSCs

Wharton's Jellies were collected from the infants born with cesarean incision. All protocols were approved by the Ethic Committee of Shiraz University of Medical Sciences, and in accordance with the university ethical guidelines. Informed consent forms were obtained from their parents. The umbilical cords were transferred to the laboratory in cold phosphate buffer saline (PBS) containing penicillin/streptomycin. The specimens were washed and flashed by PBS to remove the blood and the vein was cut longitudinally. The epithelium was scraped and the arteries were cut away. The rest of the umbilical cord, Wharton's jelly, was cut into small pieces. Each piece was mounted on the floor of a tissue culture plate. After 10 minutes, culture medium, Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin/streptomycin and 1% L-glutamine, was added to the explants for 10–15 days and cultured at 37°C, 5% CO_2. The culture media were replaced twice a week. The cells grown from the explants were preserved in the presence of DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine.

Identification of MSCs

To define the characteristics of MSCs, it is necessary to detect a series of specific surface markers for them. The cells at passage 3 were harvested and prepared for flow cytometry. Briefly, the non-specific binding sites of the harvested MSCs were blocked by PBS containing 1% Tween 20 (Merck, Germany) and 5% goat serum (Merck). Then, the cells were exposed to fluorescein isothiocyanate-conjugated mouse anti-CD44, rabbit anti-CD144 (both from Abcam, UK, Cambridge), allophycocyanin conjugated mouse anti-CD90 (MACS Miltenyi Biotec, CA, USA), phycoerythrin-conjugated rat anti-CD73, mouse anti-CD34, -CD106 and pre-CP-conjugated mouse anti-CD105 antibodies (all from Abcam) and finally the cells were fixed with 4% paraformaldehyde. The frequencies of various antibody-positive cells were analyzed on a four color FACSCalibur flow cytometer using the CellQuest pro software. The results were depicted as graphs using the WinMDI software. The matched mouse isotype controls (all from Abcam) were used to exclude non-specific binding sites. To do this, the cells were prepared for flow cytometry in the same condition with experimental cells but a cocktail of isotype controls conjugated with each fluorescence label was replaced with anti-CD marker antibodies.

The pluripotency capability of WJMSCs was also determined (Anzalone et al., 2010). To evaluate the ability of WJMSCs to differentiate into osteoblasts, WJMSCs were treated by the NH-OsteoDiff Medium (Miltenyi Biotec, Bergish Gladbach, Germany) for 4 weeks. Then, the culture media were aspirated and the induced cells were washed, stained with 0.5% alizarin red S (Sigma, St. Louis, MO, USA) in PBS and observed under inverted microscope (Olympus, Japan). To evaluate the adipogenic potential of WJMSCs, WJMSCs were incubated in DMEM containing human adipogenic stimulatory supplements (StemCell Technologies Inc., Vancouver Canada) for 3 weeks, stained with oil red S (Sigma) and observed under inverted microscope (Olympus).

3D and 2D cultures

WJMSCs at 4×10^5/mL were mixed with 0.25% alginate (Sigma) (Banerjee et al., 2009). A 40 μL of mixture sample was exposed to 100 μL of 50 mM $CaCl_2$ in a 96 well culture dish at 4°C for 15 minutes and then $CaCl_2$ was replaced by culture medium. The optimum concentration of $CaCl_2$ (50 mM) for determining the proliferation and viability of MSCs was chosen according to a previous study (Hosseini et al., 2013). The cells were cultured in alginate in the presence of neurogenic medium containing 10 μM retinoic acid (R2625; Sigma) and 20 ng/mL bFGF (F0291; Sigma) for 10 days. Then, the cells were harvested by replacing the culture media containing 15 mM sodium citrate. The cells in 3D cultures were then harvested and let them to attach to culture dishes for additional 1 day. The cell viability was assessed by trypan blue exclusion assay. An equal amount of cell suspension and 0.4% trypan blue were mixed and non-stained viable cells were counted using a hemocytometer. Then the cells were prepared for further assessments (immunofluorescence).

For 2D culture condition, the same numbers of cells were cultured in neurogenic medium containing 10 μM retinoic acid and 20 ng/mL bFGF for 10 days and then the cells were prepared for further assessments.

Immunofluorescence staining

The samples were fixed in 4% paraformaldehyde for half an hour. After washing with PBS, the cells were incubated in the mouse anti-tubulin (Promega, Madison, WI, USA; 1:1,000) and rabbit anti-CD271 (Millipore, Bedford, MA, USA; 1:300) for 1 hour at room temperature. The antibodies were diluted in PBS containing 5% goat serum. The phycoerythrin- and

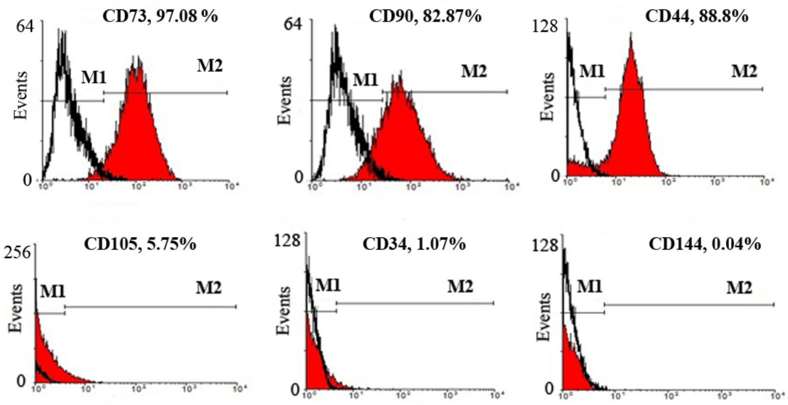

Figure 1 Identification of Wharton's jelly mesenchymal stem cells (WJMSCs).
WJMSCs can express specific surface markers CD73, CD90, CD44 and CD105 but not CD144 (endothelial cell marker), and CD34 (hematopoietic cell marker), as determined by flow cytometry. Empty histograms indicate background staining with isotype controls and red histograms indicate the frequency of the cells stained with CD markers.

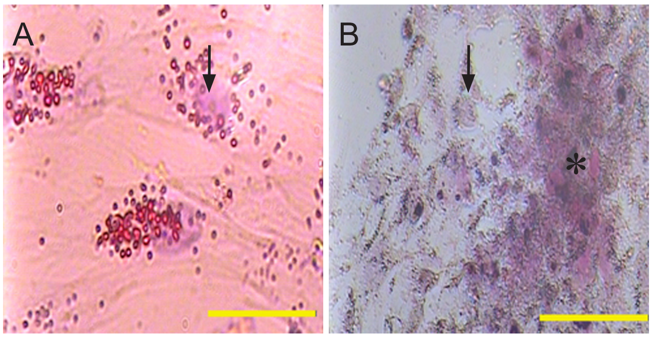

Figure 2 Wharton's jelly mesenchymal stem cells (WJMSCs) had the capability to differentiate into adipocytes (A) and osteocytes (B).
Arrows show the nuclei of the cells and star shows calcium deposit. WJMSCs could be stained with oil red S (A) and alizarin red S (B). Scale bars: 50 μm.

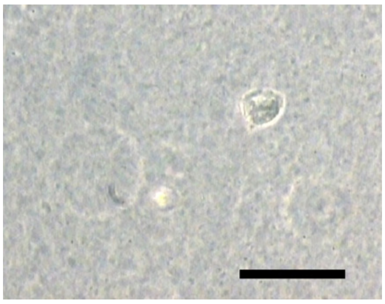

Figure 3 The mesenchymal stem cells in three-dimensional culture (Scale bar: 100 μm).

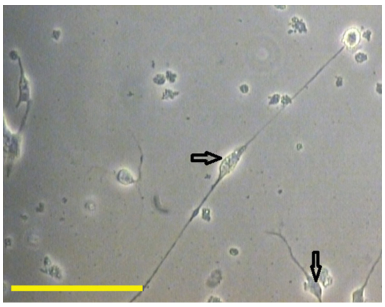

Figure 4 Morphology of the Wharton's jelly mesenchymal stem cells (WJMSCs) after culture in three-dimensional alginate scaffold in the presence of neurogenic medium for 10 days, followed by culture in conventional two-dimensional culture condition for 1 day.
The WJMSCs differentiated into neurons (arrows). Scale bar: 50 μm.

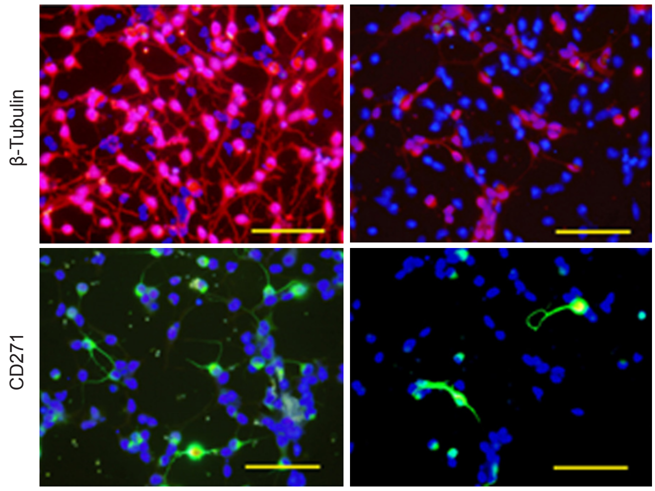

Figure 5 Detection of β-tubulin (phycoerythrin label, red) and CD271 expression (fluorescein isothiocyanate label, green) in Wharton's jelly mesenchymal stem cells (WJMSCs) cultured in three-dimensional alginate scaffold (left panels) *versus* conventional two-dimensional condition (right panels) by immunofluorescence microscopy.
The β-tubulin and CD271 expression levels were greater in the cells cultured in the three-dimensional condition than in the conventional two-dimensional condition. Scale bars: 100 μm.

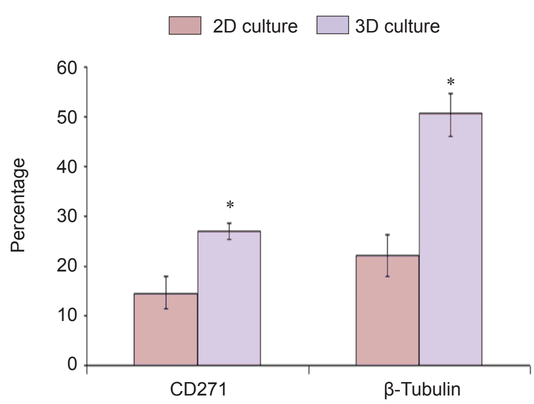

Figure 6 The percentages of CD271- and β-tubulin-positive cells in two-dimensional (2D) *versus* three-dimensional (3D) culture systems.
The data are expressed as the mean ± SD. *$P < 0.05$, *vs.* 2D culture (Mann-Whitney *U* test).

fluorescein isothiocyanate-conjugated anti-mouse and anti-rabbit secondary antibodies at 2 mg/mL were also used to label the primary antibodies. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The cells were observed under fluorescent microscopy (Nikon X66). To calculate the percentages of β-tubulin- and CD271-positive cells, ten fields of view were randomly chosen and the total number of DAPI-stained nuclei and the number of antibody-positive cells were counted.

Statistical analysis

All measurement data are expressed as the mean ± SD and were statistically analyzed by SPSS 16.0 software (SPSS, Chicago, IL, USA). The Mann-Whitney *U* test was used. A level of $P < 0.05$ was considered statistically significant.

Results

Cell characterization

The cells were shown to be positive for CD44 (88.87%), CD105 (5.75%), CD90 (82.87%) and CD73 (97.08%). The percentages of the cells positive for CD144 (0.04%) and CD34 (1.07%) were negligible (**Figure 1**). Alizarin red S and oil red O staining also revealed that the cells were capable to differentiate toward osteoblasts and adipocytes, respectively (**Figure 2**).

Cell morphology

After 10 days of culture in the 3D culture system in the presence of neurogenic medium, WJMSCs lost their processes and become rounded (**Figure 3**). The cell processes or neurites were observed after 1 day of culture in conventional 2D monolayer culture system (**Figure 4**).

Immunofluorescence staining

Immunofluorescence staining showed that WJMSCs differentiated into neurons and motor neurons in the presence of neurogenic medium (**Figure 5**). After exposure to neurogenic medium, the percentage of the cells expressing β-tubulin and CD271 was significantly greater in the 3D culture system than in the conventional 2D monolayer culture condition ($P = 0.001$ or 0.009; **Figure 6**).

Discussion

Stem cells are seeded on a soft collagen gel that can mimic the elasticity of brain tissue (Engler et al., 2006) and a 3D culture condition can promote neurite outgrowth of spinal neurons (Flanagan et al., 2002). WJMSCs spread in the 3D collagen scaffolds and show a branched appearance. 3D scaffolds alter not only cell phenotype but also gene expression pattern (Khodabandeh et al., 2015). Results from the present study showed that the cells grown in alginate scaffolds exhibited round appearance even if they were exposed to neurogenic medium. Moreover, the cells harvested from 3D alginate scaffolds exhibited more branches after 1 day of culture in the neurogenic medium than those grown in conventional 2D monolayer culture condition. A similar morphology was also detected after culturing WJMSCs (Penolazzi et al., 2010), fibroblast (Shapiro and Cohen, 1998) or adipose-derived MSCs in alginate but not in the other types of hydrogels, such as gelatin (Awad et al., 2004) or collagen (Khodabandeh et al., 2015).

Naïve WJMSCs have the ability to express nestin, neural stem cell marker, and Musashi-1, mature neuron marker, and can be considered as a good stem cell source for neural differentiation (Messerli et al., 2013). The neurogenic potential of WJMSCs was reported to be higher than that of MSCs isolated from the other sources (Balasubramanian et al., 2013). WJMSCs have the capability to differentiate into various types of neuronal cell lineages, such as dopaminergic neurons (Datta et al., 2011) and oligodendrocytes (Zhang et al., 2009). Results from this study also showed that WJMSCs could differentiate toward motor neurons.

Neural stem cells cultured in the alginate scaffold are nestin-positive cells and have differentiation potential, and it was suggested that alginate scaffold is a feasible environment for neural stem cell expansion in bioreactors (Li et al., 2006). Cytokines released from neural stem cells also remained intact when they were cultured on alginate scaffold (Purcell et al., 2009). The soft hydrogels, such as alginate, have been also shown to be an appropriate modulus for cell differentiation toward neural lineage. Neural stem cells cultured in alginate scaffolds showed a higher expression of β-tubulin because the elasticity of the scaffold was more similar to nervous tissues such as the brain (Banerjee et al., 2009). Neural differentiation of embryonic stem cells was also reported to be more obviously increased when they were cultured in the 3D alginate scaffolds compared with in conventional 2D monolayer culture system (Bozza et al., 2014). Data from the present study also showed the WJMSCs exposed to neurogenic medium and cultured in 3D alginate scaffolds expressed a higher level of both neuron and motor neuron markers than those cultured in the 2D culture condition. The morphology of the differentiated neurons from WJMSCs in alginate scaffolds was similar to the differentiated neurons derived from neural and embryonic stem cells cultured in the same scaffold. Alginate facilitated the differentiation of dopaminergic neurons from embryonic stem cells (Kim et al., 2013). We showed that alginate scaffold was also an appropriate niche for motor neuron differentiation.

The alginate stiffness has been shown to influence the differentiation of embryonic stem cells (Candiello et al., 2013). It has been demonstrated that chemical and mechanical properties of alginate scaffolds influence cell shape and the differentiation capability toward neurogenic cell lineage. Alginate can also induce embryonic stem cells to differentiate into neurons without administration of any exogenous growth factors (Banerjee et al., 2009).

In conclusion, the alginate can be considered as an appropriate scaffold to support WJMSCs differentiation toward neurons. Alginate can provide a mechanical framework similar to the brain. Therefore, culturing WJMSCs in an alginate scaffold may provide a good system for application in regenerative medicine.

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Author contributions: *SMH conceived and designed this study, were responsible for data collection, analysis and interpretation, and wrote the paper. AV conceived and designed this study, collected the data. NN, RR and ZR also participated in data collection. TTK was in charge of fundraising, provided assistance in technique and material use, authorized the study, and provided critical revision of the paper for intellectual content. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*

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