



The Protective Effects of Cultured Mesenchymal Stem Cells onto the Surface of Electrospun Poly-L-Lactide Acid Scaffolds Coated with *Matricaria Chamomilla L.* Oil in Streptozotocin-Induced Diabetic Rabbits

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Abstract

Background: Although *Matricaria chamomilla L.* oil has been shown to contribute to the differentiation of mesenchymal stem cells (MSCs) into insulin-producing cells, the molecular mechanisms underlying its effect in the treatment of Type 1 Diabetes mellitus (T1DM) is still not available.

Objectives: The purpose of this study was to evaluate the effect of cultured MSCs on the surface of electrospun Poly-L-Lactide acid (PLLA) scaffolds coated with *Matricaria chamomilla L.* oil in treatment of T1DM and clarify its possible mechanisms.

Methods: The current experimental study was performed using a total number of 36 male New Zealand white rabbits in the Experimental Animal Unit of Shahid Beheshti University of Medical Sciences Tehran, Iran, in 2018. A rabbit model of T1DM was established through a single intraperitoneal injection of streptozotocin (STZ) (80 mg/kg body weight)¹ dissolved in 0.2 mL of normal saline. For 21 days after implantation of cultured MSCs on the surface of electrospun PLLA scaffolds coated with *Matricaria chamomilla L.* oil, blood glucose levels, plasma insulin levels, plasma and pancreatic glucagon-like peptide 1 (GLP-1) were evaluated.

Results: The STZ-treated rabbits exhibited decreased levels of blood glucose ($P < 0.01$ vs. other groups except control), increased levels of plasma insulin ($P < 0.01$ vs. other groups except control), plasma and pancreatic GLP-1 ($P < 0.001$ vs. control, and $P < 0.05$ vs. other groups except control) at 21 days after implantation of cultured MSCs on the surface of electrospun PLLA scaffolds coated with *Matricaria chamomilla L.* oil.

Conclusions: Collectively, our finding showed the presence of *Matricaria chamomilla L.* oil might improve survival and differentiation MSCs in insulin producing cells that can be attributed to antioxidant properties of its bioactive compounds.

Keywords: Diabetes Mellitus, Glucagon-Like Peptide 1, Insulin Producing Cells, *Matricaria*, Mesenchymal Stem Cells, Oil, Type 1

1. Background

Type 1 Diabetes mellitus (T1DM) is an autoimmune disease that occurs due to the destruction of insulin-producing beta cells, and its main characterization is chronic hyperglycemia (1). The detailed knowledge about the initiators of this disease is not still available. A possible solution to control this disease is to keep blood sugar levels in specific ranges. To this end, patients need some lifestyle adjustments. Sometimes, these lifestyle adjustments are not sufficient, and therefore treatment options are recom-

mended (2). Commonly, the best solution for control of hyperglycemia in patients with T1DM is insulin injection (3). Aside from benefits, insulin injection might have several drawbacks such as the slower absorption at higher doses, possibility of its delayed peaking, and weight gain in patients (4). Previous studies have shown that the primary mode for the death of beta-cells in early T1DM is apoptosis that is mediated by cytokines (5). GLP-1 is a gut hormone that is secreted by endocrine cells in the intestinal mucosa and plays an essential role in the improvement of glucose-induced insulin secretion and subsequently the control of

blood glucose (6). Previous studies have shown that GLP-1 could hamper apoptotic death of beta-cells caused by glucose concentration elevated in a protein kinase B dependent mechanism (7). Currently, stem cell-based therapies have garnered much attention from the scientific community to treat T1DM through the replacement of the lost insulin-producing cells (8). It has been reported that regenerated beta cells possess the ability to exhibit robust glucose-responsiveness and maintain euglycemia (9). Unique properties of mesenchymal stem cells (MSCs) such as low immunogenicity, easy attainability, profound immunomodulatory effects, and ability to differentiate into diverse lineages make them good candidates for the treatment of T1DM (10). On the other hand, natural products and herbal medicines widely have been used for the treatment of different diseases (11-13). Chamomile is one of the ancient medicinal herbs that belong to the daisy family (Asteraceae/Compositae) (14). Chamomile is widely used as cosmetics and medicinal preparations because it possesses various classes of bioactive ingredients such as terpenoids and flavonoids (15, 16). Recently, a clinical trial study has been shown that Chamomile might have beneficial effects on serum lipid profile and glycemic control in patients with type 2 diabetes mellitus (17). Chamomile tea also contributes to the reinforcement of endogenous antioxidant defenses and inhibition of lipid peroxidation in patients with type 2 diabetes mellitus (18). Additionally, a recent study showed that electrospun PLLA scaffolds coated with *Matricaria chamomilla* L. oil could promote differentiation of MSCs into insulin-producing cells (19).

2. Objectives

Here, we went on to examine the effects of MSC-derived insulin-producing cells and *Matricaria chamomilla* L. oil on the treatment of type I diabetes in a rabbit model and explore possible molecular mechanisms.

3. Methods

3.1. Apparatus, Drugs, and Chemicals

Matricaria chamomilla L. oil, Propidium iodide, and STZ were obtained from Sigma (Sigma Aldrich Company, USA). Rabbit Insulin ELISA Kit was acquired from MyBioSource Company (San Diego, USA). Glucometer elite and glucometer strips were purchased from Bayer Company (Leverkusen, Germany). Rabbit GLP-1 ELISA Kit was purchased from MyBioSource Company (San Diego, USA). In current study, all pieces of equipment including Elisa Microplate

Washer (Stat Fax 2600, AWARENESS, and USA), Elisa Microplate Reader (Stat Fax 2100, AWARENESS, and USA), homogenizer (IKA T18, Basic ULTRA TURRAX, and USA), Incubator (Mettler, Germany), glucometer (GlucoTrend 2, Roche company, Germany), and CH-30 optical microscope (Olympus, Tokyo, Japan) were calibrated before the use.

3.2. Animals and Ethical statement

The current experimental study was performed in the Experimental Animal Unit of Shahid Beheshti University of Medical Sciences Tehran, Iran, in 2018. A total number of 36 male New Zealand white rabbits with a mean weight of 2.5 kg were purchased from Razi Institute, Iran. In this experimental study, the protocols and experiments were conducted in consistent with the ethical norms approved by the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences (Ethical approval number: 1392.49270). The animals were maintained in a controlled temperature room ($25 \pm 1^\circ\text{C}$) on a 12/12 light-dark schedule with relative humidity about 40% to 60%. The animals were allowed free access to standard pellet chow and water ad libitum. The Sample size was calculated based on the following formula:

$$n = \Psi^2 \left(\frac{\sum (S_i^2)}{K} \right) / \left[\frac{\sum (X_i - \frac{X_1+X_2+X_3}{K})^2}{(K-1)} \right]$$

$\alpha = 0.05$, $\beta = 0.10$, K: number of groups, $\Psi_{\alpha, \beta, K-1, \infty} = 2.52$, X_i , S_i : mean ($X_1 =, X_2 = \dots$) and standard deviation ($S_1 =, S_2 = \dots$).

3.3. Isolation of Adipose-Derived Mesenchymal Stem Cells

For isolation of adipose-derived mesenchymal stem cells, the animals were intraperitoneally (IP) anesthetized utilizing a mixture of 40 mg/kg ketamine and 5 mg/kg xylazine. The abdominal region was opened through a mid-line incision and 100 mL of adipose tissue was dissected from the perivesical region. The adipose tissue converted into small pieces in cold phosphate-22 buffered saline (PBS, pH = 7.4) using a mechanical method. After homogenization and centrifugation at 1250 rpm for 5 min, the supernatant was removed and the pellet was digested with 0.1% collagenase type I at 37°C for 1 hour. Once again, the samples were centrifuged at 1250 rpm for 5 min. After removal of the supernatant, cells were cultured at 37°C , 5% CO_2 in an appropriate volume of DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin to obtain the required cell density.

3.4. Assessment of Osteogenic and Adipogenic Differentiation

Oil red O staining was applied to determine adipogenic differentiation. Cells were cultured with adipogenic induction medium including 10 ug/mL insulin, 50 uM indomethacin, 100 ug/mL 3-isobutyl-1-methylxanthine, 10^{-6} M dexamethasone in alpha-MEM medium supplemented with 10% FBS in a 5% CO₂ incubator for 3 weeks. Alizarin red S staining was used to evaluate the osteogenic differentiation capacity of MSCs. In brief, MSCs were cultured with the osteogenic induction containing 50 ug/mL ascorbic acid, 10 mM glycerophosphate disodium, 10^{-7} M dexamethasone in alpha-MEM medium supplemented with 10% FBS in a 5% CO₂ incubator for 4 weeks.

3.5. Preparation of PLLA Scaffold

PLLA was dissolved in chloroform and mixed with DMF at the ratio of 4.25:0.75. Plastic syringes (5 mL) were used for loading and connecting to a 21-gauge needle. Finally, PLLA Scaffolds were fabricated through the establishment of a positive voltage between the needle and collector. In order to coat the surface of scaffolds with Chamomile or corn oils, they were immersed in oils for 24 hours.

3.6. Scanning Electron Microscopy (SEM)

To evaluate the morphology and structure of scaffolds, scanning electron microscopy (KYKY, China) was used. In brief, scaffolds were cut into 1×1 cm² samples, and then their gold Sputtering was performed in a vacuum condition.

3.7. Cell Culture on the Surface of the Scaffold

Ethylene oxide was applied to sterilize scaffolds prior to cell seeding. PLLA Scaffolds were immersed in DMEM which supplemented with 10% FBS at 4°C for 24 hours. Isolated MSCs from adipose tissue at passages 3 and an adjusted cell density of 1×10^5 per well of 24-well plates were cultured onto the surface of pure PLLA scaffolds, Chamomile oil coated scaffolds and corn oil coated scaffolds in DMEM supplemented with 10% FBS, 1% streptomycin and penicillin in a humid chamber with 5% CO₂ at 37°C. In order to check cell seeding, Propidium Iodide (PI) staining was performed after 24 hours.

3.8. Experimental Design

The New Zealand white rabbits were randomly (simple randomization) assigned into six cohorts: A group of healthy rabbits (control; n = 6), a group of rabbits only received a single intraperitoneal injection of STZ (80 mg/kg

body weight)¹) dissolved in 0.2 mL of normal saline (diabetic; n = 6), a group of diabetic rabbits only received PLLA scaffolds coated with Chamomile oil (PLLA+ Chamomile oil; n = 6), a group of diabetic rabbits received PLLA scaffolds coated corn oil with MSCs (PLLA+ corn oil + MSCs), (PLLA+ corn oil; n = 6), a group of diabetic rabbits received PLLA scaffolds coated Chamomile oil with MSCs (PLLA+ MSCs; n = 6), a group of diabetic rabbits received PLLA scaffolds with MSCs (PLLA+ Chamomile oil + MSCs). In order to confirm the induction of diabetes in rabbits, blood glucose levels were measured at 72 hours after intraperitoneal injection of STZ. A drop of blood was inserted on a glucometer strip, and the strip was read using a glucometer. Animals with blood glucose levels of 220 mg/dL with glycosuria were accepted to entry to study because they were considered to be diabetic. Animal activities, eating habits, and blood glucose levels were observed on a regular basis.

3.9. Glucose Tolerance Test (GTT)

Prior to the experiment, the animals were fasted overnight. Then, all the groups received 4 g/kg glucose via oral administration. Blood glucose was measured in 1 hour after intraperitoneal injection of STZ, 1, 2, 7, and 21 days after implantation of scaffolds. Evaluation of blood glucose level was performed via the glucose oxidase method using a glucometer.

3.10. Sample Processing

3.10.1. Serum Collection

Blood samples from the blank controls and MSC-treated rabbits were collected and centrifuged at 3000 rpm for 10 minutes to separate the serum samples. Serum samples were stored at -80°C until use.

3.10.2. Tissue Homogenization

At the end of the experimental period, the pancreas tissues were quickly dissected and removed from the body on ice. Tissues were washed with cold saline solution and were weighed. Using a homogenizer (IKA T18, Basic ULTRA TURRAX, and USA) at 5000 rpm for 3 min, 100 mg of the tissue was homogenized within 1 mL phosphate buffer. Then, the homogenized solution was centrifuged; the supernatant was separated and stored at -80°C until use. All the mentioned above stages were carried out at 4°C to reduce the destruction of proteins.

3.11. Assessment of Serum Insulin Levels

Twenty-one days after implantation of scaffolds, the animals were fasted overnight. Then, all the groups received 4 g/kg glucose via oral administration. A radioimmunoassay kit was used to determine serum insulin concentration. Measurement of the insulin in serum samples was based on antigen-antibody reaction (competition between radiolabeled ^{125}I -insulin and unlabeled insulin).

3.12. Assessment of Plasma and Pancreatic GLP-1

Twenty-one days after implantation of scaffolds, plasma and pancreatic GLP-1 was measured using Rabbit GLP-1 ELISA Kit as instructed by the manufacturer.

3.13. Statistical Analysis

All the data were expressed as the mean \pm standard deviation. Analysis of data was performed using GraphPad Prism-5 statistic software (LaJolla, 7 CA, and USA). Kolmogorov - Smirnov, and Shapiro - Wilk tests were used to examine the normality of data. Comparison of variations among groups was carried out using a one-way analysis of variance (ANOVA) followed by the Tukey test for post hoc analysis. Values were considered statistically significant when $P < 0.05$.

4. Results

4.1. Evaluation of Osteogenic and Adipogenic Differentiation

The ability of MSCs for differentiation into adipocytes was confirmed using Oil red O staining (Figure 1A). Likewise, Alizarin red S demonstrated that isolated MSCs possess capability formation of calcium deposits and mineralization (Figure 1B).

4.2. Evaluation of Scaffolds Morphology

As shown in Figure 2, SEM images showed that the diameter of fibers was in the micron size range of 0.34 μm to 0.38 μm .

4.3. Evaluation of Cell Seeding on the PLLA Scaffolds

In order to check cell seeding on the PLLA scaffolds, PI staining was performed. As depicted in Figure 3A, our results showed that cell seeding on the PLLA scaffolds was successful.

4.4. Evaluation of Blood Glucose Levels

Our results demonstrated that blood glucose levels in the STZ-treated rabbits were significantly higher than control in all days after surgery. Likewise, our results showed that the injection of STZ increased glucose levels in a time-dependent fashion. The reduction in blood glucose levels was found in STZ+PLLA+MSCs, STZ+Chamomile@PLLA, and STZ+Corn oil@PLLA+ MSCs but did not reach significant differences. Statistically, differences in blood glucose in STZ+ Chamomile@PLLA+ MSCs was observed in 14-21 days after surgery compared to other groups (Figure 3B).

4.5. Evaluation of Serum Insulin Levels

To confirm MSCs on the surface of Chamomile@PLLA can differentiate into insulin producer cell in the pancreatic region, we measured serum insulin levels 21 days after surgery. As shown in Figure 4, our results showed that serum insulin level in the STZ-treated rabbits was considerably lower than control at 21 days after surgery. Implantation of Chamomile@PLLA containing MSCs markedly increased serum insulin levels compared with STZ and other groups at 21 days after surgery.

4.6. Evaluation of Plasma and Pancreatic GLP-1

As shown in Figure 5A and B, significant increases in plasma and pancreatic GLP-1 was found in the STZ-treated rabbits at 21 days after surgery. Implantation of Chamomile@PLLA containing MSCs significantly increased plasma and pancreatic GLP-1 compared with other groups.

5. Discussion

Although the discovery of insulin in 1921 created great promise in effective treatment of type 1 Diabetes mellitus owing to its ability for the control of blood glucose levels, exogenous insulin therapy, and daily subcutaneous insulin pump is not completely capable to mimic the patterns of physiological pancreatic insulin secretion (20). On the other hand, it may create life threatening hypoglycemic episodes that, in turn, result in the occurrence of some macro and microvascular side effects including neuropathy, cardiovascular disorders, retinopathy, and nephropathy (21). Researchers have examined many novel therapeutic agents and methods to treat different diseases (22-24). In recent years, many new therapeutic options have introduced to treat type 1 Diabetes mellitus and other diseases (25-28). Currently, Stem cell-based therapy has been suggested as a promising candidate for the treatment of type

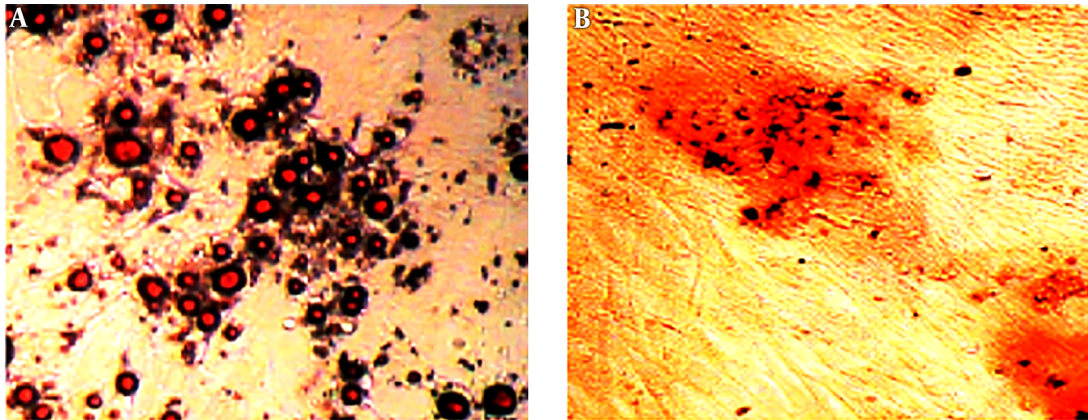


Figure 1. A, Formation of some lipid droplets in cultured MSCs was approved by Oil red O staining. B, Formation of calcium deposits and mineralization of cultured MSCs was confirmed using Alizarin red S staining (magnification 400×). MSCs = Mesenchymal Stem Cells

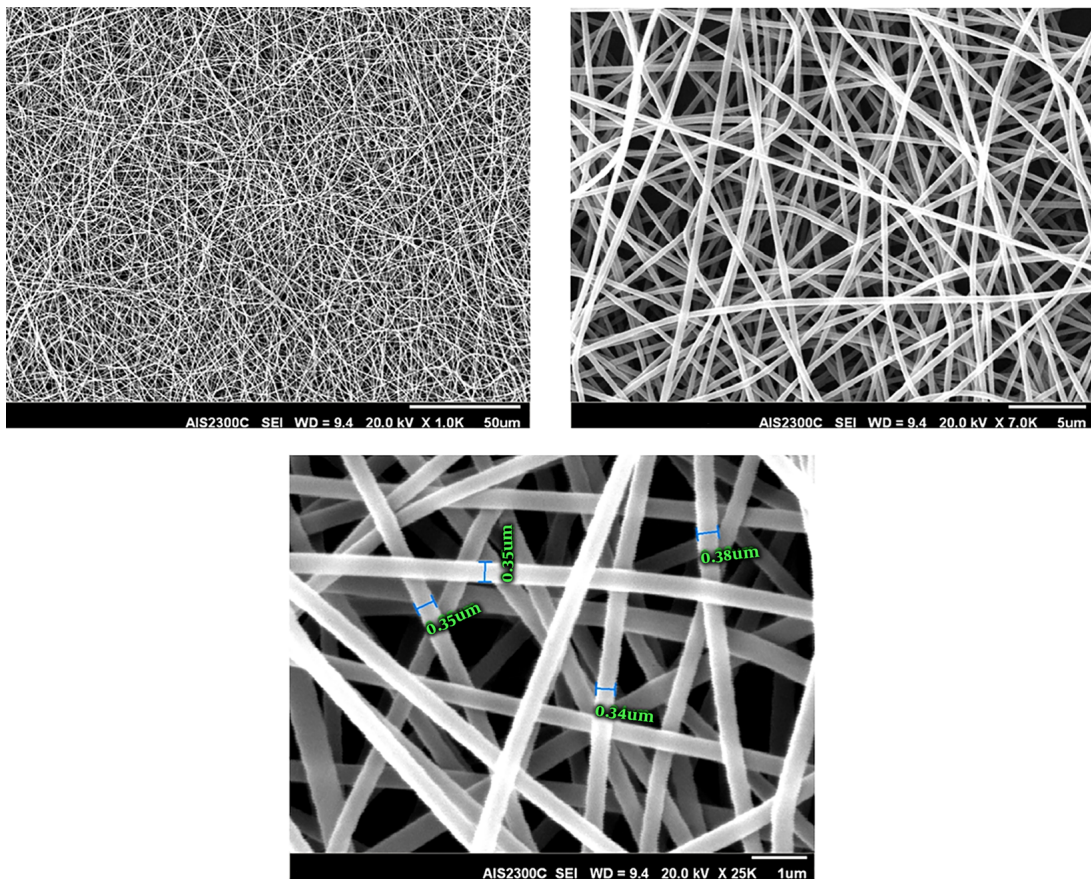


Figure 2. Scanning Electron Microscopy (SEM) images of PLLA scaffold showed that the diameter of fibers was in the micron size range of 0.34 µm to 0.38 µm. PLLA = Poly-L-Lactide acid

1 Diabetes mellitus because some evidence has shown that some stem cell lines such as MSCs have the ability to differ-

entiate into insulin producing-cells (1, 29-31). In this study, we first isolated MSCs of rabbit adipose tissue and then ex-

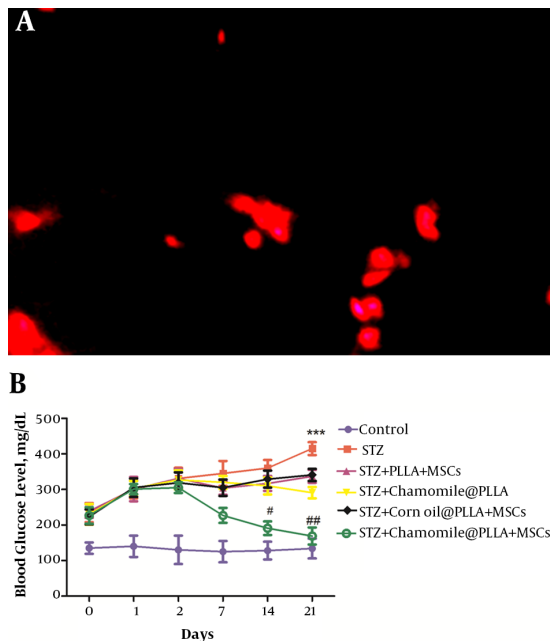


Figure 3. A, Propidium Iodide (PI) staining confirmed the presence of cells on the PLLA scaffolds (magnification 400 \times). B, Chamomile@PLLA+ MSCs significantly decreased blood glucose levels (** $P < 0.001$ vs. control in all days after surgery, # $P < 0.05$ and ## $P < 0.01$ vs. other groups except control). MSCs, Mesenchymal Stem Cells; PLLA, Poly-L-Lactide acid; STZ, Streptozotocin.

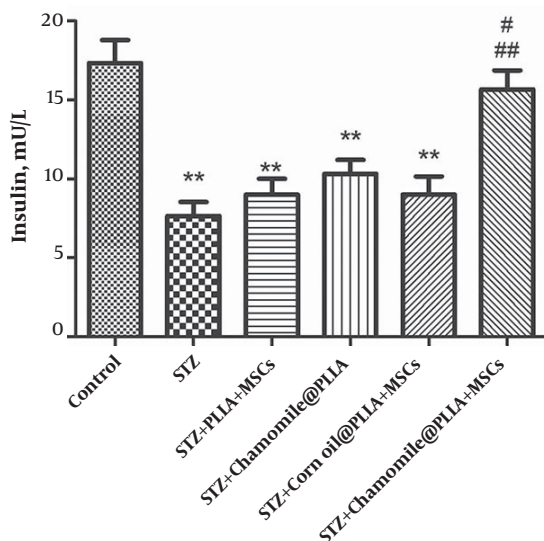


Figure 4. Chamomile@PLLA+ MSCs markedly increased serum insulin levels 21 days after surgery (** $P < 0.01$ vs. control at 21 days after surgery, # $P < 0.05$ vs. Chamomile@PLLA group, ## $P < 0.01$ vs. other groups except for control). MSCs, Mesenchymal Stem Cells; PLLA, Poly-L-Lactide acid; STZ, Streptozotocin.

amine their ability for osteogenic and adipogenic differentiation. According to a previous study (32), our results

showed that isolated MSCs possess the ability to form lipid droplets and calcium deposits. In a previous study, Fazili et al. showed that AMSCs on the surface of Chamomile oil coated poly (L-lactic)-collagen-based scaffold can differentiate into insulin-producing cells after implantation in rabbits but they did not investigate the effect on blood glucose level and plasma insulin level (19). In this study, we confirmed that implantation of AMSCs on the surface of Chamomile oil coated poly (L-lactic)-collagen-based scaffold into the pancreatic region of STZ-treated rabbits resulted in a significant decrease in blood glucose level. Our results verified that Chamomile oil plays an important role in the differentiation of AMSCs into insulin-producing cells because MSCs + PLLA and Corn oil@PLLA+ MSCs failed to reduce blood glucose level significantly. In agreement with our results, a previous study demonstrated that oral administration of *Matricaria chamomilla* L. ethanolic extract for 14 days dose-dependently decreased blood glucose level STZ-treated rabbits (33). Compared with this previous study, our result exhibited better performance because we only decorated scaffold with Chamomile oil. Also, we demonstrated that AMSCs on the surface of Chamomile oil coated poly (L-lactic)-collagen-based scaffold significantly increased plasma insulin. In agreement with our findings, above mentioned previous study indicated that oral administration of *Matricaria chamomilla* L. ethanolic extract for 14 days increased the number of insulin-producing cells in STZ-treated rabbits (33). Previous studies have demonstrated that the expression of GLP-1 by pancreatic cells increase stimulates insulin secretion by increasing proliferation and inhibition of apoptosis of beta cells (34-36). A central question is whether antioxidant properties of bioactive ingredients present in Chamomile oil hamper death of differentiated cells in the pancreas and contribute to the treatment of STZ-treated rabbits. To obtain greater insights, we measured plasma and pancreatic levels of GLP-1. Our result showed a significant increase in plasma and pancreatic levels of GLP-1 in STZ-treated rabbits. A higher increase in plasma and pancreatic levels of GLP-1 was found in Chamomile@PLLA+ MSCs. In keeping with our result, a previous study showed that STZ-treated rats showed higher plasma and pancreatic levels of GLP-1 than control. They attributed this issue to islet cells and concluded that GLP-1 contributed to beta cell survival (37). Likewise, in agreement with our findings, many previous studies have shown that bioactive ingredients present in Chamomile oil increase cell survival through inhibition of apoptosis (38-40). One of the strong points of this study was the exploration of possible molecular mechanisms by

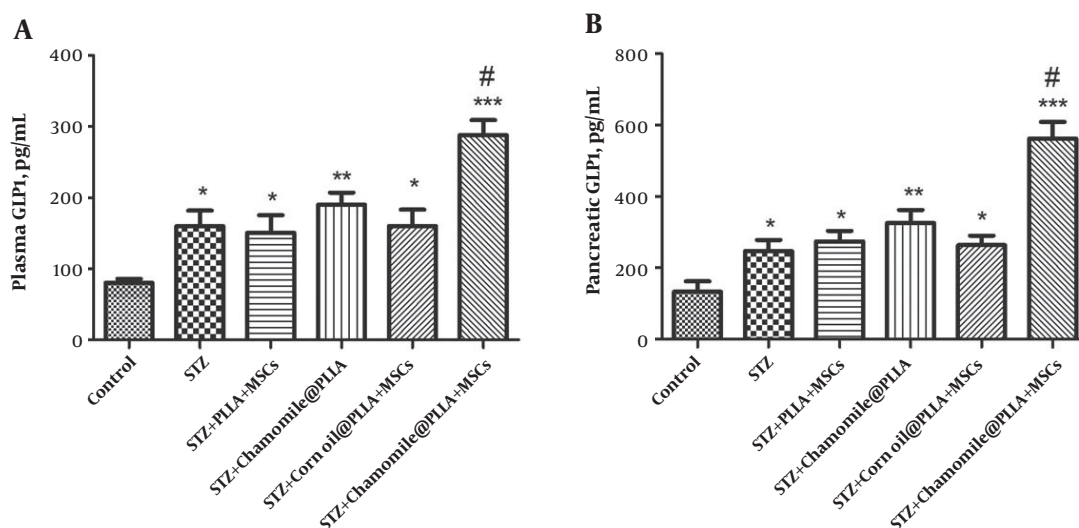


Figure 5. An increased level of plasma and pancreatic GLP-1 were found the STZ-treated rabbits. The effect of Chamomile@PLLA and Chamomile@PLLA+ MSCs were higher than other groups at 21 days after surgery (* $P < 0.05$ and ** $P < 0.01$, and *** $P < 0.001$ vs. control at 21 days after surgery, # $P < 0.05$ vs. other groups except for control). MSCs, Mesenchymal Stem Cells; PLLA, Poly-L-Lactide acid; STZ, Streptozotocin.

which cultured MSCs on the surface of electrospun PLLA scaffolds coated with *Matricaria chamomilla* L. oil which contributed to the treatment of T1DM. The main limitations of the present study were the lack of measurement of the gold standard proteins such as Bax and Bcl₂ to confirm the inhibition of apoptosis in treated rabbits.

5.1. Conclusions

In sum, our result demonstrated that the presence of Chamomile oil on the PLLA contributed to differentiation to insulin producing-cells through inhibition their death in a GLP-1 dependent mechanism.

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Footnotes

Authors' Contribution: Seyed Abdulmajid Ayatollahi contributed to conception and design. Saeid Saghahazrati, Bagher Minaei and Farzad Kobarfard contributed to all experimental work, data and statistical analysis and interpretation of data. Seyed Abdulmajid Ayatollahi was responsible for overall supervision. Seyed Abdulmajid Ayatollahi drafted the manuscript, which was revised by Saeid

Saghahazrati. All authors read and approved the final manuscript.

Conflict of Interests: The authors declare no conflict of interest.

Ethical Considerations: In this experimental study, the protocols and experiments were conducted in consistent with the ethical norms approved by the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences (Ethical approval number: 1392. 49270).

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