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Enhanced Differentiation of Wharton's Jelly-Derived Mesenchymal Stem Cells in Insulin-Producing Cells by the Extract of *Nigella sativa* Seeds

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Abstract

Background: With regards to the high potential of medicinal plants in the production of biopharmaceuticals, one can rely on the promising prospect of insulin production via plant resources.
Objectives: This study was conducted with the aim of using plant extract for insulin-producing cells.
Methods: This was a quasi-experimental study using critical case sampling. Six samples were gathered from the umbilical cord (Wharton's jelly) in a governmental university affiliated hospital, Sari, Iran in 2017 after successful isolation of mesenchymal stem cells. Initially, *Nigella sativa* seeds extraction was performed to prepare the extract for cellular differentiation. Next, dithizone (DTZ) staining was used to evaluate insulin production, and insulin level was examined by the enzyme-linked immunosorbent assay (ELISA). Data were analyzed was analyzed with the SPSS version 16 software using independent sample t-test.
Results: The mean of the amount of insulin secretion was 92.33 ± 5.13 ng/ml for the intervention and 0.33 ± 0.15 ng/ml for the control group. The results showed that there was a significant difference in the average insulin in the culture obtained from *Nigella sativa* seeds between control and intervention groups ($P = 0.001$). In addition, via the ELISA kit and specific dithizone staining, insulin-producing cells were proven.
Conclusions: In this regard, it could be concluded that the extract of *Nigella sativa* seeds was capable of including differentiation of mesenchymal stem cells derived from Wharton's jelly to Insulin-Producing Cells.

Keywords: Diabetes Mellitus, Insulin, Mesenchymal Stem Cell, *Nigella sativa*

1. Background

Diabetes mellitus is the most common metabolic disorder around the world (1). According to the international diabetes federation (IDF), it is estimated that 382 million people worldwide have diabetes (2-4). Furthermore, umbilical cord and Wharton's jelly (the vessels out of the umbilical cord) are better sources for Mesenchymal stem cells (MSCs), since they are globally accessible tissues and their gathering does not lead to any ethical harm, as they are ordinarily disposed as human waste (5-7). mesenchymal stem cells are seen as the most important candidate for cellular therapy. This fact is well-documented by the research literature carried out in vitro, under preclinical and clinical conditions (8).

Flavonoids have been studied extensively due to their antioxidant properties. In addition, these compounds have been used to induce cellular differentiation (9-12). In a study conducted on Myrtus leaf extract (2016), it was clarified that the extract of this plant significantly reduced glucose levels in diabetic rats (13).

Nigella sativa (*N. sativa*) belongs to the botanical family of Ranunculaceae and commonly grows in Eastern Europe, Middle East, and Western Asia (14, 15). The most important bioactive compounds of *N. sativa* include alkaloids, thymoquinone, riboflavin, pyridoxine, niacin, folic acid, proteins, and minerals (14, 15).

The differentiation of MSCs to Insulin-Producing Cells (IPCs) is one of the new methods for the treatment of diabetes, particularly diabetes type I (16).

In regards to the ever-increasing demand for the manufacture of insulin, it has led to the development of inexpen-

sive, cost-effective, high-capacity, and high-quality products associated with the manufacturing units. Since there is a high potential of plants to produce biomaterials by using molecular farming, the production and supply of insulin from plants could also be a promising prospect for the human society. Up till now, many researches have been conducted to generate mesenchymal stem cells and identify their differentiation to pancreatic beta cells by using different molecules; however, previous studies have failed to produce complete pancreatic beta cells.

The present study aimed at isolating mesenchymal stem cells from Wharton jelly and differentiating them using the extract of *Nigella sativa* seeds in cellular culture to insulin-producing cells (IPCs). Thus, a new

2. Methods

2.1. Collection of Human Umbilical Cord

The present quasi-experimental study used the critical case sampling method and human umbilical cord samples were collected in thoroughly sterilized conditions from six mothers after delivery/childbirth at the obstetrical department of Imam educational and therapeutic hospital in Sari city, North of Iran in 2017. Since the umbilical cord is a waste, an informed consent was taken from all six mothers with caesarian delivery.

2.2. Statistical Analysis

The mean and SD were calculated for amount of insulin secretion in the intervention and control group. The normality of groups was examined. The t-test was applied for comparison of the amount of insulin secretion between the intervention and control groups. The homogeneity of variance between the two groups was examined with Levene's test for equality of variance. To estimate the parameter and significance levels more accurately, the bootstrap technique and a sample size of 1500 was used. All data were entered and analyzed in the SPSS statistics for Windows, version 16.0, (SPSS Inc. Chicago, ILL, USA) software. The significance level was predetermined at P = 0.001.

2.3. Wharton's Jelly Culture

First, all clots and arteries of umbilical cord were isolated under laminar hood via a 10-mm plate. Then, the gelatinous substance within the umbilical cord, known as Wharton jelly, was cut to small pieces mechanically and rinsed several times with phosphorous buffered saline (PBS) at 1250 RPM for 5 minutes. For lysing the remaining RBC, 2 mL of hypertonic chloride and ammonium (USA, Pharmin Gen) was added to each sample and was rinsed after 10 minutes. The cell pellet included cell separations from the placenta tissue transferred to a T75 Flask and was cultured in high glucose-Dulbecco's modified eagle medium-F12 (HG- DMEM-F12) (USA, Gibco) supplemented with 15 % fetal bovine serum (FBS) and 50 μg/mL penicillin-streptomycin. The plate was incubated in 5% CO_2 at 37°C.

2.4. Mesenchymal Stem Cells Isolated from Other Umbilical Cord Cells

The medium was changed after 24 hours and the suspended particles (non-mesenchymal cells) migrated from the medium and the mesenchymal cells adhered to the flask and turned to spindle-like shapes. When the confluency of the cells reached 80% to 90%, the mesenchymal cells were harvested using 0.25% trypsin, including 1 ml/M EDTA (Sigma, USA). The suspension was centrifuged at 1250 RPM for 5 minutes. The cell pellet was rinsed twice and centrifuged. Finally, it was filtered using a grade 70 micron filter. In order to approve MSCs' identity, positive markers of stem cell surface, i.e. CD105, CD90, CD44, and negative markers, i.e. CD34 and HLA-DR, were evaluated by flow cytometry. Conjugating related antibodies, Fluor chromes FITC, PE, and PerCP were also applied to control Isotypes PerCP, PE, and IgG1-FITC. Additionally, WJ-MSCs were cultured in appropriate medium to evaluate the differentiation capacity of MSCs towards the adipocytes, osteocytes, and chondrocytes. Subject to the confirmation of adipocytes differentiation, the cells were stained with oil-red-O (Sigma, USA). In contrast, for the differentiation of osteocytes, the differentiated cells were stained with alizarin red and thereof calcium deposition was confirmed. For the differentiation of chondrocyte, cells were stained with Alcian blue.

2.5. The Preparation Method of Plant Extraction

The seeds of *Nigella sativa*, which were purchased from a local market, were identified at the herbarium of pharmacy school, Mazandaran University of Medical Sciences, Iran. The hydro-alcoholic extract was prepared as described previously (17).

Powdered seeds (100 g) of *N. sativa*, including 355 meshes were milled and sieved, then incubated in 300 mL of ethyl alcohol 70% in a Soxhlet extractor. The extraction with ethanol was continued on the residue and then concentrated in a rotary evaporator and kept at -20°C before use. In order to obtain the desired concentrations, the extract rates of 0.5, 1, and 2 g were dissolved in 1000-mL of drinking water, respectively (18). Fifteen grams of the product was added to 250 mL of solvent (ethanol-water 1 to 7) from this plant and placed in water at 100°C for 40 minutes. Then the extract was dilated with several millipore of filter paper. The extract was acidified by acidic chloride

and stirred by the range of 100 mL with ethyl acetate three times in each phase. The ethyl acetate extract was dried on a rotary evaporator (EYELA Japanese N1000SW220V-IP) at 40°C. Finally, the flavonoid extract of the seeds of *Nigella sativa* was exposed to -20°C storage (19, 20).

2.6. The Differentiation of Mesenchymal Stem Cells to Insulin Producing Cells (IPCs) by Extract of Nigella Sativa Seeds

The resulting cell source was placed in an incubator for 6 - 10 minutes and then differentiated by using *Nigella sativa* seeds in the cell culture after approximately day 28 of the production of the insulin producing cells (IPCs). In order to optimize the extract of the plant determined in different dilutions, this study examined the culture in order to obtain the optimum dilution. In the present study, 300 μg/mL of plant extract was used in culture at a ratio of 1 to 10 (plant extract to culture).

The culture of differentiated cells was tested by using a standard enzyme linked immunosorbent assay (ELISA) Kit (Demeditec Diagnostics GmbH, Germany-DE2935). Insulin infusion was investigated in the extract of *Nigella sativa* seeds.

2.7. Differentiation of Mesenchymal Stem Cells to Insulin-Producing Cells

Following isolation and confirmation of MSCs, the optimization of *Nigella sativa* at various dilutions was checked in the culture and the optimum dilution was obtained. Then the induction of treatment was conducted during 3 to 5 days.

2.8. Dithizone Staining

Fifty milligrams of dithizone (Merck) was dissolved in 5 mL of dimethyl sulphoxide (Sigma, USA) and the final solution was kept as a stock at -20°C. Then, 10 μM of dithizone solutions were added to the cells and they were incubated for 15 minutes. Insulin-producing cells are cells, which turn red as a result of staining.

3. Results

The mean of the amount of insulin secretion was 92.33 $\pm$ 5.13 in the intervention and 0.33 $\pm$ 0.15 ng/ml in the control group. Shapiro-wilk test of normality (Table 1) showed that for intervention and control groups, the dependent variable of the amount of insulin secretion was normally distributed (P = 0.001). The result of Levene's test showed that the variance between the 2 groups weren't equal and independent sample t-test was performed with equal variance not assumed (Table 2). The result of the t-test showed that there was a significant difference between the mean of the amount of insulin secretion between the 2 groups (P = 0.001).

Table 1. Test of Normality for Amount of Insulin Secretion in Intervention and Control Groups

Variables	Shapiro-Wilk Test of Normality		
	Statistics	df	Sig.
Intervention	0.95	3	0.57
Control	0.96	3	0.64

Abbreviation: df, Degree of Freedom.

Table 2. Independent Sample T-Test for Evaluation of Mean Difference of Amount of Insulin Secretion Between the Two Groups

Variables	Value
t-value	31.04
df	2
Sig. (2-tailed)	0.001
Mean difference	92
Std. error difference	2.96
95% confidence interval of difference	
Lower	79.26
Upper	104.73

The result of the bootstrap technique used in this study showed that estimation of parameter was exact and had precision (Table 3).

Table 3. Bootstrap for Independent Sample Test

Variables	Value
Bias	0.1
Std. error	2.76
Mean difference	92
95% confidence interval of difference	
Lower	79.26
Upper	104.73

In the current study, Wharton Jelly's mesenchymal stem cells (WJ-MSCs) were differentiated to insulin-producing cells (IPCs). For this purpose, after isolation and differentiation of WJ-MSCs to IPCs, a morphological and molecular analysis was performed.

3.1. Morphological and Phenotypical Characterization of Wharton Jelly's Mesenchymal Stem Cells

At the end of the expansion phase, the cultured WJ-MSCs became homogenous with spindle-shape and akin to fibroblast-like to be arranged in monolayers (Figure 1).

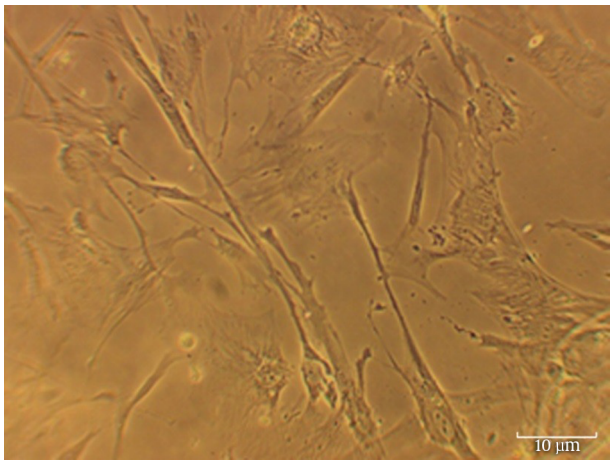


Figure 1. The Wharton's Jelly Mesenchymal Cells Were Grown from the Edge of Tissue Explants and Homogenous Population of Fibroblast-Like Mesenchymal Stem Cells at Passage 2 (× 100)

3.2. Immunophenotyping of Wharton Jelly's Mesenchymal Stem Cells

The immunophenotypes of WJ-MSCs were analyzed by flow cytometry. The results showed that WJ-MSCs were positive for CD90 (99.30%), CD105 (96.37%) and CD44 (83.11%), and were negative for hematopoietic marker CD34 (0.72%) and HLA-DR (0.85%) (Figure 2).

3.3. Dithizone (DTZ) Staining

In order to evaluate insulin production in clusters at day 21 of differentiation, the researchers used dithizone (DTZ) staining. The stock solution was prepared as previously described (16), thereby 50 mg of DTZ was completely dissolved (Sigma , USA) in 5 mL of dimethyl sulfoxide (DMSO, Sigma, USA). The solution was stored at -20°C in a dark bottle. In the staining phase, the working solution was prepared by diluting the stock solution (pH 7.8) at a ratio of 1:100 in culture medium. Three milliliters of the working DTZ solution was added to each one and the plates were incubated for 30 minutes at 37°C. The plates were then rinsed 3 times with PBS and crimson-red differentiated clusters; therefore, they were examined with an inverted phase contrast microscope (Figure 3).

3.4. Enzyme Linked Immunosorbent Assay Test

In order to determine the amount of insulin secreted by the differentiated cells after day 28, the cell culture medium was examined using the ELISA kit technique. The results showed that the differentiated cells, in comparison with the undifferentiated cells, secreted insulin. The researchers could successfully differentiate MSCs to insulin-producing cells. However, there was a significant difference between the level of insulin in the supernatant of the culture obtained from the extract of *Nigella sativa* seeds in comparison with the negative control group (P = 0.001). The insulin secretion level of 92.3 u/mL was obtained by the extract of *Nigella sativa* seeds.

4. Discussion

Medicinal herbs are effectual in the treatment of many diseases, including diabetes (10, 21).

In the present study, after successful isolation of mesenchymal stem cells (MSCs) and confirmation by fixing fibroblastic cells to the flask floor and checking the CD, the positive and negative markers were determined by flow cytometry in the next step in order to differentiate to pancreatic beta cells by using the extract of *Nigella sativa* seeds. By optimizing its concentration at different dilutions in culture, the researchers were able to obtain the proper production of insulin cells (IPCs). The significant signs of the pancreatic beta cells were observed in the insulin secretion. In the present study, the amount of insulin secretion by using the extract of *Nigella sativa* seeds was determined to differentiate the mesenchymal stem cells (MSCs) and insulin producing cells (IPCs) using the ELISA Kit; the level of insulin secretion was 92.3 u/mL, while in the negative control group, there was a very low level of insulin. Differentiation to pancreatic β-cells was achieved with the extract of *Nigella sativa* seeds and its concentration was optimized by a variety of dilutions in the culture. Then, this study succeeded to produce IPCs. It was examined by ELISA and DTZ exclusive dyeing.

In summary, the extract of *Nigella sativa* seeds could induce the differentiation of mesenchymal stem cells derived from Wharton jelly to IPCs with an appropriate insulin release.

It is suggested that in order to examine the performance of the IPCs obtained in this research by the extract of *Nigella sativa* seeds in the therapeutic procedures, cell therapy and transplantation of these cells in animals with diabetes and the evaluation of glucose level improvement and its effectiveness should be investigated in future work compared with common insulin. In respect to its role, it is an essential element in the effectiveness of the internal environment, which could be distinguished from induced treatments outside of the body. Due to the advantage of the role of the bodily internal environment, which can be applied in cellular differentiation from bodily external environments, it is an important point. Also in the future,

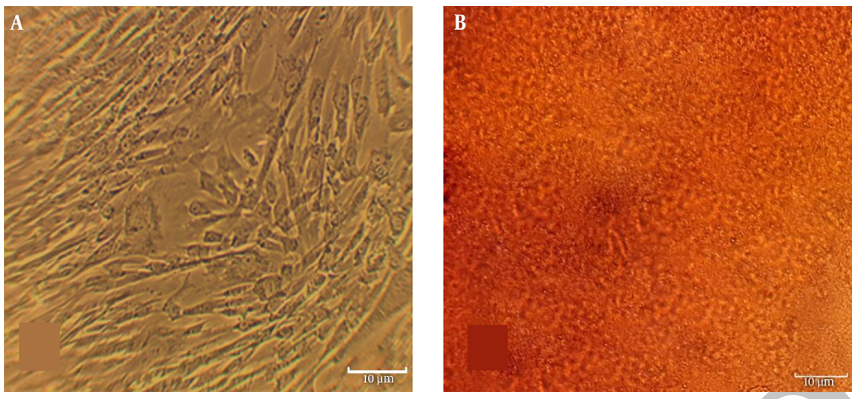
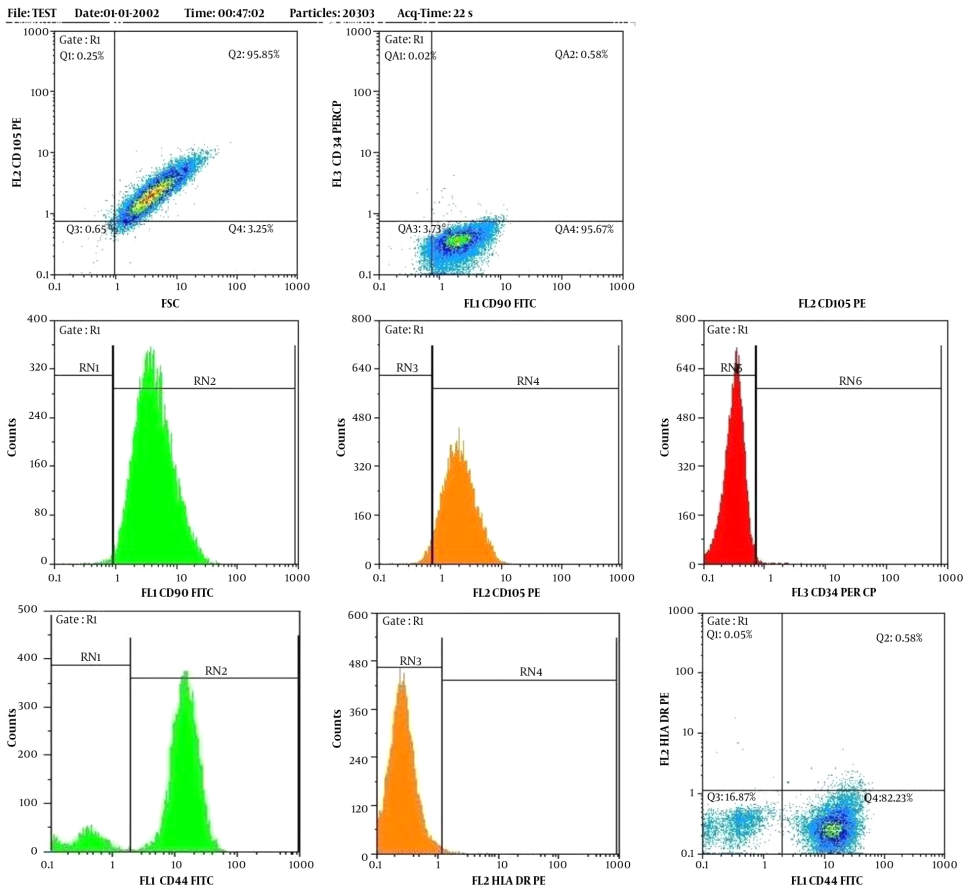


Figure 2. Flow Cytometry Analysis of Mesenchymal Stem Cells Derived from Wharton's Jelly

Figure 3. Dithizone (DTZ) Staining: Observed with Inverted Microscope and Magnified × 100



A, Untreated cells (negative control); B, Intervention cells with insulin granules come in red brick.

the focus of the research should be on anti-diabetic mechanisms of *Nigella sativa* seeds.

4.1. Conclusion

According to the present study, it could be concluded that *Nigella sativa* seeds have an effect on the process of dif-

ferentiation of the mesenchymal stem cells derived from Wharton's jelly (WJ-MSCs) toward insulin producing cells (IPCs).

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Footnotes

Authors' Contribution: All authors read and approved the final manuscript.

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