

## Neural Differentiation of Wharton's Jelly Stem Cells in Three-Dimensional Chitosan Based Culture Environment

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### ABSTRACT

**BACKGROUND AND OBJECTIVE:** Wharton Jelly Stem Cells (WJSCs) can be a good option for differentiating and regeneration of nervous system damage. Three dimensional (3D) cell cultures by providing a body-like environment have more advantages than the two dimensional (2D) cell cultures. The aim of this study was to investigate the neural differentiation of these cells in a 3D chitosan based culture environment.

**METHODS:** This experimental study was performed in 4 groups of 2&3D with and without differentiation media on WJSCs. First, to construct the hydrogel, hydroxyl ethyl cellulose was added to chitosan-beta-glycerophosphate solution (8.4: 0.8) (HEC: CH-β-GF). Human WJSCs after isolation by enzymatic method from wartons' jelly of born infant in Imam Khomeini hospital in Tehran and characterization with flow cytometry, were cultured  $5 \times 10^5$  cell in each well of 24-well plate in a 2D and 3D environment using the hydrogel in neural differentiation media for 4 days. Then, the neural differentiation of WJSCs was evaluated by quantitative analysis of β-Tubulin III, Nestin and β-actin (internal control) genes expression by Real Time PCR (RT-PCR).

**FINDINGS:** The results of RT-PCR showed that expression of β-Tubulin III and Nestin genes in WJSCs was significantly increased by the influence of the neural differentiation media in both 2D (more than 4 folds) and 3D (more than 2 folds) culture conditions ( $p < 0.005$ ). But the expression of β-Tubulin III and Nestin in 3D cell culture condition (more than 1.5 folds) was greater than that in the 2D cell culture condition under the influence of the neural differentiation media ( $p < 0.01$ ).

**CONCLUSION:** The results showed that neural differentiation of WJSCs in a chitosan based 3D environment is higher than 2D.

**KEYWORDS:** Wharton's jelly, Stem cells, Neural Differentiation, Chitosan.

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## Introduction

The use of cell based and stem cell therapies in the treatment of diseases and injuries of the nervous system has been promising (1,2). An important source of stem cells is the Wharton Jelly stem cells. Features such as availability, high proliferation without the risk of developing tumors, their high differentiation potential to transform into different types of tissues, the possibility of autologous transplantation for one's own or allogeneic for another person are the reasons for extensive study of these cells in tissue engineering and regenerative medicine (3).

In addition, stem cells extracted from Wharton Jelly have higher differentiation potentials to become neuronal precursors than bone marrow and adipose tissue (4) and survive, support and repair nerve cells by secreting neurotrophic factors and cytokines (4, 5). In a study, transplant of cells after differentiation into neurons in the cerebral ischemia model, there has been a significant improvement (6). One of the most important points in using these cells is culturing and differentiating them in the laboratory. In this regard, the use of 3D culture with imitation of 3D body and natural environment has many advantages (7). The use of 3D culture in different studies of neuronal cell or neuronal differentiation has had different results than 2D culture. Several studies have reported the use of three-dimensional culture in modeling the nervous system for viral infection or screening for cell differentiation (9, 8). Chitosan-based hydrogels are scaffolds used in tissue engineering that can mimic natural extracellular matrix conditions and provide a three-dimensional environment for cell growth (10).

The use of Wharton jelly stem cells with hydrogels that can provide the 3D environment needed to grow them, can be a viable strategy for the treatment of nerve tissue damage (11,12). Chitosan (CH) is a natural polysaccharide derived from chitin. Biocompatible and biodegradable chitosan has anti-free radical and neuroprotective properties. It also facilitates adhesion and growth of the nerve cell and has an adjustable degradation rate (13) and antibacterial property (14). Therefore, it can be a suitable scaffold for 3D culture and neural differentiation of stem cells. For the production of CH temperature-dependent hydrogels, it can be linked to  $\beta$ -GPs and cellulose derivatives such as hydroxyethyl cellulose (HEC) (15).  $\beta$ -GP causes the acidity of CH to close to the physiological range and prevents its rapid gelation (16). The use of HEC improves the mechanical properties and initiates the

hydrogel gelation process (17,18). Different markers are used to evaluate the neural differentiation of stem cells. One of these markers is the  $\beta$ -Tubulin III protein, which is found specifically in neurons and can be examined at the beginning of neuronal differentiation. Another marker indicating the onset of neuronal differentiation is Nestin (19). Considering the above-mentioned evidence and the importance of investigating the differentiation of these cells in 3D media and since no studies have been reported so far, this study aimed to evaluate and compare the neuronal differentiation of Wharton jelly stem cells in CH - $\beta$ -GP-HEC hydrogels as a three-dimensional culture medium compared to two-dimensional culture.

## Methods

**Study type and groups:** This experimental study was performed in 4 groups of 2D culture of Wharton jelly stem cells in the presence of nerve differentiation medium and its absence (medium lacking differentiators) and three-dimensional culture of Wharton jelly stem cells in the presence of nerve differentiation medium and its absence (medium lacking differentiators).

To evaluate and compare neural differentiation, two expression variables of  $\beta$ -Tubulin III and Nestin as two markers and criteria for measuring neural differentiation and  $\beta$ -actin gene (as internal control and benchmark gene or reference gene) were evaluated by Real time PCR. Wharton jelly stem cells were isolated from neonatal cord at Imam Khomeini Hospital after obtaining written consent and with the approval of Ethics Committee of Tehran University of Medical Sciences (Code: 2406TUMS.rec.).

**Wharton Jelly stem cell separation:** Cord specimens were placed under sterile conditions containing normal saline and transferred to the laboratory. To eliminate contamination, the cord was rinsed with 70% alcohol for 30 seconds. Then, it was washed with PBS containing penicillin, streptomycin, amphotericin and gentamicin. Wharton jelly was separated from the cord by a sterile razor blade and divided into pieces less than 5 mm square.

The fragments were transferred to complete culture medium (containing glucose, 20% FBS and 1% penicillin/ streptomycin) and incubated in 95% humidity and 5% CO<sub>2</sub> at 37 ° C. Cells were separated by culture of a piece of Wharton jelly and adhered to the

bottom of the flask. Wharton jelly remnants were removed 24 h after the first wash with saline phosphate buffer.

**Wharton Jelly stem cell identification:** Three passage cells were used to confirm the identity of Wharton jelly stem cells extracted by flow cytometry (BD FACS Calibur Bioscience, USA) for expression of CD105, CD90, CD73, CD45, and CD34 markers by specific antibodies and results were analyzed using FlowJo software version 1, 7 and 6.

**Hydrogel preparation:** To make the hydrogel, first weighed 225 mg of CH powder and after sterilizing in 0.1 ml of 0.1 M hydrochloric acid was placed on the shaker with 140 RPM for several hours to completely dissolve. In the next step, 2.25 g of weighted  $\beta$ -GF was dissolved in 3.5 ml of de-ionized water and sterilized with a filter.

The sterile solution of CH and  $\beta$ -GF were both kept on ice for 15 min separately, then the  $\beta$ -GF was dropwise and gently stirred on the CH solution while the CH solution was stirred. After adding  $\beta$ -GF, the final volume of CH and  $\beta$ -GF solution reached 15 ml. 125 mg HEC (autoclaved) was dissolved in 10 ml PBS. Cold HEC was added to CH- $\beta$ -GF solution at a ratio of 4.8: 0.8 (HEC: CH-  $\beta$ -GF). Cell addition was performed concurrently with the addition of HEC.

**Evaluation of hydrogel gel morphology and gelation process:** To determine the gelation time of low temperature HEC solution was added to CH- $\beta$ -GF and the resulting hydrogel was transferred immediately (constant temperature 37 ° C) and gelation time was investigated. To investigate the morphology of the hydrogel, it was dried by freeze drier and gold coated with Sputter Coater (Technics, Hummer II, Japan) and imaged with HITACH-S4160 electron microscope.

**Evaluation of neural differentiation of Jelly-Wharton stem cells:** In order to evaluate the neuronal differentiation of Wharton jelly stem cells in 2D medium, these cells were cultured in nerve

differentiation medium for 4 days. But in the 3D culture group, these cells were subjected to neuronal differentiation after exposure to hydrogel. The differentiation medium included DMEM / F12, 10% KSR serum, 20 ng/ml EGF growth factor, 20 ng/ml bFGF growth factor and 10 mg/ml heparin. After 4 days, the cells of both groups and control groups without differentiation medium were evaluated morphologically by invert phase contrast microscopy.

Real time-PCR was also performed using the primers for Nestin,  $\beta$ -Tubulin III and  $\beta$ -actin genes (as internal control) (Table 1). The cDNA kit according to the manufacturer's protocol was also used for cDNA synthesis. A negative control was assumed for real-time PCR for each. Three replicates were prepared for each sample. Each sample consisted of 10  $\mu$ L Syber green, 1  $\mu$ L Rox, 0.7  $\mu$ L F primer, 0.7  $\mu$ L R primer, 1  $\mu$ L cDNA, 6.4  $\mu$ L RNase free water and the volume of each reaction was 20  $\mu$ L. In the negative control all the above compound was present except cDNA. The reaction was performed on the ABI Step One device according to the manufacturer's protocol.

40 cycles were considered for each cycle of Real Time PCR. After each run of PCR, the accuracy of each amplification was confirmed by the Melting curve using the specific temperature of the Melt of product specific to each gene. The expression levels of target genes (Nestin and  $\beta$ -Tubulin III) were compared to the reference gene ( $\beta$ -actin) using  $\Delta\Delta$  CT formula.

**Statistical tests:** All data were analyzed by SPSS software version 23 using one-way analysis of variance, and graphs were draw with Excel 2016 software. To analyze the results of Real-Time PCR, raw data were converted to interpretable data by REST 2009 software. The expression levels of target genes (Nestin and  $\beta$ -Tubulin III) were compared to the reference gene ( $\beta$ -actin) using  $\Delta\Delta$  CT formula. One-way analysis of variance was then used and  $p<0.05$  considered significant.

Table 1. Sequences of forward and reverse primers

| Gen                 | Forward and reverse primers                        |
|---------------------|----------------------------------------------------|
| $\beta$ -actin      | Forward primer (5'-3'):acatcaaggagaagctgtgctac     |
|                     | Reverse primer (3'-5'):cttcgatgaggagtggaaggtagt    |
| Nestin              | Forward primer (5'-3'):ggcttctctcagcatcttgg        |
|                     | Reverse primer (3'-5'):aaggctggcataggtgtgc         |
| $\beta$ -tubulinIII | Forward primer (5'-3'):cagagcaagaacagcagctactt     |
|                     | Reverse primer (3'-5'):gtgaactccatctctgctccatccctc |

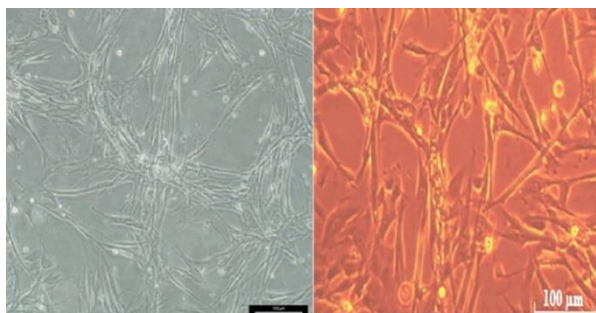
## Results

**Identification of Wharton jelly stem cells:** These cells initially appeared rounded and adhered to the bottom of the flask after 24 hours and appeared to resemble fibroblasts (Fig 1). Flow cytometry analysis (Fig 2) showed the highest expression of positive CD44 stem cell markers with 98.6% and the remaining positive markers CD105, CD90, CD73 with 91.4%, 98.3% and 95.9%, respectively. In addition, negative markers CD45 and CD34 were expressed 4.75% and 2.74%, respectively.

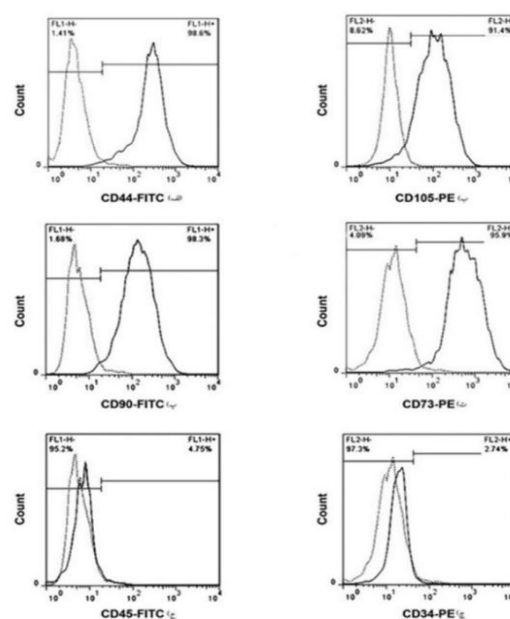
**Porous morphology of hydrogel:** The structure of the hydrogel after its removal was investigated by SEM microscopy (Fig 3). The results showed that the structure of this hydrogel was completely porous and the pores were interconnected.

**Hydrogel gelation time:** The results of gelation time determination showed that the gel began to gelation immediately after transfer to 37 °C and completed in about 25 minutes.

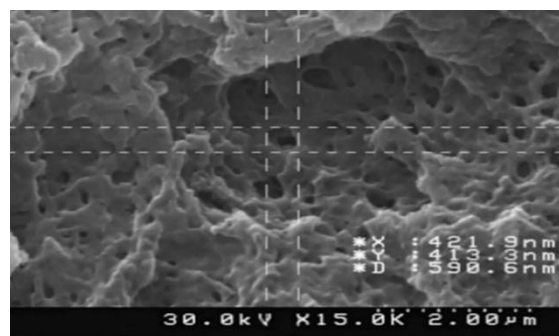
**Evaluation of the neural differentiation genes expression in 2D and 3D cultures:** Quantitative expression of Nestin and  $\beta$ -Tubulin III genes (Fig 4) showed increased expression of these genes in the presence of neuronal differentiation medium both in 3D and 2D culture conditions. Expression of these genes was increased (more than 2-fold) in three-dimensional culture conditions and in neuronal differentiation medium ( $p < 0.005$ ). Moreover, expression of these genes in two-dimensional culture conditions and presence of neuronal differentiation medium was increased (more than 4-fold) compared with control group without differentiation medium ( $p < 0.005$ ). Chitosan based hydrogel and presence of neuronal differentiation medium showed similar increase (more than 1.5 fold) compared to similar two-dimensional conditions ( $p < 0.01$ ).



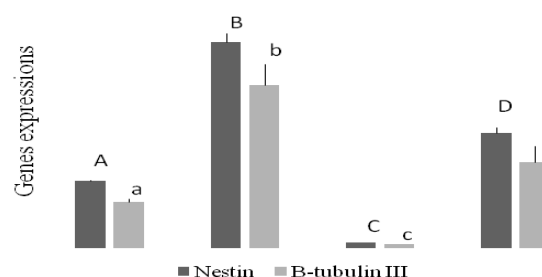
**Figure 1.** Fibroblast-like morphology of Wharton jelly stem cells in DMEM/F12 medium on the right and nerve cells induced Wharton jelly stem cells in differentiation medium after 4 days on the left



**Figure 2.** Flow cytometry analysis of Wharton jelly stem cells with positive marker including: (a) CD44 (98.6%) b) CD105 (91.4%) c) CD90 (98.3%) and d) CD73(95.9%) and negative markers included: (e) CD45 (75.4%) (f) CD34 (2.74%)



**Figure 3.** Porous morphology of CH- $\beta$ -GP-HEC hydrogels after water removal by electron microscopy



**Figure 4.** Results of Nestin and  $\beta$ -Tubulin III gene expression in study groups after 4 days. Three D without: 3D cell culture (in hydrogels), Three D with: 3D cell culture in neural differentiation medium, Two D without: 2D cell culture, 2D with: 2D cell culture in the presence of neuronal differentiation medium

## Discussion

The results of this study showed that expression of neuronal differentiation markers ( $\beta$ -Tubulin III and Nestin) in 3D culture conditions using CH- $\beta$ -GP-HEC chitosan basic hydrogel was increased compared to 2D culture medium. Wharton jelly stem cells were isolated by the culture of small fragments, a common method of isolating these cells (20). The cells isolated in this way, like other mesenchymal cells, were found to resemble fibroblasts after sticking to the bottom of the culture medium. Flow cytometry studies in line with other studies on the identity of these cells showed that the cell surface markers of CD45 and CD34 were not expressed by the cells. However, surface markers of mesenchymal stem cells including CD90, CD73, CD105, and CD44 were expressed (20).

The results of the hydrogel morphology evaluation showed that this hydrogel, like other basic chitosan hydrogels, has a porous structure and its pores are interconnected. The porosity allows the cell to be positioned and the connection between the pores leads to the transfer of food and waste. The porous structure of CH- $\beta$ -GP-HEC hydrogels can provide suitable conditions for stem cell growth and proliferation. Other studies of these hydrogels have reported similar results (21). The gelation time of CH- $\beta$ -GP-HEC hydrogel at 37 ° C is about 25 minutes. However, the gelation time for CH- $\beta$ -GP hydrogels was reported to be 6 minutes (22). Rapid gelation can disrupt the cellular positioning process and cause shock to them.

The time taken for CH- $\beta$ -GP-HEC hydrogels seems to be a very good one. According to the results of MTT test, there was no significant difference in survival between control group and cells in contact with hydrogel. appropriate concentration of  $\beta$ -GP used can be mentioned as a reason for the lack of hydrogel toxicity. The strong interaction between water molecules and CH prevents its mass or gelation. Electrostatic repulsion of the CH chains is neutralized by phosphate group of glycerophosphate salt. In very small amounts of glycerophosphate salts, the resulting gel returns to a soluble state with the lowering of the temperature; because the very low salt content cannot overcome the electrostatic charge between the chitosan chains (23). One of the common ways to overcome this problem is to increase the amount of glycerophosphate salts (25, 24). The main problem with this method is the increase in toxicity at high concentrations of

glycerophosphate. Cross linking agents such as HEC can be used to reduce the glycerophosphate intake and increase the bio-compatibility of these hydrogels (26, 24). The appropriate combination of CH- $\beta$ -GP-HEC hydrogels has made this hydrogel a suitable environment for differentiating cell types into different classes. In one study, the potential of chondrogenic differentiation of this hydrogel was evaluated for adipose-derived stem cells, and the results showed that the porous structure of this hydrogel could provide a suitable environment for the growth and differentiation of adipose tissue derived mesenchymal stem cells to chondrocytes (21). Real-time PCR results showed that the three-dimensional hydrogel medium without nerve differentiation medium increased the expression of Nestin and  $\beta$ -Tubulin III gene in Wharton jelly stem cells. Expression of these neural markers during the 4-day period that eventually induces neural protein synthesis indicates the initiation of differentiation of Wharton jelly stem cells into neurons (19).

In the presence of hydrogel and neuronal differentiation medium, Wharton jelly stem cells had the highest expression of Nestin and  $\beta$ -Tubulin III compared to the other groups. This increase was due to the synergistic effect of the presence of CH- $\beta$ -GP-HEC hydrogel as a three-dimensional culture medium and a neuronal differentiation medium, which is due to the usefulness of this hydrogel in neuronal differentiation of Wharton jelly stem cells. In addition to enjoying the benefits of three-dimensional culture in providing natural and near-body conditions due to the high cost of preparing the nerve differentiation medium, hydrogels that can help induce nerve differentiation are economically viable. It is concluded from this study that 3D culture in CH- $\beta$ -GP-HEC hydrogel contributes to neuronal differentiation of Wharton jelly stem cells and can be used as a suitable 3D culture medium in the laboratory for proliferation and differentiation of these cells or as a carrier for injecting and transplanting these cells to different parts of the nervous system.

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