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Research Article

Construction and Transfer of PEI-miRNA-126/210 Polyplex into Human Umbilical Vein Endothelial Cells with Investigation of Its Effect on Islets Survival and Function

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Abstract

Background: Type 1 diabetes is an autoimmune disorder characterized by the loss of pancreatic islets. Islet allotransplantation is a potentially beneficial therapeutic approach for diabetes. Islets suffer a variety of cellular insults including ischemia and partial vascular loss during isolation, resulting in a significant reduction in viability prior to transplantation. The present study aimed to investigate the effect of angiogenic microRNA (miRNA)-126 and -210 on islet function and viability in an indirect way.

Methods: Poly Ethylenimine (PEI)-miRNA-126 and -210 polyplexes were constructed at various Nitrogen/Phosphate (N/P) ratios. After confirmation by gel retardation and ethidium bromide dye exclusion assay, its cytotoxicity and transfection efficiency were analyzed by MTT and fluorescent assays, respectively. After that, the selected polyplexes were used to transfect Human Umbilical Vein Endothelial Cells (HUVECs) in vitro and were indirectly co-cultured with islet cells for three days. Real-time polymerase chain reaction and enzyme-linked immunoassay were conducted to verify the regulation of target genes and the functionality of the islets.

Results: The findings showed that PEI could condense miRNAs at N/P=5. The viability of the HUVECs was decreased by increasing the amount of PEI. Additionally, ployplex-126 and -210 led to a decrease in the expressions of target genes, phosphoinositol-3 kinase regulatory subunit 2, sprouty-related EVH1 domain-containing protein 1, and ephrin-A3 in the islets. Moreover, the expressions of *Bax* and *Bcl2* and their ratio in the treated groups by polyplex-126 and -210 led to better survival and function of the islets, with a higher expression of insulin and response to glucose stimulations. Furthermore, polyplex-210 could downregulate the anti-angiogenic protein, thrombospondin 1, compared to the other groups. Finally, the secretion of C-peptide was higher in polyplex-210 than in the other groups, adjusted for insulin secretion.

Conclusion: The results indicated that angiogenic miRNAs could promote the survival and function of islet cells by interacting with their targets.

Introduction

Type 1 diabetes is an autoimmune disorder caused by the destruction of insulin-producing beta cells in pancreatic islets.[1] Allogeneic islet transplantation is a promising potential option for diabetes treatment. Despite the effectiveness of conventional clinical transplants based on the Edmonton protocol, it is limited by cell dysfunction during long-term transplantation and most recipients return to insulin replacement.[2,3] In fact, islets are subject to numerous cellular damages during the enzymatic digestion step. These include ischemia, mechanical stress, loss of basement proteins, and partial disruption of intra-islet Endothelial Cells (ECs) that lead to a considerable loss of cell viability before transplantation.[4,5] Therefore, prompt and proper revascularization is essential for the survival and function of transplanted islets.[6,7] Although transplanted islets are considered avascular, freshly isolated islets maintain angiogenic due to ECs within the islets. Various inducers such as Vascular Endothelial Growth Factor (VEGF), can stimulate these cells to form blood

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vessels via angiogenic sprouting.[8-10]

Survival and function of islets can be improved by pre-vascularization prior to transplantation. It can aid islet-to-host inosculation, as well.[11] ECs play critical roles in tube formation, vascular remodeling, and vascular integrity maintenance.[12,13] Among ECs, Human Umbilical Vein Endothelial Cells (HUVECs) have the most enhanced ability to initiate tube formation.[14,15] During angiogenesis, ECs respond to various growth factors such as basic Fibroblast Growth Factor (bFGF), VEGF, Thrombospondin 1 (THBS-1), cytokines, and nitric oxide, pro-angiogenic mRNAs, and microRNAs (miRNAs) to modulate the formation of new vessels.[16-19]

miRNAs as noncoding RNA molecules (18–25 nucleotides) regulate some cellular activities through the posttranscriptional inhibition of gene expression.[20,21] Among these miRNAs, miRNA-126 is an ECs-specific microRNA that acts as a key regulator in promoting angiogenesis and vascular integrity. For instance, the upregulation of this miRNA contributed to angiogenesis after vascular injury or hypoxia. This function was done by downregulating the Phosphoinositol-3 Kinase Regulatory Subunit 2 (PIK3R2) and Sprouty-Related EVH1 Domain-Containing Protein 1 (SPRED1), as the negative regulators of the VEGF pathway.[22-24] miRNA-210 also plays a key role in the cellular response to hypoxia, regulating cell survival, migration of VEGF-derived ECs, and enabling endothelial cells to build capillary structures by increasing EC tube formation and migration. In the presence of hypoxia, blocking miRNA-210 inhibits these processes and causes apoptosis. The direct suppression of Ephrin-A3 (EFNA3), one of the targets of miRNA-210, causes these consequences.[25,26] The low efficiency of miRNA delivery, as the main obstacle to using miRNAs as a therapeutic agent, has led to efforts to improve it.[27] Several studies have demonstrated the potential of non-viral vectors such as Poly Ethylenimine (PEI), FuGene, and lipofectamine for genetic modification.[28-30] However the ability of these materials for primary cell transfection remains sub-optimal.

PEI or polyaziridine, as a bioavailable polymer, is composed of a secondary amine group and aliphatic dual carbon separators. PEI converts DNA into positively charged particles. These particles bind to the anionic components of cell surface and enter the cell by endocytosis.[31,32] PEI has weak-base buffering properties. In addition to its proton acceptor behavior, PEI reduces the nuclease activity and protects nucleic acids from degradation within the endosomal vesicles.[33] PEI also has the proton sponge effect for endosomal lysis to release nucleic acids into the cytoplasm.[34,35]

The current study aims to indirectly investigate the effects of the condition media released from transfected HUVECs with polyplex (PEI/miRNA)-126 and -210 on the survival and angiogenesis of islet cells.

Materials and Methods

Preparation and characterization of polyplex

Gel retardation assay

PEI (3500-4500 KDa, Sigma-Aldrich, USA) solution (0.01% in HBD buffer (HEPES (Sigma-Aldrich, USA) 20 M) + Dextrose 5% (pH=7.4) were mixed with different ratios (1, 2, 5, 7, and 10) of Fam labeled (as a fluorescent dye) miRNA (100 pmol/ml, Azar Gene Mazyar, Iran)[36] and were incubated at room temperature for 30 min. Different polyplex mixtures were run in an electrophoresis apparatus (Uh10, Uvitech, UK) at 60 V in agarose gel (2.5% (w/v), 30 min). After that, they were visualized by a UV lamp (Doc-800X, Uvitech, UK).[37]

Ethidium bromide dye exclusion assay

To determine the optimal Nitrogen/Phosphate (N/P) ratio for loading and condensing nucleic acid sequences, Ethidium bromide (EtBr) dye exclusion assay was performed.[38] For this assay, polyplexes were prepared according to the aforementioned method in 96-well plates. After a 30-min incubation at 22 ºC, 50 µl of EtBr (Sigma-Aldrich, USA) solution (final concentration: 0.5 µg/ml) was added to each sample and mixed gently. After a 15 min incubation, the fluorescence intensity was read at the excitation/emission wavelength of 510/595 nm. Naked miRNA sequence and deionized water were considered positive and negative controls, respectively.

Cell culture

Cellular uptake of polyplex

The HUVECs (purchased from National Cell Bank, Pasteur Institute, Iran) were grown in DMEM-F12 media (Gibco, Germany) via 10% Fetal Bovine Serum (FBS, Gibco, UK) and 1% Penicillin-Streptomycin (Pen/Strep, Sigma, Germany) at 37 °C with 5% CO2. At the 70–90% confluent, HUVECs were washed with PBS and detached with trypsin at 37 °C for 5 min. Totally, 50000 HUVECs were seeded in plates. After a 24-h incubation at 37 °C and 5% CO_2, the cells were treated with the culture medium containing optimum polyplex to evaluate the ability of this polyplex to cross the cell membrane. After 4 h, the cells were washed with PBS and the transfected cells were analyzed under a fluorescent microscope (CKX53, Olympus, Japan). The fluorescent images were then analyzed using IMAGE J software by selecting one cell at a time in an image and measuring the area, integrated density, and mean gray value using the formula for Corrected Total Cell Fluorescence (CTCF), as described by Schindelin *et al.*[39] and McCloy *et al.*[40]:

CTCF= integrated density–(area of selected cell × mean fluorescence of background readings)

MTT cytotoxicity assay

A total of 50000 HUVECs were cultured in 24-well plates at 37 °C and 5% CO2 and after a 24-h incubation, the cells were treated with the culture medium containing various amounts of the selected polyplex at the final concentrations of PEI (5, 2.5, and 1×10^{-4} %) to evaluate the cytotoxicity. The HUVECs were considered the control group. After 72 h, the medium was replaced with 200 µl MTT (0.5 mg/

ml, in PBS) (Sigma-Aldrich, USA) and was incubated for a further 3 h. Finally, the medium was aspirated and the remaining formazan crystals were solubilized in 200 µl/well Dimethyl Sulfoxide (DMSO). The absorbance was measured at 570 nm and the background absorbance was corrected at 630 nm. Cell viability was calculated as a percentage relative to untreated control cells.[41]

Cell culture and polyplex transfection

Totally, 50000 HUVECs were cultured in the upper section of 24-trans-well plates (pore size = 0.4 µm, Corning, USA) and were transfected by optimum polyplex-126 and -210 after 24-h. After 4 h, the inserts were washed with PBS, incubated in the complete medium mentioned above, and maintained for further experiments.

Islet isolation

Adult BALB/c mice weighing 20-25 grams were maintained under a 12 h light/12 h dark condition at 22-24 °C and had free access to pelleted food and tap water. All the animal experiments were approved by the Departmental Committee for Care and Use of Laboratory Animals (code: IR.SUMS.REC.1397.900). The mice were anesthetized with ketamine (100 mg/kg IP). After dissecting the abdominal cavity, pancreas tissues were separated from peripheral tissues and islets of Langerhans were isolated by collagenase P (Roche, Germany), as previously described with some modifications.[42,43] After the separation of pancreas tissues, they were quickly dissected into collagenase P solution (3 mg/1mL Hank's Balanced Salt Solution (HBSS)). After the incubation of the digested pancreas tissue at 37 ºC for 15 min, they were washed by medium A (HBSS, 1% HEPES, and 2% FBS) twice. After that, the islets isolation and purification were done by means of Lymphodex (INNO-TRAIN Diagnostik GMBH, Kronberg, Germany). Islet size, purity, and Islet Equivalents (IEQs) were determined by microscopic sizing on a grid after staining with 1, 5-diphenylthiocarbazone (Sigma, Germany). Afterwards, the islets were incubated in 5% CO2 in RPMI 1640 (Sigma, Germany) supplemented with 10% FBS and 1% Pen/Strep in the lower section of a 24-trans-well plate at 37 ºC for 24-h. They were then used for indirect co-culture experiments

Studied groups

Totally, 1000 IEQs were assigned to each study group as follows: Group A, indirect co-culture of islet cells with untransfected HUVECs as the control group; Group B, indirect co-culture of islet cells with transfected HUVECs with polyplex-126; and Group C, indirect co-culture of islet cells with transfected HUVECs with polyplex-210. The experiments were done in triplicate in all the study groups. In the following experiment, the conditioned medium of HUVECs was transferred to the bottom wells containing islet cells for three days. At the end of the treatment, the condition media of the islet cells were collected and the islet cells were maintained for the next experiments.

Laboratory tests in the pancreatic islets indirectly treated with polyplex

Gene expression measurement

The expressions of BAX, BCL2, VEGF, THBS-1, insulin, C-peptide, SPRED1, PIK3R2, and EFNA3 genes were evaluated by Real-Time Polymerase Chain Reaction (RT-PCR) (n=3). The RNAs of the islet cells were extracted by RNA-Sol isolation kits (Alphabio, Canada). Then, cDNA was synthesized using PrimeScript TM RT reagent kits (Takara, Japan). The primer design was done by NCBI tool Primer-BLAST, and GAPDH was considered the housekeeping gene. The primer pairs have been presented in Table 1. The amplification of the gene was evaluated by SYBR® Premix Ex TaqTM II (Takara, Japan) using Applied Biosystems Step One Plus™ Real-Time PCR system (ABI, USA) in 40 cycles. The fold changes of each gene were calculated by the Livac ($2^{-\Delta\Delta CT}$) method. Melt curves were

Table 1. Gene sequences for the real time PCR.

Gene	Sequences	5′→3′	Length(bp)
Bax	Forward Reverse	CATCGCACTCACCCCTGTAG ATCCATCCCCCTGACTCTCC	130
Bcl-2	Forward Reverse	AGGATAACGGAGGCTGGGAT AGGGTCTTCAGAGACAGCCA	91
VEGF-α	Forward Reverse	GTCCTCTCCTTACCCCACCT CACACACAGCCAAGTCTCCT	102
THBS-1	Forward Reverse	CTGGACTTGCTGTAGGTTATGAT GTCATCATCTCTCTCGGTGTTG	83
Insulin	Forward Reverse	GCTCTCTACCTGGTGTGTGG GTGCCAAGGTCTGAAGGTC	133
SPRED-1	Forward Reverse	CTCAAGTGGTGGATGGCTAC AAGTCCGCACAGCCATTCT	96
PI3KR2	Forward Reverse	GCTGAGTGGCAAACGAGA TGCGGTAGATGACACAGT	113
EFNA3	Forward Reverse	GCTCTGCCCCTTCCTATGAC AGAGTGTGTGGGCAAAGAGG	149

Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; VEGF, vascular endothelial growth factor; THBS: thrombospondin; SPRED, sproutyrelated EVH1 domain containing; PI3KR,phosphoinositide-3-kinase regulator; EFNA3,ephrin A3;bp, base pair

also analyzed to confirm the specificity of reaction at the end of the program.

Measurement of protein secretion

To determine the effect of the polyplex function more efficiently, the condition media of the islet cells were collected after 72-h. The VEGF (ng/L), THBS-1 (ng/L), and C-Peptide (ng/ml) proteins were measured via mouse Enzyme-Linked Immunoassay (ELISA) kits (Bioassay, China) based on the manufacturer's instructions.

Insulin stimulation indices

In order to assay the insulin stimulation indices, the pancreatic islets were exposed to 2.8 mM glucose in RPMI 1640 supplemented with 0.5% Bovine Serum Albumin (BSA). After 1 h, the medium was collected. Next, the islets were incubated in 20 mM glucose in RPMI 1640 supplemented with 0.5% BSA for 1 h and the medium was collected. The concentrations of insulin (IU/L) in the collected culture media were determined via ELISA kits (Bioassay, China) and a microplate spectrophotometer (BioTek Epoch™, USA) at 450 nm. The ratio of the secreted insulin concentration at 20-2.8 mM was calculated as the stimulation index.[44,45]

Statistical analysis

All statistical analyses were performed using the SPSS 19.0 software (SPSS Inc., Chicago, USA). The results were expressed as mean ± Standard Deviation (SD). The data were analyzed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test. $P<0.05$ was considered statistically significant.

Results

Characterization of polyplex

Gel retardation assay

As shown in Figure 1a, the cationic charge was insufficient to neutralize and condense the miRNA at low N/P ratios (1 and 2). However, the migration of miRNA was restricted in the agarose gel by increasing the N/P ratio up to 10. Accordingly, no migration occurred in the gel at N/P=5. Overall, a complete neutralization of the negatively charged surface of miRNAs was observed through a complete polyplex formation (Figure 1a).

Ethidium bromide dye exclusion assay

The fluorescence intensity of the incorporated EtBr was assessed at different N/P ratios. As shown in Figure 1b, increasing the N/P ratio resulted in a decrease in the fluorescence intensity and an increase in the presence of condensation, indicating oligonucleotide sequestration by polycations. Although PEI was the efficient nanocarrier in capturing miRNA, the difference was more pronounced at N/P=5.

Cell culture

Cellular uptake of polyplex

According to Figure 2, the polyplex labeled as Fam entered the HUVECs and showed a dot-like pattern in the cytoplasm. This complex stayed in the cell after washing with PBS. The level of CTCF and cellular fluorescence intensity of each random cell was averagely 27500 and 57200 respectively.

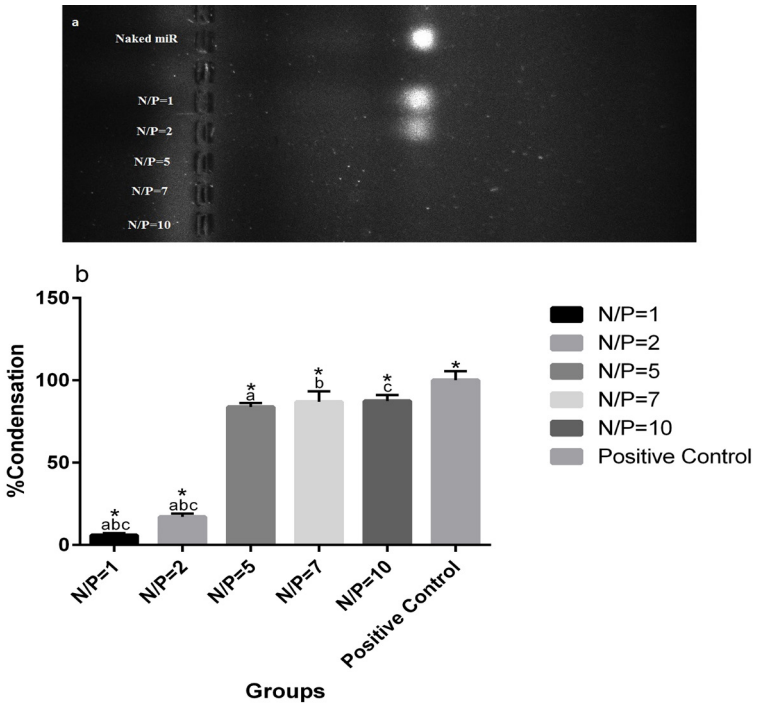

Figure 1. a: Gel retardation assay for miRNA and **b:** Ethidium Bromide dye exclusion assay. Groups with the same superscripts were significantly different at α=0.05 ($p<0.05$). Symbol shows differences between the treated groups and the control group, and alphabet shows significant differences between the treated groups. N, nitrogen; P, phosphate

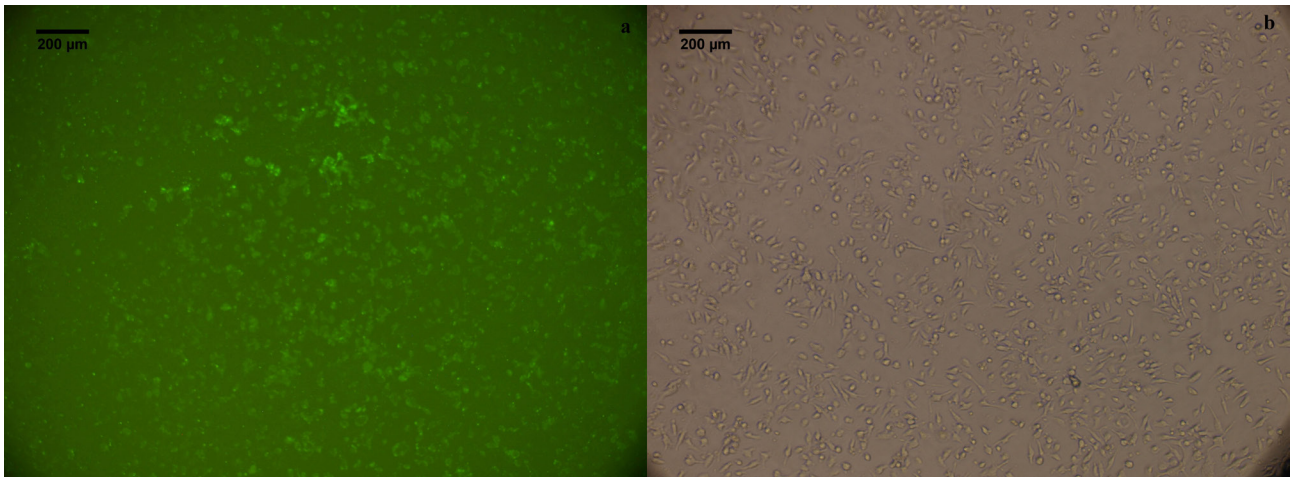

Figure 2. Fluorescence microscopy images of the cellular uptake of polyplex. **a:** fluorescent and **b:** bright fields.

MTT cytotoxicity assay

Based on Figure 3, the viability of the HUVECs treated with different concentrations of PEI significantly decreased at the final concentration of 5×10^{-4}% compared to the 2.5 and 1×10^{-4}% concentrations. Besides, no significant difference was observed between the 2.5 and 1×10^{-4}% concentrations in this regard. These results demonstrated that an increase in the amount of PEI was accompanied by an increase in the cytotoxicity of the cells.

Laboratory tests in the pancreatic islets indirectly treated with polyplex

Gene expression analysis

Isolated islets (Figure 4) treated indirectly with transfected HUVECs by polyplexes were functionally analyzed after 24 and 72-h. The expressions of SPRED-1 and PI3KR2, as miRNA-126 targets, were higher after 24-h than after 72-h. In fact, polyplex-126 could inhibit its targets after 72-h with the subsequent THBS downregulation and VEGF upregulation, which determined the function of miRNA-126. On the other hand, miRNA-210 downregulated its target, EFNA, after 72-h and resulted in the same trend. However, no significant difference was detected between the two groups ($p>0.05$) (Figures 5a and b).

The expressions of BAX and BCL2 as well as their ratios in the treated groups were in favor of the survival and upregulation of insulin gene expression (Figures 5c, d, and e).

Measurement of protein secretion

The VEGF protein secretion was similar in all the study groups. The secretion of THBS was higher in the polyplex-126 group compared to the other groups. In this respect, the polyplex-126 and -210 groups were significantly different from the control group ($p<0.05$). The secretion of C-peptide was higher in the polyplex-210 group in comparison to other groups. Additionally, a significant difference was observed between the polyplex-210 group and the control group in this regard ($p<0.05$) (Figure 6a).

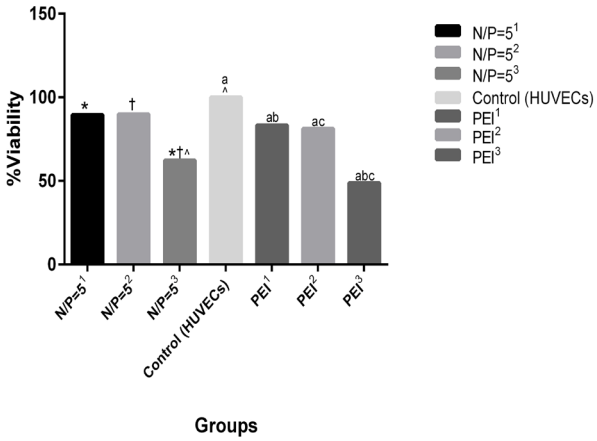

Figure 3. MTT cytotoxicity assay. Groups with the same superscripts were significantly different at α=0.05 ($p<0.05$). N/P, nitrogen/phosphate; PEI, polyethyleneimine; HUVEC, human umbilical vein endothelial cell. 1, 2, and 3: final concentrations of PEI; i.e., 1×10^{-4}%, 2.5×10^{-4}%, and 5×10^{-4}%.

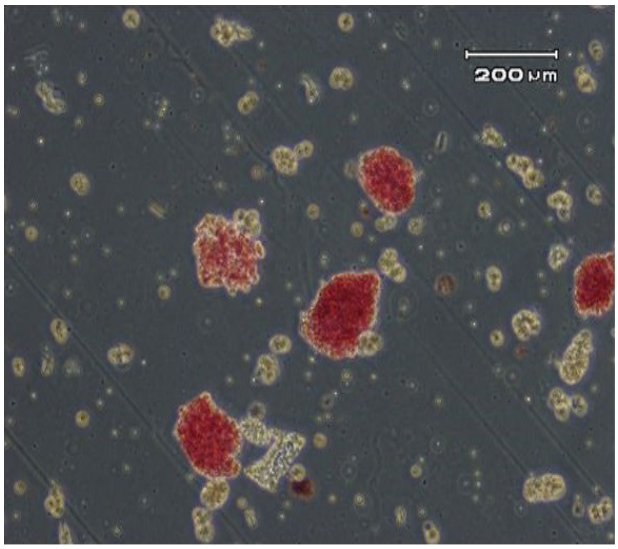

Figure 4. 1, 5-diphenylthiocarbazone staining of the mouse islets after digestion. Bright fields of microscope.

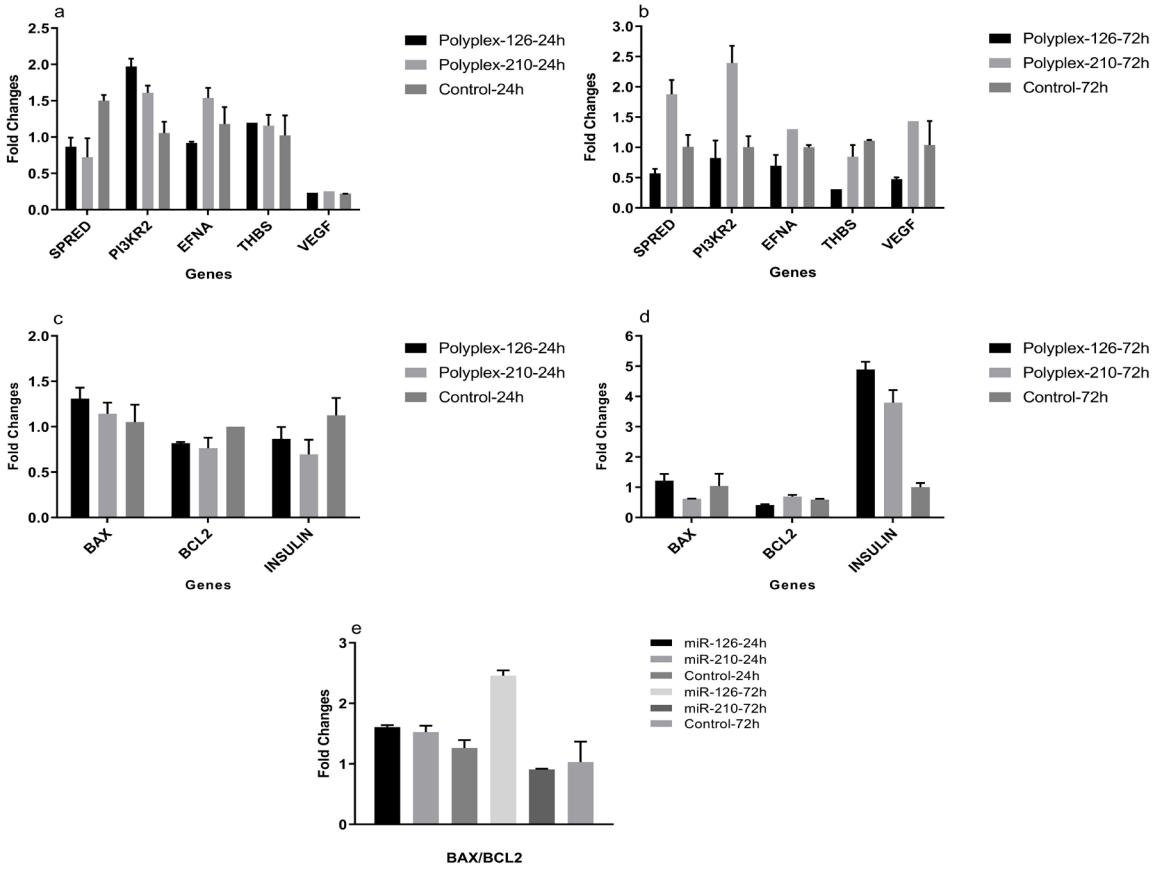

Figure 5. Gene expression in the pancreatic islets. There was no significant difference among the groups (p>0.05).

Insulin stimulation indices

Subsequent to the islets incubation for 72-h, the findings showed a significant difference between the groups treated with polyplex-126 and -210 and the control group concerning insulin secretion indices ($p<0.05$). However, no statistically significant difference was observed between the treated groups in this regard ($p>0.05$) (Figure 6b).

Discussion

During islet isolation, mechanical and enzymatic digestion causes the loss of microvessels and the transplanted cells over time. As a result, it is important to provide crucial revascularization for the maintenance of transplanted islets and return to their normal performance.[4-7] Intra-islet ECs are necessary for vascular integrity and have a function in angiogenesis as well as in response to ischemia or injury.[46] These cells play a crucial role in normal islet function, as well. In diabetes-induced rodents, islet grafts accompanied by ECs demonstrated greater engraftment capacity and improved islet function.[47,48] Various inducers such as VEGF can trigger islet ECs to produce vessels through angiogenic sprouting.[8-10] Hypoxia upregulates VEGF-A expression in islets,[49] which is critical for capillary tube formation by intra-islet ECs, preservation of pancreatic β-cells, and islets revascularization after transplantation.[50,51] In this way, miRNAs are also involved in angiogenesis.

The limited efficacy of miRNA transfection is a fundamental obstacle to using miRNAs as a therapeutic agent.[27] PEI is a non-viral transfected method that converts nucleic acid into positively charged particles. These particles bind with the endosomal lytic activity that is unique to the anionic components of the cell surface.[31,32] On the other hand, the cytotoxicity of PEI is an insurmountable barrier to successful gene transfer.[52] In this study, the polyplex formation after adding miRNA to PEI (as the nanocarrier) was investigated by using agarose gel electrophoresis. The ideal N/P molar ratio of the nanomaterials varied depending on their chemical nature and structure. At the N/P ratio=5, no bands were detected in the agarose gel and PEI could condense miRNA. This result was subsequently confirmed by the EtBr dye exclusion assay. After that, an assessment of the viability of ECs indicated that higher PEI concentrations were accompanied by an increase in the ECs' death and were thus not suitable for gene delivery. These results could be explain by the amine functional groups of the polycations resulting in interactions with negatively charged molecules on the cell surface.[52]

miRNA-126 and -210, as angiomiRs, acted by inhibiting their targets (PIK3R2 and SPRED1 for miRNA-126 and EFNA3 for miRNA-210). In this way, some signaling pathways were activated, eventually leading to VEGF upregulation.[22,25] According to the present study findings,

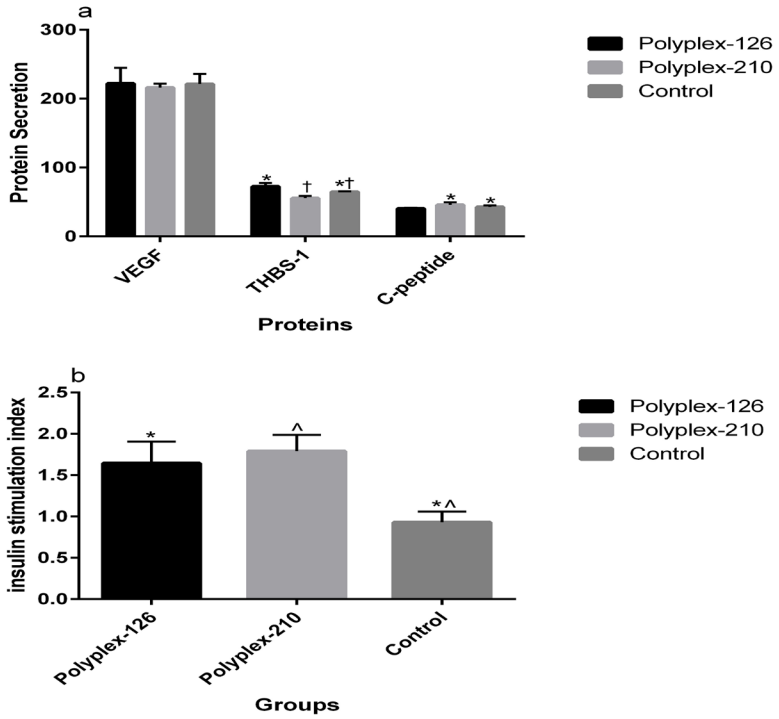

Figure 6. a. Measurement of protein secretion and **b.** Insulin stimulation indices. Groups with the same superscripts were significantly different at α=0.05 ($p<0.05$).

the transfected HUVECs with polyplex-126 and -210 that were indirectly co-cultured with mouse isolated islets resulted in a decrease in the expressions of the target genes related to these miRNAs in the islets. These findings were also proved in the studies carried out by Fish *et al.*[23] and Chen *et al.*[53] They explained that miRNA-126 could exert proangiogenic effects and promote survival and vascular regeneration by targeting the negative repressors of the VEGF pathway like SPRED-1 and PI3KR2. The response of endothelial cells to VEGF was regulated by miR-126 alteration. miRNA-126 knockdown also resulted in the loss of vascular integrity and hemorrhage during embryonic development in zebrafish.[23] miRNA-210 downregulated its target, EFNA3, and enhanced the expressions of VEGF and VEGFR-2. The decrease in the expression of these targets reduced the THBS expression and resulted in an increase in the expression of VEGF. All these changes led to the formation of capillary-like structures that determined the function of these miRNAs.[54] Zhang *et al.*[55] showed that miR-210 could upregulate VEGF in ischemic conditions and increase the survival rate.Moreover, Jansen *et al.*[56] reported that delivery of miRNA-126 by means of microparticles into ECs improved vasculogenesis via SPRED-1 in an endothelium denudation mouse model, resulting in endothelial repair by its migration and proliferation.

The current study results revealed that the expressions of Bax and Bcl2 and their ratios led to a better survival and function of islet cells as well as a higher expression of insulin and response to glucose stimulations in the groups treated with miRNA-126 and -210. Chen *et al.*[53] also reported that downstream proteins such as Bcl-2 in the PI3K/Akt pathway were remarkably changed via the inhibition or overexpression of miRNA-126 in ECs both *in vivo* and *in vitro*.[53]

In the present research, miRNAs augmented VEGF protein secretion in the mouse islets, which was consistent with the findings obtained by Zhang *et al.*[51] They found that microvasculature degree and insulin content was improved by VEGF transduction.[51] Furthermore, miRNA-210 could downregulate the antiangiogenic protein, THBS1, by increasing the secretions of C-peptide and insulin, which might be associated with a better prognosis of islet function after transplantation.

Conclusion

Prior to transplantation, neovascularization can increase islet survival and function by promoting islet-to-host inosculation, which is essential for proper engraftment.[11] This was supported by the present *in vitro* study, which demonstrated that the transfection of HUVECs with miRNA-126 and -210 could promote the angiogenesis status of islet cells through the downregulation of SPRED-1, PI3KR2, and EFNA-3 target genes. These islets could survive more and respond better to glucose stimulation, as well.

Ethics Issues

Approval was obtained from the Ethics Committee of the Departmental Committee for Care and Use of Laboratory Animals, Shiraz University of Medical Sciences, Shiraz, Iran (code: IR.SUMS.REC.1397.900).

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Author Contribution

FSS: Conceived of the presented idea, developed the theory and performed the computations, carried out the experiment, performed the analytic calculations, investigation, writing original draft, writing-review & editing, visualization, accountable for all aspect of the work; AMT: Investigation, writing-review & editing, visualization, supervision, project administration, accountable for all aspect of the work; MHK: Writing-review & editing, visualization, accountable for all aspect of the work; RY: Writing-review & editing, visualization, accountable for all aspect of the work; BG: Writing-review & editing, visualization, accountable for all aspect of the work; MH: Carried out the experiment, writing-review & editing, visualization, accountable for all aspect of the work; IHAA: Writing-review & editing, visualization, accountable for all aspect of the work; NA: Conceived of the presented idea, developed the theory and performed the computations, resources, writing-review & editing, visualization, supervision, project administration, funding acquisition, accountable for all aspect of the work.

Conflict of Interest

The authors declare that they have no conflict of interests.

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