Original Article

Differentiation of Human Wharton's Jelly Mesenchymal Stem Cells into SOX17 Expressing Cells Using a Wnt/ß-catenin Pathway Agonist on Polylactic Acid/Chitosan Nanocomposite Scaffold

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Abstract

Objective: The β-catenin signaling pathway promises the potential for differentiation of stem cells into definitive endoderm (DE) cells as precursors of beta cells. Therefore, it can be considered as an inducer for cell replacement therapies in diabetes. The main goal of this research is to successfully culture and induce differentiation of human Wharton's jelly mesenchymal stem cells (hWJMSCs) into Sox17-expressing cells using a Wnt/β-catenin pathway agonist (SKL2001) plus nanoparticles on a polylactic acid/chitosan (PLA/Cs) nanocomposite scaffold.

Materials and Methods: In this experimental study, the nanocomposite was prepared through an electrospinning method and hWJMSCs were isolated through an explant technique. The morphology and the cell viability were evaluated by scanning electron microscopy (SEM) and 3-(4, 5- Dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Here, we present two differentiation protocols: the first one is induction with SKL2001; and the second one is with a combination of SKL2001 and zinc oxide nanoparticles (nZnO). Real-time quantitative reverse transcription (QRT-PCR) and immunocytochemistry analysis are carried out to examine the expression of specific markers in the differentiated cells.

Results: The nanocomposite had appropriate biocompatibility for cell adhesion and growth. While the hWJMSCs cultured on the PLA/Cs scaffolds differentiated into DE cells in the presence of SKL2001, introducing nZnO to their environment increased the differentiation process. Analyses of DE-specific markers including *SOX17, FOXA2*, and gooscoid (GSC) genes in mRNA level, indicated significantly high levels of expression in the SKL2001/nZnO group, followed by SKL2001 group compared to the control.

Conclusion: Our results show the beneficial effects of the Wnt/β-catenin pathway agonist in three-dimensional (3D) cultures in cell replacement therapy for diabetes.

Keywords: Differentiation, Nanoparticles, Tissue Engineering, Wharton's Jelly, Wnt/β-Catenin Pathway

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Introduction

Treatment of chronic endoderm-derived organ failures, such as hepatic cirrhosis and diabetes, is rather challenging since no complete cure is known (1). Today, organ transplantation is the major treatment for these disorders. However, it is accompanied with many difficulties, including shortage of donors, transplant rejections, surgical complications, and high costs. Today, cell therapy has been considered as an alternative approach to remove some of these barriers (2).

Mesenchymal stem cells (MSCs) are multipotent and differentiate into various cells and tissues, as these cells have been found in most adult tissues (2, 3). Recently, the application of human wharton's jelly MSCs (hWJMSCs) in therapy has come to attention. Because of high potency, multipotent properties amongst adult stem cells, fast and relatively inexpensive extraction, and their non-tumorigenic state, hWJMSCs have been suggested as a promising candidate for cell therapy (4). Suitably for our studies, it has been shown that hWJMSCs may be induced to differentiate into a variety of cell types including definitive endoderm (DE), beta cells, and hepatocytes (5, 6).

The first and basic developmental step in formation of endodermal organs is the induction of DE (7). Notably, the pancreas, lungs, liver, and other organs of the gastrointestinal tract are derived from DE during embryogenesis (8, 9). Multiple signaling pathways such as Nodal, Activin A, and Wnt3a could cause differentiation of DE through signaling intermediates. In addition, the Wnt/β-catenin signaling has a critical role in cell morphology, proliferation, motility, axis determination, differentiation, and organ development. SKL2001, as an agonist for Wnt/β-catenin signaling, has been shown to inhibit the phosphorylation of β-catenin (by an alternative mechanism and independent of inhibition of GSK-3β activity), thus increasing the levels of intracellular β-catenin (10).

According to previous studies, zinc is considered as an abundant trace metal and a catalyzer for various enzymes

in the body (11, 12). Besides, zinc protects insulin from degradation and stimulates insulin biosynthesis, secretion, and its storage. Different zinc transporters, such as zinc transporter-8, exist in pancreatic β-cells and affect insulin secretion. Also, zinc improves insulin signaling by several mechanisms, such as increasing insulin receptor phosphorylation, inhibition of glycogen synthase kinase-3 (GK), and increasing PI3K activity (11). Indeed, based on recent reports, zinc oxide nanoparticles (nZnO) reduce blood glucose and significantly increase blood insulin in diabetic animal models, when compared to ZnO (12).

On the other hand, not only intracellular signaling pathways, but also cell interactions with the extracellular matrix (ECM) are taken as critical parameters in behavior, adhesion, morphology, migration, proliferation, and differentiation of the cells (13). In tissue engineering, a suitable scaffold can provide this platform. Also, the electrospinning technology can be applied to fabricate three-dimensional (3D) synthesized scaffolds. The electrospun scaffolds with diameters of tens to hundreds of nanometers are designed to mimic the ECM in cell and tissue culture (10, 14).

Thus in this study, we used SKL2001 as a Wnt/β-catenin pathway agonist alone or in combination with nZnO to induce differentiation of hWJMSCs into *SOX17*-expressing cells (as DE like-cells) on PLA/Cs three-dimensional scaffolds.

Material and Methods

All animal procedures and experimental tests were approved by the Animal Ethics Committee of Shahid Chamran University of Ahvaz (93042515).

Preparation of scaffold by electrospinning

In this experimental study, the electrospinning technique was employed to fabricate polylactic acid/chitosan (PLA/Cs) scaffolds (Electronic, FNM, Iran). To obtain 2.5 (w/v) solutions, PLA and chitosan (Cs) were added in hexafluoroisopropanol (HFIP) and acetic acid, respectively. These solutions were blended in the ratio of 7:3 (PLA:Cs) to make a scaffold. The solution was shaken for 12 hours and inserted into a 10-ml plastic syringe, and connected to a high voltage (14-18 kV) at 25°C Aluminum foil was used to collect spray drift (at a distance of 10 centimeters). The electrospun fibrous membranes were dried in a vacuum oven for two days and separated from aluminum foil at the time of use (15, 16).

Cell seeding on PLA/Cs nanocomposite scaffold

The scaffold was cut into discs with a diameter of 1.6 centimeters and placed in 24-well plates. After that, the scaffolds were sterilized by ultraviolet irradiation for 2 hours and floated in Dulbecco's modified eagle medium (DMEM, Gibco, USA) medium supplemented with 1 µg/ml amphotericin B and 3% pen/strep overnight at 37°C. Then, hWJMSCs were cultured at a density of 6×10^4 cell/scaffold and incubated at 37°C and 5% CO_2.

Scanning electron microscopy

The morphology of the prepared PLA/Cs scaffolds was studied with scanning electron microscopy (SEM). The average diameter of the mats was measured by analyzing SEM images using Image J software (National Institutes of Health, USA). The diameter distribution was measured by examining at least 100 samples. To observe hWJMSCs cultured on PLA/Cs nanocomposite scaffolds, the samples were fixed with 2.5% glutaraldehyde for 2 hours. Then they were washed in phosphate buffer saline (PBS, Sigma, USA) and dehydrated in ethanol series (30, 50, 70, 80, 90 and 100%) at 37ºC for 15 minutes per solution. Then the scaffolds were sputter-coated with gold and studied by a SEM (model Philips XL-30, Netherland), operated at 15 kV.

Isolation and identification of hWJMSCs

Human WJMSCs were obtained as described previously (17, 18). In summary, human umbilical cord was obtained after delivery from term natural births. The cord blood was removed immediately, then it was cut into 1-centimeter pieces and washed. The pieces were soaked in PBS supplemented with 3% (v/v) pen/strep for 24 hours. The stem cells were isolated by explant cultures as each piece was cut carefully with a scalpel, then the vessels were removed and wharton jelly was collected. Then the jelly was sliced into 2-millimeter pieces, cultured in tissue culture flasks and maintained for 14 days to allow for cell migration and expansion. DMEM low glucose medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin was used and the medium was changed every 3 days as the cells were passaged by 0.25% trypsin. Also, hWJMSCs were characterized by flow cytometry for cell surface markers. Briefly, hWJMSCs were incubated with specific antibodies including CD146 (endometrial stem cell markers, 1:200, Santa Cruz, USA), CD90 (1:200, Santa Cruz, USA), CD105 (1:100, Santa Cruz, USA), CD34 (hematopoietic marker, 1:100, Santa Cruz, USA), and CD31 (endothelial marker, 1:100, Santa Cruz, USA) for 60 minutes at room temperature and analyzed by flow cytometry (Becton Dickinson, USA) after washing. Also, for differentiation of hWJMSCs into adipocytes and osteocytes, the cells were seeded at a concentration of 5×10^4 cells/well in a 24-well plate. When the cells reached 80% confluency, the medium was replaced with adipogenic- or osteogenic-inducing media as described previously (17). The differentiated cells were stained with Oil Red or Alizarin Red to detect adipocytes or osteocytes, respectively.

Assessment of cell viability

The viability of the hWJMSCs seeded on the PLA/CS scaffolds was assessed by the MTT (3-(4, 5-Dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide) reduction assay. 6×10^4 cells were cultured on each scaffold

and incubated for 24 hours at 37°C in an incubator with 5% Co_2. Afterward, 300 µl of 0.5 mg/ml MTT solution was added to each well, and the plates were incubated for 3-4 hours at 37°C then the medium was removed, and DMSO was added to dissolve the formazan crystals. The samples were shaken by a mechanical shaker, next the absorbance was read at the wavelength of 490 nanometers in a microplate reader (Fax 2100, USA).

Acridine orange staining

For acridine orange/ethidium bromide double staining (Sigma, USA), 1 mg/ml ethidium bromide dye and 1 mg/ml acridine orange dye were prepared and mixed at a 1:1 ratio. After that, the cells were seeded on scaffolds (after 48 hours), were stained for 3 minutes and then examined under a fluorescent microscope (Olympus, Japan).

Human WJMSCs culture and differentiation into definitive endoderm cells

The cultured cells on PLA/Cs scaffolds were induced to differentiate using two different protocols. The first: the hWJMSCs were cultured on PLA/Cs scaffold and treated with 20 µM SKL2001 (Sigma, USA) and 0.2% FBS for 6 days. The second: the cells were cultured on PLA/Cs scaffold and treated with 20 µM SKL2001 in combination with 50 µg/ml nZnO (Loletics Germany, ≥70 nm avg.) for 6 days. As a control, hWJMSCs were cultured on the PLA/Cs scaffolds in the absence of differentiation factors for 6 days. DMEM medium was used during the differentiation phase. This medium was supplemented with FBS at 0.2% and 10% concentrations in the experimental and control groups, respectively. The medium in cell culture plates was replaced with fresh medium every two days.

RNA extraction and reverse transcriptase-polymerase chain reaction

The mRNA expression was examined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). On day 6 days of the culture, the cells were lysed by QIAzol lysis reagent (Qiagen, Hilden, Germany) and the total RNA was extracted according to the manufacturer's instructions. The extracted RNA (3 µg) was reverse transcribed by the TaqMan Reverse Transcription Kit (Applied Biosystems, CA, USA). QRT-PCR reactions were performed in 6-well plates in a StepOne™ Real-Time PCR machine (Corbett, Australia) using primers that are shown in Table 1. QRT-PCR was performed by SYBR green Supermix (Ampliqon, Denmark) and the applied protocol was: initial denaturation (95°C for 30 seconds), amplification (95°C for 5 seconds and 60°C for 33 seconds), and melting (95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 1 second). The target genes' threshold cycle (Ct) was obtained from the StepOne software, and the values were normalized by *GAPDH*.

Immunofluorescence staining

The samples were fixed with paraformaldehyde (4%, Sigma, USA) for 40 minutes and permeabilized with 0.1% Triton X-100 in PBS at room temperature. Then the samples were blocked for 1 hour with 5% bovine serum albumin (BSA, Sigma, USA) at room temperature and stained with primary antibodies against human *FOXA2* (1: 500, Polyclonal rabbit IgG, Millipore, Germany, AB4125) and human *SOX17* (1: 20, Polyclonal Goat IgG, R&D, USA, AF1924) overnight at 4°C. After that, the samples were stained with secondary antibodies [Alexa fluor 594 donkey anti-rabbit (1:200, Gibco, USA, A-21207) or Alexa fluor 488 donkey anti-goat (1:200, Gibco, USA, A-11058)] for 60 minutes at room temperature. The cell nuclei were stained with DAPI (1 µg/ml, Sigma, USA, D8417) for 5 minutes and the samples were imaged under a fluorescent microscope (Olympus, Japan).

Statistical analysis

The data are presented as means ± standard deviation (SD) of three replications. Statistical analyses were carried out by a one-way ANOVA method followed by unpaired Student's t test and P≤0.05 was designated as significant difference. We used SPSS software version 16.

Table 1: Sequences of the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR*)* primers

Gene	Primer sequence (5´-3´)	Accession number	Product length (bp)
Forkhead box A2 (*FOXA2*)	F: GAGACAAATCTCAGCCTCCCA R: CGTTCTCGAACATGTTGCCC	NM_153675.3	110
SRY (sex determining region Y)-box 17 *(S0X17)*	F: TCAGCAAGCAGCTGGGATAC R: AACTGCAATTCTTCGGCAG	NM_003140.3	162
Goosecoid homeobox (*GSC*)	F: AACGCGGAGAAGTGGAACAA R: AGCATCGTCTGTCTGTGCA	NM_173849.3	158
GAPDH	F: CCATGGGGAAGGTGAAGGT R: AGTGATGGCATGGACTGTG	NM_002046.7	548

Results

Characterization of hWJMS cells

Human WJMSCs readily adhered to the bottom of the flask and were extractable. After 2 weeks, the cells were fully attached to the bottom of the flasks and had grown to 80% confluency. At this point they were passaged one last time and reached to the third passage, after which they were used for treatment. As shown in Figure 1, under the inverted microscope, the cells appeared to be normal with elongated and spindle-like shapes (Fig.1A). To confirm multipotency of the isolated hWJMSCs, they were treated with osteogenic and adipogenic inductive media, and their differentiation was confirmed by Alizarin Red, and Oil Red O staining, respectively (Fig.1B, C). In addition, when evaluating their cell surface markers by flow cytometry, our results indicated that the cells were expressing CD105, CD90, and CD146 markers, but not CD31 and CD34 (Fig.1D).

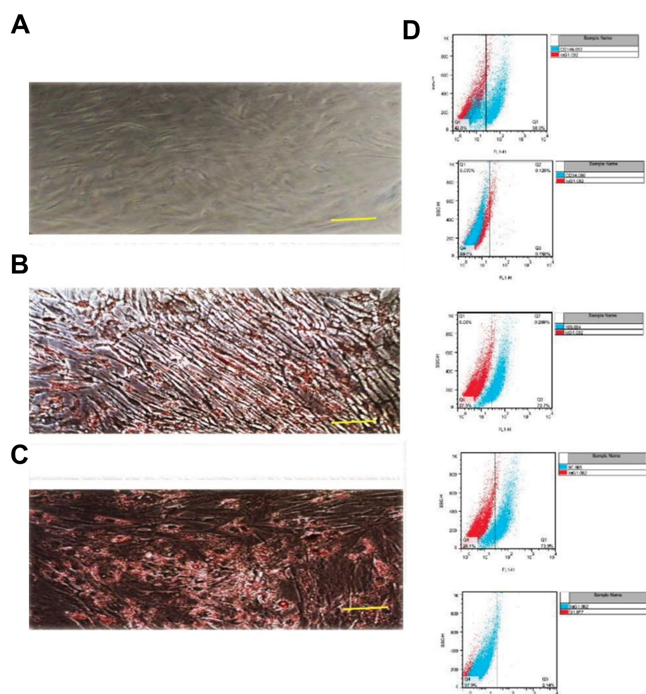

Fig.1: Characterization of human Wharton's jelly mesenchymal stem cells (hWJMSCs). **A.** Typical observation of hWJMSCs under phase-contrast microscopy. **B.** Differentiation of hWJMSCs into osteoblasts as shown by Alizarin Red staining. **C.** Differentiation of hWJMSCs into adipocytes as shown by Oil Red O staining. **D.** Flow cytometric analysis of the isolated hWJMSCs for MSC (CD146, CD105, and CD90), and hematopoietic (CD34) and endothelial (CD31) markers (scale bar: 100 µm).

Morphology of the electrospun PLA/Cs nanocomposite scaffold

We produced the PLA/Cs nanocomposite scaffolds by a solution blending method. As shown in Figure 1, the nanocomposite was a homogeneous scaffold with high porosity and average diameter of 70 micrometers. Also, the average thickness of this scaffold was about 460 micrometers. Indeed, the SEM images indicated random fibers without any beads with an improved porous network. Also, the study of culturing the adherent cells on the scaffold revealed that numerous hWJMSCs were attached and scattered on PLA/Cs scaffold after 72 hours following the seeding (Fig.2). These results proved that the PLA/Cs scaffold effectively supported cellular adhesion and growth of the hWJMSCs.

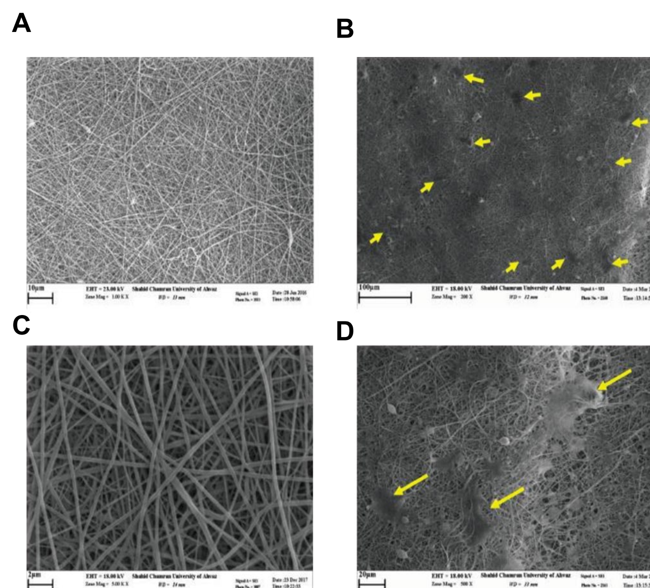

Fig.2: Scanning electron micrographs show the morphology of the plated human Wharton's jelly mesenchymal stem cells (hWJMSCs) on polylactic acid/chitosan (PLA/Cs) scaffold on day 3 after seeding. **A.** The fibers of PLA/Cs scaffold were randomly entangled to form a strong, flexible, and porous 3D matrix. **B.** The fibers of PLA/Cs scaffold with higher magnification, **C.** The plated hWJMSCs on PLA/Cs scaffold, **D.** The plated hWJMSCs on PLA/Cs scaffold with higher magnification. Yellow arrows show the plated hWJMSCs on the scaffold.

Measurement of cell viability

In this study, we evaluated cell viability of hWJMSCs on PLA/Cs scaffolds using the MTT assay for 6 days. Three days after plating the cells, no significant difference was observed between the viability of cells on PLA/Cs scaffold and the monolayer culture ($P<0.05$). However, on days 4 and 6 of the culture, the viability of the cells on PLA/Cs scaffolds significantly increased ($P<0.05$) compared to the monolayer culture. Therefore, it suggests that there is a time-dependent increase in stability and viability of the hWJMSCs on the nanocomposite scaffold compared to the monolayer culture (Fig.3A).

Acridine orange staining

Acridine orange is a double staining, such that live cells turn green, while apoptotic cells are orange, and if cells are in late stages of apoptosis, the nuclei get fragmented, compacted, and red. Our result suggested that the cultured cells on the PLA/Cs scaffold were normal, clear, green and without shrinkage, proving their viability (Fig.3).

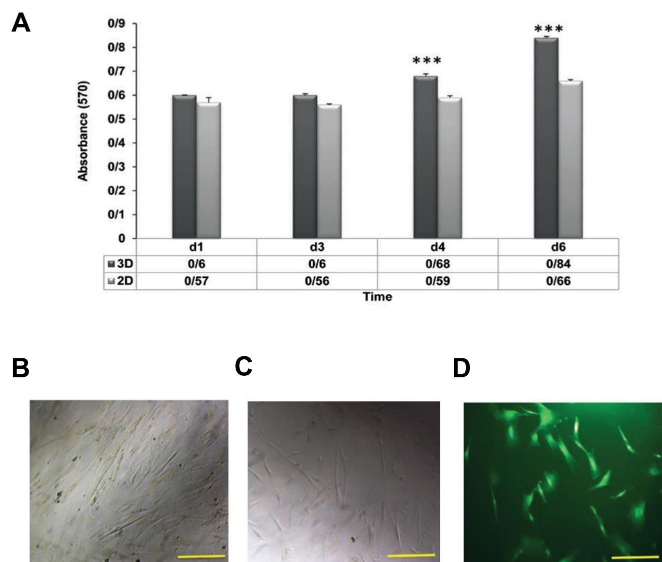

Fig.3: MTT assay and morphological study of hWJMSCs by an inverted microscope. **A.** Formosan absorbance has been expressed as a measure of cell viability from the hWJMSCs cultured on nanocomposite scaffold for 6 days. **B.** Passage 1 hWJMSCs after 3 days in culture, **C.** Passage 3 hWJMSCs after 7 days, **D.** The cell viability assay using acridine orange/ ethidium bromide staining of hWJMSCs was performed on the cells cultured on PLA/Cs scaffold by fluorescent microscopy after 2 days (B, C, and D represent ×200, ×200, and ×100 magnification, respectively, scale bar: 100 µm). hWJMSCs; Human Wharton's jelly mesenchymal stem cells, PLA/Cs; Polylactic acid/chitosan, and ***; P<0.05 and values are mean (n=3).

Differentiation of hWJMSCs into definitive endoderm cells

To distiguish DE cells from hWJMSCs, two protocols (SKL2001 or SKL2001/ nZnO) were used and their efficiencies were compared. As shown in Figure 4, in response to SKL2001 induction, the expression of the hallmark genes of DE i.e. *FOXA2*, *SOX17*, and *GSC* were increased about 130, 159, and 90-fold, respectively. Also, the induction of hWJMSCs with SKL2001/nZnO resulted in a significant increase in the expression levels of *FOXA2*, *SOX17*, and *gsc* genes to 141, 204, and 92 folds, respectively. The expression levels of *FOXA2* and *SOX17* were also significantly higher (P<0.05) than their expressions in the cells induced by SKL2001 alone, but no obvious difference in *GSC* expression was found between these two groups (Fig.4). In this research, mRNAs expression of the DE marker genes in both experimental groups was significantly more than that in the control group. Based on these data, treatment with SKL2001 or especially with SKL2001/nZnO could obviously induce the differentiation of hWJMSCs into DE cells.

Immunocytochemical technique was performed for further evaluation of the more effective protocol i.e. SKL2001/nZnO. This analysis of the hWJMSCs-derived DE sample suggested that the main population of DE cells expressed SOX17 and FOXA2 proteins within the nuclei. Therefore, treatment with SKL2001/ nZnO could successfully induce differentiation into DE cells (Fig.5).

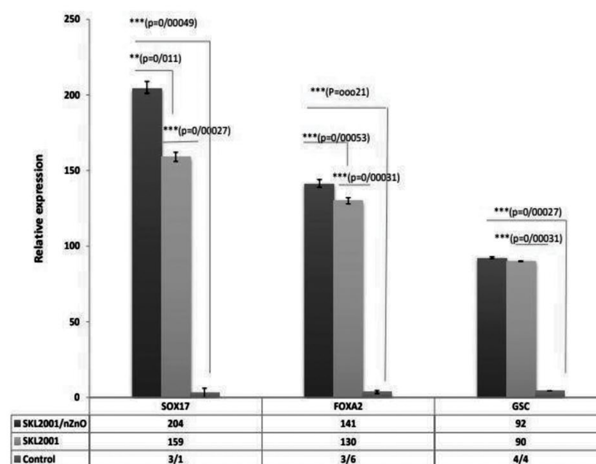

Fig.4: Quantitative expression analysis of DE cells derived from hWJMSCs cultured on PLA/Cs scaffold after 6 days. The results are collected from 3 independent experiments with 2 internal replicates per experiment. Differences observed were statistically significant when P≤0.05. Comparison of the gene expression levels of DE markers (*FOXA2*, *SOX17*, and *GSC*) in two experimental groups. hWJMSCs; Human Wharton's jelly mesenchymal stem cells, PLA/Cs; Polylactic acid/chitosan, DE; Definitive endoderm, ***; P<0.001, and **; P<0.01 untreated cells were considered as a control group.

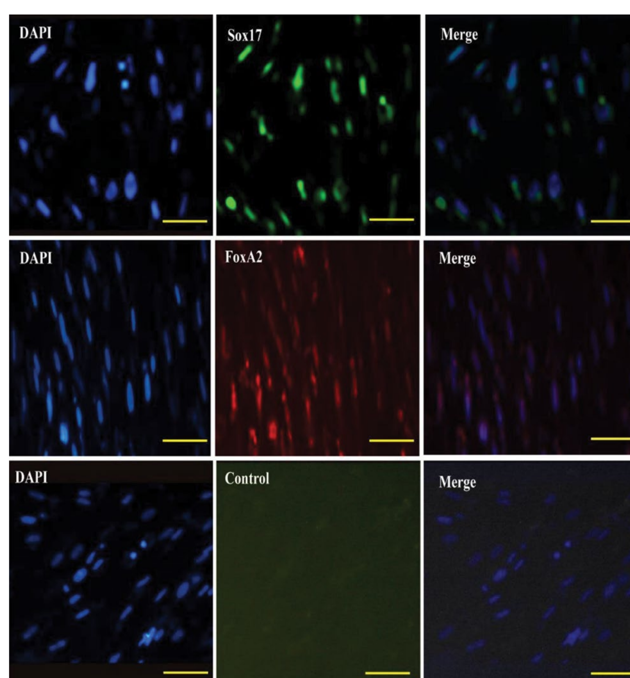

Fig.5: Immunocytochemistry performed for analyzing SOX17 and FOXA2 as endoderm-specific proteins by differentiated hWJMSCs on the scaffold after 6 days of culture. The staining of nuclei was performed by DAPI (×400 magnification, scale bar: 100 µm).

Discussion

In the present study, we have assessed the potentials of differentiation of hWJMSCs into DE cells using SKL2001 as a small molecule and nZnO as a nanoparticle on an electrospun nanocomposite scaffold. The analysis of qRT-PCR demonstrated that the expression of endodermal marker genes such as *FOXA2*, *SOX17*, and *GSC* in experimental groups were significantly more than the control group. Using immunocytochemistry, we also evaluated the expression levels of the protein products of these genes.

We can define tissue engineering as a multidisciplinary approach that provides a promising strategy for regenerative medicine (19). Various research projects have demonstrated the advantages of tissue engineering as a suitable therapeutic strategy for induction of cell differentiation (20, 21). Interestingly, these studies have proven that the use of tissue engineering improves cell proliferation, survival, and cell-cell interactions comparing to monolayer cultures (22-24). Today, the administration of synthetic polymers to prepare scaffolds has significantly increased, which is due to their suitable mechanical properties, cost-effectiveness, and convenient fabrication processes. Furthermore, polymer blending methods have been used to improve the hydrophilic properties of synthetic polymers and the adhesion of cells to scaffolds (25-28). In our study, PLA/Cs, as a blended scaffold, was a biocompatible and suitable scaffold in promoting cell viability and attachment. Also, the infiltration and extension of the cells into the PLA/Cs scaffold was confirmed by SEM studies. Furthermore, according to our results, the DE differentiation with high efficiency in the expression of *SOX17* was due to the treatment of hWJMSCs seeded on PLA/Cs with SKL2001 and nZnO.

It has been proved that the formation of DE can be considered as the first and most critical stage in the generation of stem cell-derived hepatocytes, beta cells, and other endoderm derived organs (29-31). D'Amour et al. (29) in 2006 presented a protocol for efficient induction of DE cells. Because of the high efficiency of this protocol, generally it has been used by many research groups (32, 33). Based on the recent studies, to induce DE cells from different stem cells, several factors including Nodal, Wnt3a, Activin A, and some of the small molecules have been used (31). For example, Borowiak et al. (34) showed that inducer of DE1 as a small molecule could increase DE differentiation, comparable to those induced by Activin A. In the present study, our results indicated that SKL2001/nZnO combination offers a practical method for DE differentiation from hWJMSCs in a 3D culture.

Previous studies have reported that zinc is effective in glucose metabolism, promoting hepatic glycogenesis by acting on insulin pathways and improving glucose utilization. Zinc plays an essential role in biosynthesis, secretion, and storage of insulin because it protects insulin structure. Moreover, beta cells contain several zinc transporters that stimulate insulin secretion (35). Also, nZnO has antidiabetic effects and it can decrease blood glucose, inhibit glucokinase (GK) activity, increase insulin level, and stimulate the expression of glucose transporter 2 in diabetic rats (12). Besides, in some studies, it has been reported that zinc ions act as signalling molecules (36, 37). Thus, we can divide the intracellular zinc functions into two categories: i. Protein binding zinc, contributing to enzyme's activity and structure protection, and ii. Labile zinc, with is non-binding to proteins and acts as a signal transferring molecule (38). Based on the existing evidence, nZnO may be conducive to the differentiation of DE through signaling pathways, especially by inhibiting GK activity. In this study we showed that the small molecule SKL2001 has synergistic effects with nZnO in induction of DE cell formation. We conclude that in this induction pathway, nZnO has synergies with SKL2001 via activation of β-catenin signaling, since nZnO can activate Wnt/β-catenin indirectly by inhibiting GK.

The Wnt/β-catenin has important functions in differentiation processes of various cells such as MSCs. Gwak et al. (39) in 2012 introduced SKL2001 as a novel agonist of the Wnt/β-catenin pathway after screening 270 000 synthetic chemical compounds. They identified the molecular mechanism of SKL2001, which leads to release of β-catenin, by opening up the Axin/β-catenin bond.

Maschio et al. declared that Wnt/β-catenin agonist plays a crucial function in cell differentiation and proliferation during embryogenesis. They specifically demonstrated that there is a relationship between the Wnt/β-catenin pathway and type 2 diabetes. Also, they found that the unregulated Wnt/β-catenin pathway leads to the disruption of beta cells in the early phase of diabetes (40).

Conclusion

Our findings indicated that the PLA/Cs nanocomposite scaffolds provide a protective and suitable environment for hWJMSCs' growth and viability. Here we showed for the first time that the small molecule SKL2001 as a Wnt/β-catenin pathway agonist could induce differentiation of hWJMSCs into DE cells. Also, our results showed that the treatment of hWJMSCs with SKL2001 combined with nZnO had a synergistic effect on DE cell induction. It has been suggested that we can provide an efficient method with the functional differentiation of DE cells via combining a suitable scaffold with essential supplements and a reliable cellular source. These results be used for further differentiation into pancreatic and hepatocytes cells.

Acknowledgments

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Authors' Contributions

E.H.; Contributed to conception, design, all experimental work, and were responsible for overall supervision. S.T.; Contributed to data and statistical analysis, interpretation of data, and drafted the manuscript. All authors read and approved the final manuscript.

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