Iran Red Crescent Med J. 2022 July; 24(7):e1419 doi: 10.32592/ircmj.2022.24.7.1419
Published online 2022 July 20 **Original Article**

Enhancement of the Differentiation Potential of Adipose Tissue-Derived Mesenchymal Stem Cells into Insulin-Producing Cells Using Flavonoid Compounds in Diabetic Rats

Sima Mashayekh[1], Mahnaz Azarnia[2], Esmail Fattahi[1,*] and Reza Moghadasali[3]

[1] Department of Biology, Ayatolla Amoli Branch, Islamic Azad University, Amol, Iran
[2] Department of Biology, Kharazmi University, Tehran, Iran
[3] Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

* ***Corresponding author:*** Esmail Fattahi, Department of Biology, Ayatolla Amoli Branch of Islamic Azad University, Amol, Iran. Tel: +989113255311; Email: esmail_fattahy@yahoo.com

Received 2021 August 17; **Revised** 2021 October 13; **Accepted** 2022 June 08.

Abstract

Background: Differentiation of stem cells into pancreatic beta cells is a global challenge in reconstructive medicine for the treatment of diabetes. Studies have shown that compounds derived from walnut green skin can differentiate stem cells into beta cells. Flavonoid compounds appear to be the main cause of this differentiation.
Objectives: This study examined the effect of flavonoid compounds in walnut green skin on the differentiation of pancreatic beta cells.
Methods: The present study conducted the differentiation of adipose tissue-derived mesenchymal stem cells (AD-MSCs) into insulin-producing cells under flavonoid extract at doses of 50 and 100 mg/ml for three weeks. For diabetic rats, streptozotocin was injected intraperitoneally at a dose of 60 mg/kg. To evaluate cell differentiation, this study utilized morphology, dithizone (DTZ) staining, insulin-proinsulin production, insulin beta-receptor by the immunofluorescence method, and insulin measurement by the ELISA method. Serum glucose, cholesterol, and lipids were measured by enzymatic colorimetric, enzyme kit, and autoanalyzer, respectively. The expression of pancreatic cell-specific genes, including Pancreatic and Duodenal Homeobox 1 (Pdx1) and Neurogenin-3 (Ngn3), was also assessed by real-time PCR.
Results: Differentiated cells transformed from spindle-shaped to circular or clustered cells under the influence of flavonoid compounds with their specific DTZ staining being positive. The expression of insulin-proinsulin markers, beta receptors, and insulin secretion was also confirmed. Decreased blood lipids and glucose, as well as increased expression of Pdx1 and Ngn3 insulin-producing genes, were significant in the treatment groups ($P<0.05$).
Conclusion: The findings showed that flavonoid compounds could effectively induce the differentiation of AD-MSCs into insulin-producing cells.

Keywords: Adipose-derived mesenchymal stem cells, Beta-pancreatic cells, Differentiation, Flavonoids, Walnut green skin

1. Background

Diabetes is the most common metabolic disease with an estimated 300 million sufferers worldwide by 2025. It is a chronic and progressive disorder that results from insulin resistance in peripheral tissues, such as muscles, liver, and adipose tissue, with a relative violation of insulin secretion (1).

Research has shown that diabetic patients develop long-term disorders, such as neuropathy, nephropathy, and cardiovascular disease. To maintain pancreatic beta-cell mass, there should be a balance between neogenesis, proliferation, and apoptosis. Identification of pancreatic stem cells and mechanisms that control their differentiation and proliferation are of great importance for the development of new methods in the treatment of diabetes (2).

In recent years, the use of stem cells in the treatment of diabetes has received special attention. Studies with a history of nearly 50 years have shown that stem cells can divide frequently and remain undifferentiated, but can differentiate into different cell types if there is a specific stimulus in the environment. Therefore, attempts have been made to use stem cells to replace malfunctioning and malignant cells at the level of laboratory studies, as well as in a small number of clinical trials (3,4).

To obtain stem cells that could differentiate into beta cells, adult pancreatic stem cells and then, bone marrow, liver, as well as small intestine, were examined, leading to relatively good findings in animal experiments. Although limited, human experiments showed that differentiated beta cells could not completely cure patients and even caused numerous complications for them. These cells pose some challenges, including technical problems, the short lifespan of beta cells, the production of teratomas with malignant capacity, and preventing the excretion of transplanted cells (5).

Due to the limited number and ability of mature stem cells to proliferate and the difficulty of isolating them, scientists in the last decade have focused on the possibility of using adipose tissue-derived mesenchymal stem cells (AD-MSCs) since they grow faster, compared to umbilical cord and bone blood stem cells. The most critical advantage of AD-MSCs is that they can be easily obtained from

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patients. Additionally, simple culturing, rapid proliferation, and maintaining pluralistic characteristics for a long time after several passages show that these cells can be a good choice for therapeutic purposes (6).

Walnut is a very important and valuable tree found in many parts of the world. It belongs to the angiosperms, the subclass of dicotyledons, the suborder of Amentales, the family of Juglandaceae, and the genus of Jiglans with shaft fruit. All parts of walnut are used for different purposes; its leaves for treating rheumatic pain, fever, diabetes, and anemia, its roots for diabetes, its flowers for the treatment of malaria and rheumatic pain, and its kernel for the treatment of kidney stones. Moreover, due to the presence of Omega-3, it is used as a tonic for the senses and a sexual enhancer (7). The green skin of the fruit also contains many medicinal compounds and has a beneficial effect on human health. Thirteen phenolic compounds have been identified in walnut, including hydroxycinnamic acids (chlorogenic acid, caffeic acid, ferulic acid, and sinapic acid) hydroxybenzoic acids (gallic acid, alginic acid, protocatechuic acid, syringic acid, and vinylic acid), flavonoids (catechin, epicatechin, and myricetin), as well as Juglans (8).

Flavonoids in walnuts are a group of polyphenolic compounds of plant origin and a low molecular weight that are naturally distributed in fruits, leaves, and other plant organs. Flavonoids have antioxidant, anti-inflammatory, anti-aging, anti-cancer, and anti-diabetic properties and protect the body against coronary heart disease, as well as metabolic disorders (9).

According to the findings of previous studies, hydroalcoholic extract of walnut flowers increases the production of insulin due to its antioxidants, including flavonoid compounds, by producing beta cells and lowering blood sugar (10).

2. Objectives

Despite the beneficial effects of walnut on the treatment of diabetes, no research has investigated flavonoid compounds of green walnut skin to differentiate AD-MSCs to insulin-producing cells in pancreatic tissue. This study aimed to increase the effectiveness of AD-MSCs assisted cell therapy in streptozotocin-induced diabetic rats and evaluate the expression of some central genes in pancreatic transcription and development, including Pancreatic and Duodenal Homeobox 1 (Pdx1) and Neurogenin-3 (Ngn3).

3. Methods

In this experimental study, 30 adult male rats of the Wistar breed with an approximate weight of 200±20 g were purchased and transferred to the animal room in the Kharazmi University of Tehran, Tehran, Iran. The animals were kept in standard cages (Razi factory, Iran) under the same conditions at 21±2°C and with an optical cycle of 12 h of light and 12 h of darkness, as well as the same amount of water and food until the experiment. Ethical rules for keeping and working with animals were followed at all stages of the experiment. The rats were then divided into six groups of five: control or healthy rats (G1), diabetic rats with streptozotocin (G2), diabetic rats treated with differentiated beta cells under the influence of flavonoids of walnut green skin (treatment group 1 or G3/dose 50 mg/kg), diabetic rats treated with differentiated beta cells under the influence of flavonoids of walnut green skin (treatment group 2 or G4/dose 100 mg/kg), diabetic rats treated simultaneously with the injection of flavonoids and beta differentiated cells from AD-MSCs (treatment group 3 or G5/dose 50mg/kg), and diabetic rats treated simultaneously with the injection of flavonoids and beta differentiated cells from AD-MSCs (treatment group 4 or G6/dose 100 mg/kg).

For diabetic rats, streptozotocin (ZellBio GmbH, Germany) was used as a single dose and intraperitoneally at a rate of 60 mg/kg dissolved in cold saline. To ensure that the animals were diabetic, blood was drawn from their tails 72 h after the injection. The blood glucose level was measured using a glucometer (MDSS GmbH, Germany). Diabetes mellitus was considered higher than 250 mg/dl of blood sugar (Figure 1).

3.1. Collecting and Extracting the Medicinal Plant of Green Walnut Skin

In the fall, when the fruit was ripe, the walnuts in Valian village (Iran) were harvested by hand randomly without any damage to the green skin, and they were completely healthy. In the next step, they were peeled and dried in an environment away from light and at room temperature. After complete drying, the green skins were carefully crushed with an electric shredder (Grinder, Japan) and were sieved to even out the particle size.

The extract of this plant was prepared by extracting pure methanol at a temperature of 60 to 70°C, and a rotary evaporator (Wiggens GmbH, Germany) was then used to separate the extract and methanol mixture. With the help of distilled water at 80°C and pure butanol extraction buffer at 80 to 100°C, flavonoids were purified from the remaining compounds in the extract, and pure methanol was then added for a long-lasting effect.

3.2. Determination of Flavonoid Extract Concentration Using Standard Catechin Solution

Standard solutions of catechin flavonoids were used to determine flavonoid concentration. The adsorption rate of standard catechin solutions at 510

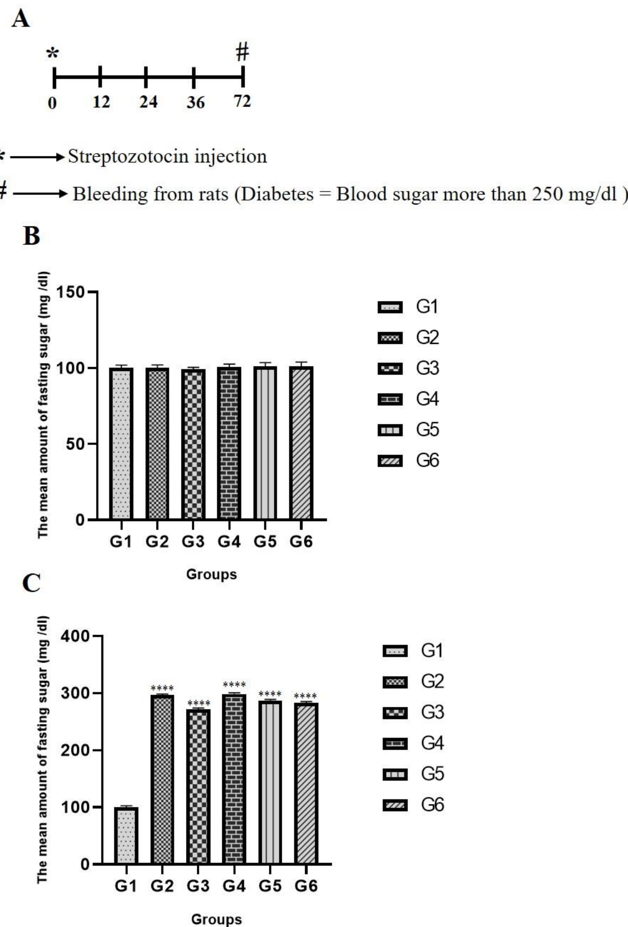

Figure 1. Development of a diabetic model using Streptozotocin in rats. A: Streptozotocin was injected and diabetic rats were checked after 72 h. B: Fasting blood sugar was measured before injecting Streptozotocin. C: Fasting blood sugar was measured after injecting Streptozotocin.

nm was measured by a spectrophotometer (Hitachi, Japan). The concentration of the extract was obtained by drawing a standard curve based on the adsorption rate of catechin solutions. In the present study, flavonoid herbal medicine was used with concentrations of 100 mg/kg and 50 mg/kg.

3.3. Extraction, Proliferation, and Purification of AD-MSC From Rats

After being killed, the rats were prepared to separate adipose tissue. By slightly cutting the peritoneal membrane, adipose tissue was separated from the lower part of the abdomen and digested by type 1 collagenase (ZellBio GmbH, Germany) at a concentration of 0.1 g. In the next step, after centrifugation, the deposition of mesenchymal stem cells (MSCs) containing 10% Fasting Blood Sugar (Sigma-Aldrich, St Louis, USA), Dulbecco's Modified Eagle Medium High Glucose (GIBCO, Thermo Fisher Scientific, USA) (Sigma-Aldrich, St Louis, USA), and mML2, glutamine, penicillin, as well as streptomycin (*in vitro* gen) (Sigma-Aldrich, St Louis, USA), were homogenized and cultured in 75 cm^2 flask. They were incubated (Memmert, Germany) at 37°C with 5% CO_2, and after 24 h, the culture medium was changed, and the MSCs were gradually purified during subsequent passages.

3.4. Identification and Proof of the Presence of AD-MSCs with Flow Cytometry Technique

After the isolation and culturing of adipose tissue cells, the presence of stem cells must be proven. For this purpose, the cells cultured in Passage II were used after summarizing and isolating to study the markers of MSCs and confirm them by flow cytometry. After the second cell passage, the cells were separated from the bottom of the flask by trypsin, the cell suspension was counted, and $1×10^6$ cells were added to each number. The vials were blocked for 30 min with 1 ml of 1% BBSA-peripheral blood smear (PBS) solution and centrifuged at 300× g for cell suspension for 5 min at appropriate concentrations of CD_{34}-PE (BD Pharmingen, clonc:562), CD_{45}-FITCL (Pharmingen, clone:2D1), CD_{90}-Fitcl (Pharmingen, clone:5E10), CD_{105}-FITCL (Pharmingen, clone:266), LgG_1-FITC (BD Phermingen, clone:Aopa31c), and IgG_1-PE (BD Pharmingen, clone:Mopc-21c) antibodies. After incubating the

cells, they were washed and centrifuged (universal 320, Iran) again with PBS for 1 h under dark and 4°C conditions. In the last step, the cell precipitation was suspended with 200 µl FACS buffer and kept at a temperature of 2-3°C until studying and reading with a flowmeter (Biorad, USA) device. Finally, data analysis and graph drawing were conducted using melting software (version 2.8).

3.5. Differentiation of AD-MSCs into Insulin-Producing Cells

After the isolation of AD-MSCs, culture, proliferation, identification, and confirmation of adipose tissue stem cells by flow cytometry, differentiation of them into islet cells was performed *in vitro*. To induce differentiation, 10^6 cells were cultured in 96-well plates, and after 24 h, they were treated with flavonoid extract at doses of 100 and 50mg/ml for three weeks. During this period, changes in these cells were examined under a contrast phase microscope.

3.6. Investigation of Differentiation Cells Using Dithizone-Specific Staining

Dithizone (DTZ, Merck, CaNtik 6433055, USA) staining was used to differentiate at this stage. The DTZ-specific staining is a method that specifically identifies insulin-producing cells. In total, 80 mg of DTZ powder was first dissolved in 10 ml, and dimethyl sulfoxide was reduced to 50 ml by PBS. After filtration, 500 µl of DTZ was poured on the cells for each milliliter of culture. It was placed in an incubator for 24 h. After coloring the DTZ, 40 µl of cell suspension was placed on the neobar slide, and white cells (undifferentiated cells), as well as red cells (differentiated cells), were numbered using light microscopy (Olympus, Japan).

3.7. Investigating the Expression of Specific Beta-Cell Markers by Immunofluorescence Method

To evaluate the expression of specific markers of insulin-producing cells in AD-MSCs by immunofluorescence method, primary anti-insulin, pro-insulin, and anti-insulin beta-repair antibodies were used. Differentiated cells were stabilized in the laboratory for 20 min after being washed twice with PBS cells with 4% Paraformaldehyde. Increased permeability with Triton X-100 3% and normal serum 10% was performed in an incubator for 30 min. Afterward, PBS was washed twice and each time for 5 min. The cells were then incubated with the primary anti-insulin antibody, proinsulin, or the primary anti-beta insulin antibody for 2 h. The anti-mouse IgG antibody attached to fluorescence isothiocyanate (FITC) prepared in goats was used as a secondary antibody. The FITC turns green against UV rays. The samples were then glued with 70% glycerol, insulated with nail polish, and examined with a fluorescence microscope (Olympus, Japan).

3.8. ELISA Test

The insulin immunoradiometric test is a type of test sandwich to determine insulin levels produced by cells distinct from ELISA (Kit, Czech Insulin, IRMA). The test is performed on coated tubes. The insulin-containing serum is added to these tubes, and then, insulin binds to antibodies. Secondary antibodies that bind to I_{125} are added to the head. In this case, insulin is placed between two antibodies and becomes a sandwich. Radioactivity is then measured by a gamma counter and expressed by a standard curve device.

3.9. Transplantation of Beta Distinguished Cells in Diabetic Animals

After the induction of anesthesia by intraperitoneal injection, as well as a mixture of 10% ketamine at a dose of 50 mg/kg and 2% xylazine at a dose of 10 mg/kg, the rat rail was kept in warm water for 1 min to dilate the tail arteries, exposing the tail vein. Afterward, using PBS 5 ml insulin serum, 10^6 differentiated stem cells were injected into the veins of rats in treatment groups 1, 2, 3, and 4. In addition to injecting differentiated beta stem cells into group therapy for 20 days, flavonoids at doses of 50 mg/kg were injected into group 3, and those at doses of 100 mg/kg were injected into group 4.

Finally, 24 h after the last administration and anesthetization of the rats with chloroform, their breasts were dissected, and blood was drawn from their hearts using a syringe. After the centrifugation of blood samples, a sufficient amount of serum was prepared and kept in the freezer (Samsung RL72 SP, South Korean) at −20°C until checking the variables.

The next step was to use a surgical procedure to remove the pancreatic tissue. Small pieces measuring 0.5×0.5 cm were removed from the pancreas and immersed in 1.5 microscopes containing one ml of Ribonucleic acid (RNA) later. Afterward, the microtube and its contents were first stored in a freezer at −20°C for 24 h and then, transferred to a −70°C freezer (Arctiko, Denmark).

3.10. Steps to Perform Real-Time PCR

For the extraction of RNA, the Column kit (Yekta Tajhiz Azma, Iran) was used and all the steps were performed according to the kit protocol and using the solutions of the kit and spin column. A spectrophotometer (NanoDrop, Hitachi, Japan) was used to evaluate the purity and RNA concentration with an absorption rate of 260 to 280.

After ensuring that the extracted RNA was pure, cDNA synthesis was performed using the kit (Takta Tajhizat Azna, Iran), and all the steps were performed according to the kit instructions, as well as using the materials and solutions in the kit under the hood (Micro flow, England) and on ice.

The primers used in this study were designed based on the corresponding gene sequences obtained

Table 1. Sequence of the upper (F) and lower (R) primers of Pdx1 and Ngn3 genes

Genes	Sequences of primers	Annealing temperature	Product size (bp)
Ngn3	F:5′ TAACCAGGATTCGCACAGT3′ R:5′ GAGTCACCAGGAAGTATG3′	58	166
Pdx1	F:5′ GGTGCCAGAGTTCAGTGCT3′ R: 5′ GAGGTGGTGGTGGAGGTG3′	62	189
GAPDA	F:5′TCAACGGCACAGTCAAGG3′ R:5′CTCAGCACCAGCATCACC3′	57	113

Ngn3: Neurogenin-3
Pdx1: Pancreatic and Duodenal Homeobox 1

from the National Center for Biotechnology Information site using Allele ID6 software and then synthesized based on Pdx1 and Ngn3 gene sequences. The GAPDH gene was used as an internal control. The relevant sequence is given in Table 1.

To perform real-time PCR (RT-PCR), the SYBR green kit (Yekta Tajhiz Azma, Iran) was utilized. All steps were performed in standard conditions and according to the kit protocol. The reaction mixture after preparation was affected by the temperature program mentioned in the Kit Protocol in the Real-Time device (Qiagen 5PLEX, USA). All reactions were repeated three times, and RT-PCR Real-Time software was used to confirm the genes related to the curve analysis of the melting PCR product.

3.11 Statistical Analysis

The following factors were measured: glucose levels, triglycerides by enzymatic pigmentation using oxidase phosphate glycerol, cholesterol by enzymatic kit and autoanalyzer (Pars Azmoun-Iran), high-density lipoprotein cholesterol (HDL-C) by enzyme kit (Pars Azmoun-Iran), and low-density lipoprotein cholesterol (LDL-C) serum by kit (Pars Azmoun-Iran) using auto analysis machine. Data were analyzed using the SPSS software and the LSD-Tukey variance statistical analysis test. The results are presented as mean±SD on the graph with $P<0.05$ values considered significant.

4. Results

4.1. Morphological Changes

The AD-MSCs were obtained from adult male rats of the Wistar breed. These cells can adhere to the plastic container and form a colony in laboratory conditions. At the beginning of the extraction, these cells were spherical and after a week, they became elongated spindles. The appearance of these cells is similar to fibroblast cells. The difference is that fibroblast cells cannot tolerate successive passages and cannot also differentiate into different lineages (Figure 2).

During the differentiation, morphological changes of MSCs were investigated by invert microscopy. Before differentiation, these cells had a morphology similar to fibroblasts (spindle-shaped with short cytoplasmic exudates). After differentiation, however, their morphology changed significantly and was observed in the form of cellular or cluster masses.

4.2. Evaluation of Flow Cytometry Results of AD-MSCs

In this study, after extracting the MSCs of adipose tissue, to confirm these cells, the expression of surface receptors of MSCs derived from adipose tissue (CD_{34}, CD_{45}, CD_{90}, and CD_{105}) was investigated using flow cytometry (Figure 3).

4.3. Dithizone Dedicated Staining:

Cells differentiated with flavonoids were specifically stained with DTZ. Insulin-producing cells derived from AD-MSCs were reddened using DTZ staining. In the control group, in which cells were not treated with flavonoids, there was a limited number of red cells indicating the spontaneous differentiation of some cells into pancreatic beta cells.

In vitro, MSCs were able to differentiate spontaneously into insulin-producing cells; however, based on the findings, the percentage of differentiated cells that responded positively to DTZ was significantly different in the control group from that in the other groups. Therefore, green walnut skin flavonoid extract has a positive effect on inducing differentiation of MSCs into insulin-producing cells. The highest percentage of insulin-producing cells derived from MSCs was obtained by flavonoids at a dose of 100 mg/ml. In this statistical test, $P<0.05$ was considered significant (Figure 4).

4.4. Immunofluorescence Examination

In the above method, to study the expression of the specific gene of insulin-producing cells, the primary antibodies against insulin-proinsulin and anti-insulin beta-receptor antibodies were used separately. Insulin-producing cells from flavonoid compounds were able to express the insulin-proinsulin protein and the beta-insulin receptor (Figure 4).

4.5. ELISA Analysis

The mean of three repetition tests of normalized secretion of secreted insulin from supernatant culture medium in the three experimental groups of undifferentiated cells, differentiated cells with a dose

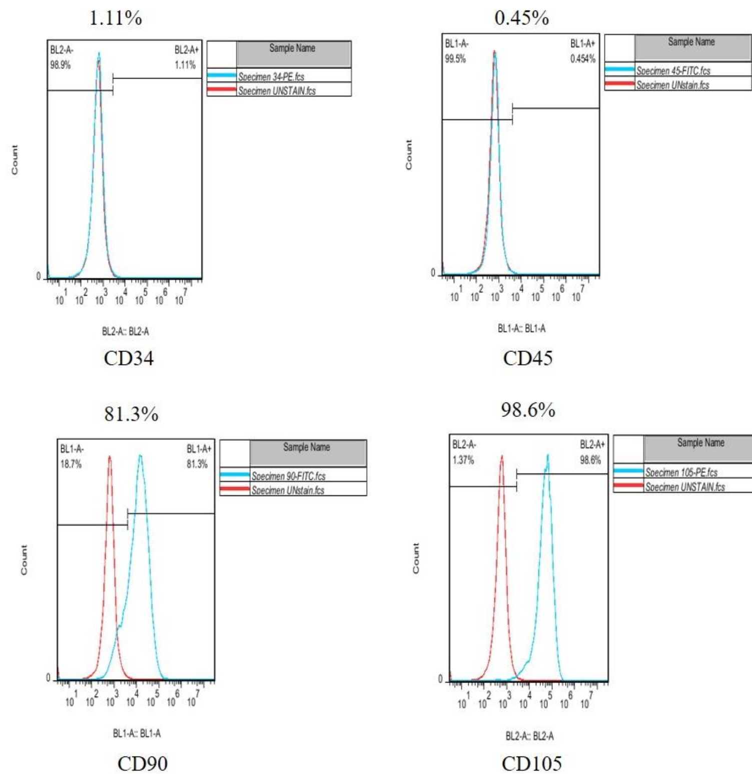

Figure 2. Flucyometry of mesenchymal stem cells. Cells are positive for CD_{90} and CD_{105} markers and negative for CD_{34}, as well as CD_{45} markers.

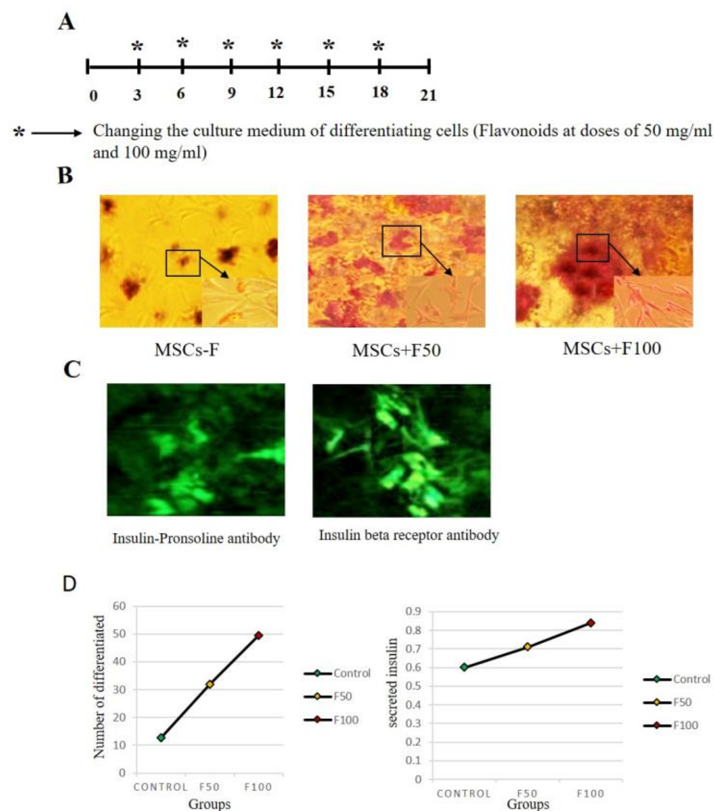

Figure 3. Evaluation of the induction of differentiation of adipose tissue mesenchymal stem cells into pancreatic insulin-producing cells. A: Alteration of differentiating cell culture medium during the differentiation induction period with flavonoid extract. B: Dithizone-specific staining in three different groups. C: Immunofluorescence from insulin-producing cells resulting from differentiation. D: Counting differentiated cells and ELISA.

100 mg/ml at a wavelength of 450 nm were 0.60, 0.71, and 0.84, respectively. The level of insulin produced in flavonoid differentiated cell groups had a significant increase, compared to that in the undifferentiated cell group (P<0.05) (Figure 4).

4.6. Effect of Flavonoids on Blood Glucose Concentration

The mean serum glucose levels in the control and diabetic groups were 106.32±2.40 and 471.02±4.03 mg/dl, respectively. In treatment groups 1, 2, 3, and 4, it was 232±5.21, 223.86±3.75, 205±3.54, and 192.72±2.77 mg/dl, respectively. Glucose levels in the diabetic group showed a significant increase, compared to that in the control group. In the treatment groups, the amount of glucose was significantly reduced, compared to that in the diabetic group (P<0.05).

4.7. Effect of Flavonoids on Blood Lipid Concentrations

Mean serum total cholesterol in the control and diabetic groups was 81.32, 21, 5.21, 101.9, 38, and 38.32 mg/dl, respectively. In treatment groups 1, 2, 3, and 4, it was 94.50±6.67, 89.5±7.52, 83.87±9.82, and 76.25±10.02 mg/dl, respectively. The mean total cholesterol in the diabetic group increased significantly, compared to that in the control group. In treatment groups 3 and 4, the mean total cholesterol showed a significant decrease, compared to that in the diabetic group; however, this decrease was not significant in treatment groups 1 and 2 (P<0.05).

The average serum LDL-C concentration in the diabetic group was 66.48±6.65, which showed a significant increase, compared to 23.05±4.01 in the control group. Additionally, the average serum LDL-C concentration in treatment groups 1, 2, 3, and 4 was 39.04±7.22, 35.21±5.97, 28.21±5.29, and 21.35±4.93, respectively, showing a significant decrease, compared to that in the diabetic group (P<0.05).

The average serum HDL-C in the diabetic group (36.53±5.05) showed a significant decrease, compared to that in the control group (48.74±8.45). The average serum HDL-C concentration in treatment groups 1, 2, 3, and 4 was 45.59±9.48, 49.68±9.21, 51.57±9.79, and 53.8±11.54, respectively, and compared to that in the diabetic group, only treatment group 1 did not show a significant increase (P<0.05).

Plasma triglyceride levels were 107.98±8.17 in the diabetic group, which showed a significant increase, compared to that in the control group (57.02±7.44). Additionally, the average triglyceride concentration in treatment groups 1, 2, 3, and 4 was 78.61±8.41, 75.03±8.42, 69.30±8.51, and 63.51±6.95, respectively, which showed a significant decrease, compared to that in the diabetic group (P<0.05)...

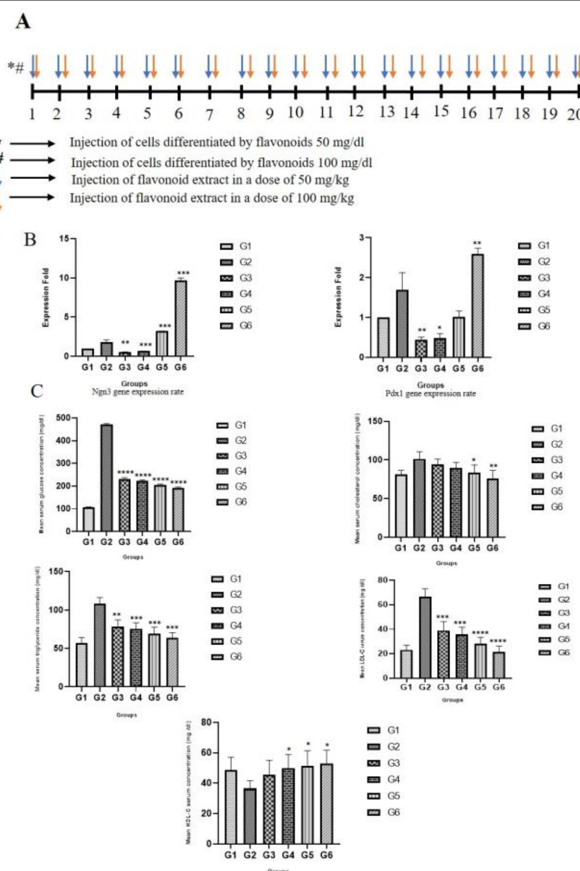

Figure 4. A: Treatment of rats with flavonoid extract and differentiated insulin-producing cells. B: Real-Time PCR. C: Comparison of mean serum blood concentrations in the studied groups.

4.8. Real-time PCR Test

After performing RT-PCR, the cDNA multiplication curve was plotted for specific Ngn3 and Pdx1 genes, and its CT was determined. Statistical analysis of Ngn3 and Pdx1 genes was also performed with the SPSS software, and since the data were normal, the One-Way analysis of variance test (ANOVA) was used.

The expression of this gene was reduced in treatment groups 1 and 2, compared to that in the control and diabetic groups, and this decrease was significant, compared to the control group. In treatment groups 3 and 4, the expression of the above gene increased, compared to that in the control and diabetic groups, and this increase was significant, compared to the control group ($P<0.05$)

The lowest expression of the gene was for treatment group 1, which received differentiated AD-MSCs with a flavonoid dose of 50 mg/ml. The highest rate of gene expression between groups belonged to treatment group 4, which received the differentiated AD-MSCs with a flavonoid dose of 100 mg/ml, including the injection of 100 mg/kg of flavonoids into rats over 20 days.

The expression of this gene was reduced in treatment groups 1, 2, and 3, compared to that in the control and diabetic groups; however, this decrease was not significant in treatment group 3, compared to the control group. In treatment group 4, the expression of the above gene increased, compared to that in the control and diabetic groups; however, it was not significant ($P<0.05$).

Evidence suggests that the expression of this gene has increased in treatment groups 3 and 4, which, in addition to receiving differentiated AD-MSCs, received flavonoid compounds separately (Figure 5)

5. Discussion

Wilson et al. (2013) studied the ability to culture and differentiate AD-MSCs and found that stem cells isolated from adipose tissue could be used as a new and important source for differentiation into different cell types (11). In a 2010 study by Taha, AD-MSCs of 12- to 14-week-old rats were isolated and evaluated by flow cytometry. Both types of neonatal AD-MSCs were isolated from adipose tissue, and AD-MSCs that were passaged several times had the multipotent capacity of differentiating into several cell categories, including bone, fat, and cartilage cell lines. They eventually concluded that adipose tissue had a population of stem cells that appear to be selective cells with high potential for future replacement therapies. In 2010, Carcia et al. injected 1×10^6 stem cells isolated from human adipose tissue into diabetic rats. This study showed a decrease in blood sugar during treatment (12). Young Zhao et al. used umbilical cord stem cells in the treatment of diabetes in 2009 and 2010. Ende et al. also showed the effect of umbilical cord stem cells attached to diabetic rats in lowering blood sugar and glucose (13).

Evidence shows that tannins and polyphenols, especially flavonoids, have anti-diabetic effects. Many plant compounds, including polyphenols, have antioxidant properties. Polyphenolic compounds, especially flavonoids, have a protective effect against damage caused by liver toxins and free radicals (14).

Studies on the plant alkaloid conophyline have shown that this alkaloid induces the differentiation of pancreatic endocrine cells in pig infants into insulin-secreting cells (15). Methanolic extract of plant leaves and calluses significantly increases beta cell production in diabetic rats and can potentially be used as herbal medicine to treat insulin-dependent diabetes mellitus (16). The effect of biologically active compounds extracted from these plants in reducing glucose is greater than chemical medicine (17). Flavonoids in epigallocatechin gallate can prevent the death of cytokine-induced cells in beta-pancreatic cells isolated from rats by inhibiting the activation of the nuclear-cappuccino factor (18).

The findings of a 2010 study by Kamili et al. showed that blue walnut leaf extract in diabetic animals significantly reduced cholesterol, LDL-C, and blood triglycerides, while significantly increasing HDL-C. The extract was considered to have a beneficial anti-diabetic therapeutic effect (19).

The findings of a 2017 study by Miri et al. showed that the roots of rattle plants lower blood serum glucose levels due to the presence of alkaloids, flavonoids, and tannins. The other reason lies in various mechanisms, such as increased insulin secretion, activation of glucose catabolism pathway, inhibition or inactivity of gluconeogenesis way, directing glucose into the cell, absorbing glucose-free and preventing them from binding to proteins, as well as preventing the absorption of glucose from the gut (20).

Puraboli (2012) confirmed that Quercetin and Morin flavonoids in the Otostegia persica plant can inhibit pancreatic lipase, in addition to their antioxidant, anti-allergic, anti-inflammatory, anti-mutational, and anti-cancer effects. The findings also implied that flavonoids in this plant exert their antioxidant effect in several ways, such as combining with free radicals, producing less active products, and inhibiting the body's metabolic enzymes, including lipoxygenase, cyclooxygenase, xanthine oxidase, and nitric oxide synthase, which are involved in the production of free radicals (21).

The results of this study showed that the use of flavonoid compounds in walnut green skin significantly reduced the concentration of glucose, total cholesterol, and triglyceride in blood, either for the treatment of rats with differentiated adipose tissue mesenchymal stem cells or for injection after treating rats with these cells. Furthermore, the

concentration of HDL-C in the treatment of these cells by diabetic rats showed a significant increase while LDL-C showed a significant decrease. Hyperlipidemia is a complication associated with diabetes mellitus that leads to quantitative and qualitative abnormalities in lipoprotein (22).

Elevated triglyceride levels alone cannot be a problem; however, since its high levels are associated with low HDL-C, high LDL-C, obesity, and diabetes, it is harmful to health. It also accelerates and intensifies the process of atherosclerosis. Studies have shown that increasing total cholesterol or LDL-C in the blood is a powerful risk factor for coronary heart disease that diabetics are prone to (23).

In this study, according to previous studies with an intraperitoneal injection of streptozotocin, it was observed that streptozotocin causes diabetes by destroying the pancreas and affecting the desired variables. Changes in the studied samples by AD-MSCs differentiated by flavonoid extract depend on the rate of destruction of pancreatic beta cells. There are two reasons for this finding. First, AD-MSCs, which were differentiated into pancreatic beta cells under the influence of flavonoids, may have replaced damaged beta cells after injection or improved damaged beta cells. Second, in addition to injecting differentiated MSCs into insulin-producing cells, simultaneous injection of flavonoid extract, as well as the replacement or improvement of damaged insulin-producing cells, has prevented the death of the remaining beta cells.

In 2003, Vesal et al. examined the anti-diabetic effect of streptozotocin on rats and found that intrathecal administration of some flavonoids to streptozotocin-induced diabetic rats reduced the dose-dependent flavonoid content of glucose while it did not have a specific effect on blood glucose in healthy animals (24).

In 2014, Fallah et al. studied the anti-diabetic effects of Momordica charantia on diabetic rabbits under treatment with naloxone-derived methanolic and ethanolic extract of Momordica charantia, which is rich in phenolic and flavonoid compounds. With a change in the number of insulin-producing cells, a 28% reduction was observed in hypercalcemia by ethanol extract (25).

Research shows that flavonoids have favorable effects on plasma glucose and lipid profile concentrations. In addition, they inhibit the differentiation of fat cells by inhibiting or altering the key genes involved in the fat production process (26). Another mechanism by which inductive material exerts its effect on the differentiation of stem cells is the change between some genes in the endocrine and exocrine parts of the pancreas.

Xu (2008) found that all endocrine cells show a temporary expression of Ngn3 in a short time when the rat fetal develops, which appears in 13.5 days of fetal development, has an expression peak after 15.5 days of the fetal, and then, disappears completely. No cell that expresses the Ngn3 gene is seen after birth in the adult pancreas (27).

Vetere et al. (2003), using a Cre-ERTM-loxP-induction system, showed that cells expressing Ngn3 ancestors are the endocrine cells of pancreatic islets and have no association with exocrine progenitor cells during fetal and adult development in rats. The Ngn3 is needed to stimulate differentiation in pancreatic endocrine cells. Another report found that the ectopic expression of Ngn3 in chick fetal converted endodermal cells in each region to α-type but not β-type (28).

Adresicol et al. (2015) investigated the expression of human insulin-producing factors and found that factor Ngn3 is a natural part of the pancreatic biology of an adult human. These factors can increase in number by using specific medicine, and converting them into differentiated cells of the pancreas can produce effective beta cells to treat diabetes (29). Rostalin et al. (2009), in a study of neurogenin as a major regulator of pancreatic endocrine development, found that treatments to increase Ngn3 expression in diabetes are means of increasing cell mass and beta mass function (30).

Limbert et al. induced an increase in Pdx1 gene expression in MSCs to differentiate them into insulin-producing cells (31).

Myazaki et al detected C-peptide and Pdx1 in stem cell-differentiated embryonic cells by immunohistochemistry. They pushed these cells toward insulin-secreting cells as they differentiated. Eventually, the expression of C-peptide increased dramatically in these cells (32).

6. Conclusion

The present study showed that rat AD-MSCs can differentiate into pancreatic beta cells *in vitro* under the influence of flavonoid compounds, and after transplantation, they cause significant changes to improve the levels of glucose, triglyceride, LDL-C, HDL-C, as well as total blood cholesterol. They also alter the expression of transcription factors related to the growth and function of pancreatic cells (Ngn3 and Pdx1 factors).

Acknowledgments

This article is an excerpt from a doctoral dissertation. In this way, esteemed professors and everyone who sincerely helped in conducting this study are appreciated.

Footnotes

Conflicts of Interest: The authors declare no conflict of interest.

Funding/Support: This study did not receive any

specific grant from funding agencies in the public, commercial, or not-for-profit.

Ethical statements: This study has been approved by the Ethics Committee of Kharazmi University with the code of ethics of IR.KHU.REC.1398.031.

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