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Preparation of Preproinsulin Gene Construct Containing the Metallothionein2A (pBINDMTChIns) and Its Expression in NIH3T3 Cell Line and Muscle Tissue of Alloxan Diabetic Rabbits

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Background: Diabetes mellitus type 1, formerly called insulin-dependent diabetes, is one of the autoimmune diseases where insulin-producing cells are destroyed by autoimmune response via T cells. The new approaches in treatment of diabetes are using the stem cells, cell transplantation of islet β cell, gene transfer by virus based plasmids, and non-viral gene constructs.
Objectives: The purpose of this study was to construct glucose inducible insulin gene plasmid and use it in the muscle tissue of the rabbit.
Materials and Methods: To achieve this goal, the preproinsulin, metallothionein2A promoter and the response element to carbohydrate genes were cloned into pBIND plasmid by standard cloning methods, to construct pBINDMTChIns. The gene cloning products were confirmed by the polymerase chain reaction (PCR) and restriction enzyme digestion template. The recombinant plasmid, containing the preproinsulin gene, was transferred into NIH3T3 cells and insulin gene expression was evaluated by reverse transcriptase PCR and western blotting techniques. Plasmid naked DNA containing the preproinsulin gene was injected into the rabbits' thigh muscles, and its expression was confirmed by western blotting method.
Results: This study shows the prepared gene construct is inducible by glucose. Gene expression of preproinsulin was observed in muscle tissue of rabbits.
Conclusions: These finding indicated that research in diabetes mellitus gene therapy could be performed on larger animals.

Keywords: Diabetes Mellitus; Preproinsulin; Rabbits; Gene Therapy

1. Background

Type 1 diabetes (T1DM), is a polygenic disorder and autoimmune disease in which insulin-producing cells are destroyed by autoimmune response via T cells (1, 2), leading to many biochemical and clinical problems, including hyperglycemia, kidney failure, retinopathy, and neuropathy (3). The classic treatment is using exogenous insulin, which is not sufficient to stable the blood glucose at normal level and could cause clinical complications (4, 5). Various procedures (such as pancreas transplantation, using stem cells, or islet cells) have been conducted to induce better results with fewer side effects. However, these methods can cause serious problems like risks related to surgery, lack of pancreas, graft rejection of the pancreas, and high cost (6, 7).

The other strategy is immunotherapy for suppressing the immune system (8-10). In recent years, viral and non-viral gene constructs such as the retro-associated, or adeno-associated viruses, have been used for T1DM gene therapy as some novel approaches (11). Although, non-viral vectors are weak, they are safer than viral vectors, and researchers tend to use these plasmids (12, 13). Despite the great efforts in engineering the insulin production, the perfect system has not been established for the optimal control of glucose. In this study, insulin plasmid gene construct was prepared for T1DM gene therapy. As the insulin production depends on exact regulation of glu-

cose levels, the plasmids were constructed for the same purpose. Vectors containing glucose 6-phosphatase and GLUT-2 promoter have already been used in cell lines and animal tissues of mice and rat (14, 15).

2. Objectives

Considering that diabetes mellitus is increasing in the world, and the classical treatment cannot improve disease appropriately; therefore, we designed pBIND eukaryotic vector consisting of the metallothionein2A promoter, the carbohydrates response element, and human preproinsulin gene (Gen Bank: JQ951950.1). We tested its responsiveness to glucose in rabbit.

3. Materials and Methods

3.1. Total RNA Extraction From Human Pancreatic Tissue and RT-PCR Reactions

Normal pancreas (from patients with brain death) was obtained from the Organ Bank of Imam Khomeini Hospital, Tehran, and immediately frozen in liquid nitrogen and saved at -80°C. Pancreatic tissue total RNA was extracted using the RNA extraction kit (QIAGEN) according to the manufacturer's protocol. Total RNA concentration was determined by Eppendorf Biophotometer and RNA integrity was confirmed by electrophoresis technique on 1.2% agarose gel (16). Synthesis of cDNA was performed by reverse transcription reaction using the 1 μg RNA, 2 μL random hexamer RT-Primer, 1x RT buffer, 50 U MultiScribeTMRT enzyme (Invitrogen™), 4.2 μL nuclease free water, 0.2 mM dNTP in 20 μL final volume. Then, the given master mix was incubated at 25˚C for 10 minutes and then at 37˚C for 120 minutes. The polymerase chain reaction was performed for gene amplification using the human preproinsulin forward and reverse primers (The primers prepared from MWG Company, Germany). The forward primer sequence was 5`-GCGGCCGCATGGCCCTGTGGATGCGC-3` and the reverse primer sequence was 5`-GGTACCCTAGTTGCAGTAGTTCTCCAG-3`. The primers contained NotI and KpnI restriction sites on the 5`ends. The forward and reverse universal primers for pBlueScriptSK (+) plasmid were 5`-GTAAAACGACGGCCAGT-3` and 5`- CAGGAAACAGCTATGAC-3`, respectively.

3.2. Cloning the Preproinsulin Gene

The pMNTCh plasmid containing metallothionein2A (MT2A) promoter and carbohydrate response element (ChoRE) was gifted by Prof. O L Kan. BamHI and NotI restriction enzymes (Takara Bio, Japan) were used for isolating MT2A promoter and ChoRE sequences. The purification of the given DNA sequences was performed by gel extraction kit (QIAGEN), ligated to BamHI and NotI digested pBIND plasmid, and named pBINDMTCh. PCR method was used to amplify the preproinsulin gene sequence. The PCR product was ligated to pBlueScriptSK (+) cloning vector by T/A cloning method and then was transformed into B21 *E. coli* strain as a competent cell. For plasmid extraction, colonies were cultured, then extracted plasmids were electrophoresed on 1% agarose gel (17). The plasmid containing preproinsulin gene was digested by NotI and KpnI restriction enzymes and inserted into the pBINDMTCh and produced pBINDMTChIns plasmid.

3.3. Cell Culture and Transfection

NIH3T3 cell line was obtained from National Cell bank of Iran (NCBI) (C156). The cells were incubated in DMEM media (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich Co.), containing 2 mM glutamine, 10% fetal calf serum (FCS) and penicillin-streptomycin (100 U/mL and 0.1 mg/mL, respectively) at 37˚C and 5% CO_2 in humidified air incubator (18). Four groups of NIH3T3 cells were used. The cells in the first group were negative control that received no substance. The second group was also negative control that received preproinsulin gene free plasmid. The third group was treated by pBINDMTChIns, and the fourth group by pBINDMTChIns plasmid and 16 mmol/L glucose. Three hours before transfection, cells were incubated with fresh medium. While NIH3T3 cells covered 50%-80% of the cultured surface, transfection was performed. Nearly 4×10^5 cells per 35 mm-plate were used for transfection. Transfection solution containing 5 μg of plasmid was incubated at 25˚C for 20 minutes and then added slowly as droplets to the plate. Plates were incubated at 37˚C for 8 hours in a CO_2 incubator. Then the medium containing calcium-phosphate was removed and the cells were washed with culture medium. Plates were fed with 2 mL of complete growth medium and 16 mmol/L glucose and were incubated at 37˚C for 24 hours until gene expression analysis were carried out. Total RNA was extracted by the Fermentas total RNA purification kit and gene expression was examined by RT-PCR and also protein expression was examined by western blotting method using anti-human insulin antibodies. At first, proteins were electrophoresed by SDS-PAGE method, and then the bands were transported to a nitrocellulose paper sheet. Uncovered surfaces of the paper sheets were blocked by milk proteins. Subsequently, the paper sheets were incubated with an antibody against insulin and secondary antibody that was conjugated with horse-radish peroxidase (HRP). Finally, the substrate was added to nitrocellulose to visualize the detected bands.

3.4. Animal Studies

3.4.1. Preparing Diabetic Animals

Male New Zealand white rabbits weighing 1300 ± 200 g were purchased from Pasteur Institute of Iran (IPI).

Animals were kept on standard chow with free access to food and water and in controlled conditions (19, 20). For environmental adaptation, rabbits were kept under the condition mentioned above for one week. Animals were divided into four groups containing five rabbits in each. The first group of rabbits (control, normal non-diabetic) were injected by 1 mL of sterile citrate buffer (pH = 4.5). Diabetes was induced in the second (diabetic control), third, and fourth groups using alloxan injection. Alloxan was dissolved in fresh sterile citrate buffer (pH = 4.5) and then the solution (175 mg/kg) was administered intravenously after overnight fasting. Diabetic animals were verified as weight loss and fasting blood glucose concentrations greater than 300 mg/dL at three days after alloxan injection (20). Glucometer (BioNime GM300) was used to measure blood sugar (glucose oxidase method). Blood sampling was performed in animals marginal ear vein before and after treatment with alloxan.

3.5. Intramuscular Injection of Plasmid

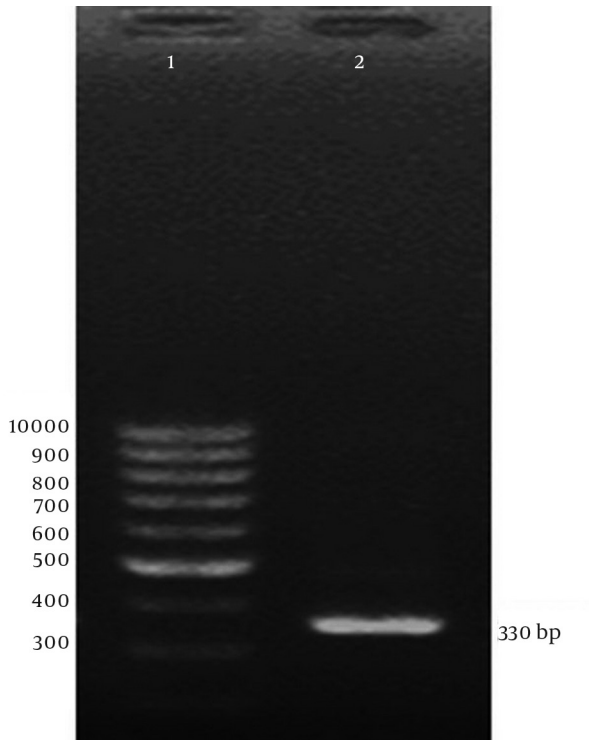
The second group of diabetic rabbits was injected with empty pBIND plasmid. The third group (as the control group) was injected by 2 mL of endotoxin-free PBS into the thigh muscle and the fourth group was injected with pBINDMTChIns plasmid. Before injection, pBINDMTChIns plasmid was tested by LAL (Limulus Amebocyte Lysate) pyrogen test (21). Four milliliter of plasmid solution containing 400 μg of the plasmid DNA was injected into rabbits' thigh muscles. Four days later, blood samples were taken from the marginal ear vein of all rabbits. Then all animals were killed by a large dose of pentobarbital (100 mg/kg IV) and their muscles were collected and immediately frozen in liquid nitrogen (19). Total RNA was extracted from the tissues, and preproinsulin gene expression was investigated by RT-PCR and western blotting using specific antibodies.

4. Results

After mRNA extraction and reverse transcription of total mRNA to cDNA, agarose gel electrophoresis was used for detecting the PCR product bands as shown in Figure 1, where lane 2 shows the amplified proinsulin gene compared with the low-range DNA ladder. Since proinsulin-gene sequence contains 330 bp, the detected band in this region (Figure 1) confirmed the correct PCR reaction and products.

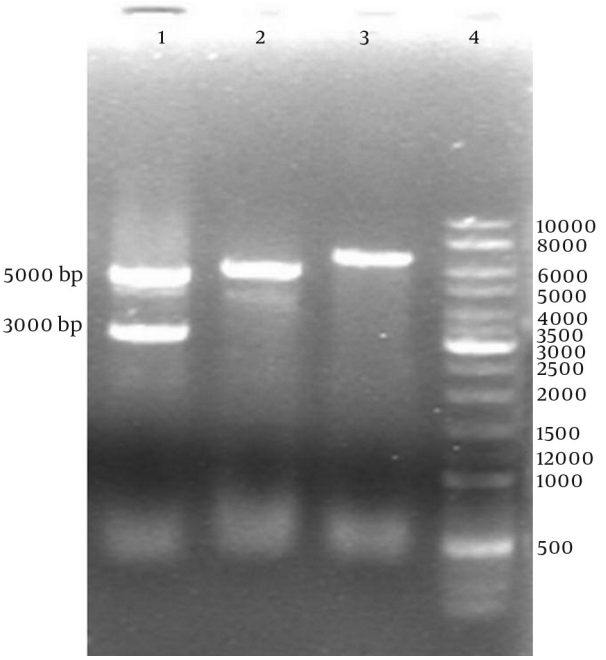
Figure 2 shows the results of electrophoresis of digested and undigested pBINDMTCh plasmid on 1% agarose gel. In Figure 2, lane 1 shows that pBINDMTCh plasmid has been digested by BamHI and NotI restriction enzymes, thus both ChoRE gene and MT2A promoter were amplified together to show a band at the 3000 bp. Lane 2 shows the linear pBIND plasmid that has been digested and become linear by NotI enzyme that is 6360 bp in length. Lane 3 shows the undigested pBINDMTCh plasmid, that it is 7817 bp in length.

Figure 1. Electrophoresis Pattern of PCR Product of Amplified Proinsulin Gene in Agarose Gel (2%)



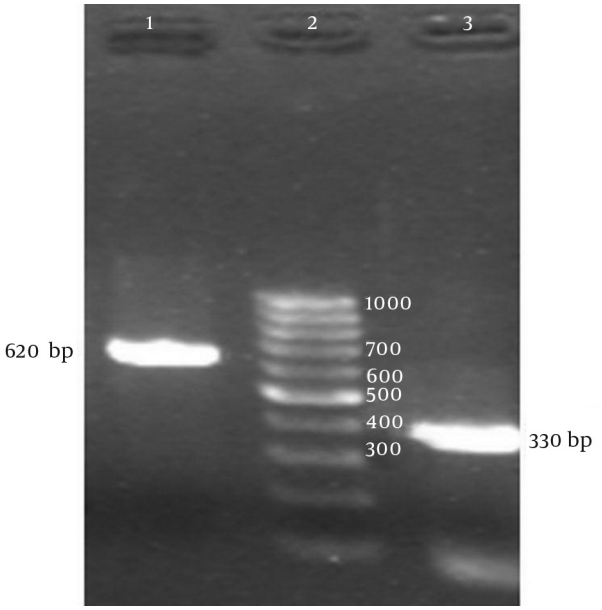
Lane 1, DNA ladder marker; Lane 2, the 330 bp PCR product of preproinsulin gene.

Figure 2. Electrophoresis Pattern of Digested and Undigested pBINDMTCh Plasmid in 1% Agarose Gel



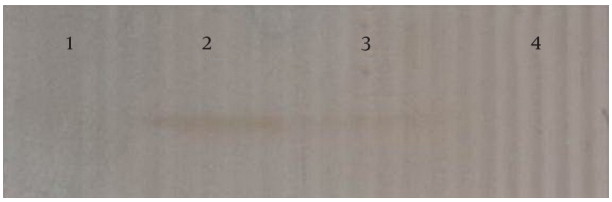
Lane 1, The BamHI and NotI digested pBINDMTCh; Lane 2, The linear pBIND plasmid that has been digested by NotI enzyme; Lane 3, The undigested pBINDMTCh; Lane 4, DNA ladder (high range).

Figure 3. Electrophoresis of PCR Products of pBlueScriptSK(+) Plasmid Containing the Preproinsulin Gene on 2% Agarose Gel



Lane 1, The PCR product of pBlueScriptSK (+) plasmid using the plasmid universal primers; Lane 2, The DNA ladder; Lane 3, The PCR product of pBlueScriptSK(+) plasmid using the special preproinsulin gene primers.

Figure 4. Protein Analysis by Western Blotting on NIH3T3 Cell Culture Expressed Preproinsulin by Anti-human Insulin Antibody



Lane 1, Negative control (only NIH3T3 cell culture); Lane 2, NIH3T3 cells contained pBINDMTChIns and 300 mM glucose; Lane 3, NIH3T3 cell transfected by pBINDMTChIns plasmid; Lane 4, Negative control (NIH3T3 cell was transfected by intact pBIND plasmid).

Figure 5. Western Blotting Analysis on Rabbit Thigh Muscle Expressed Proinsulin by Anti-human Insulin Antibody

Lane 1, Positive control (human insulin protein); Lane 2, extraction of rabbit thigh muscle transfected by pBINDMTChIns plasmid.

Figure 3 shows the results of electrophoresis of PCR products of pBlueScriptSK (+) plasmid containing the preproinsulin gene on 2% agarose gel. Lane 1, shows the PCR product of pBlueScriptSK (+) plasmid using the plasmid universal primers, that it is 620 bp in length. Lane 3, shows the PCR product of pBlueScriptSK (+) plasmid using the special preproinsulin gene primers, that its length is 330 bp. Thus, these results confirmed that the preproinsulin gene cloning has been performed correctly. Also, the DNA sequencing was performed (MWG Company, Germany) to confirm the presence of preproinsulin gene, and the gene sequence was registered in the Gene Bank (JQ951950.1) and published in world wide web (http://www.ncbi.nlm.nih.gov/genbank/).

Figure 4 shows the protein analysis by western blotting on NIH3T3 cell culture expressed preproinsulin by anti-human insulin antibody. Lane 1, shows negative control, NIH3T3 cell line without receiving any plasmid, thus there is no band in this region. Lane 2, shows the NIH3T3 cells containing pBINDMTChIns and 300 mM glucose, thus there is a relatively wide band in this region. Lane 3 shows NIH3T3 cell transfected by pBINDMTChIns plasmid. The bands in this region, confirms correct transfection and protein production. Finally, lane 4 shows the negative control, NIH3T3 cell transfected by intact pBIND plasmid. Also, there is no band in this region like the lane 1.

4.1. Results of Diabetic Animals

The average amount of non-diabetic animals' blood sugar was about 88 mg/dL. Average blood sugar level of diabetic animals in the groups 2, 3, and 4 were 345, 337, 359 mg/dL, respectively. Free preproinsulin gene pBIND plasmid was injected to the second group of animals; endotoxin-free sterile PBS was injected to the third group and pBINDMTChIns plasmid was injected to the fourth group. The blood sugar levels after injection were 350, 345 and 340 mg/dL respectively. Four days after injection, blood sampling was done, then the animals were sacrificed. RNA was extracted from muscle tissue at the injection site, and the insulin gene expression was confirmed by RT-PCR and western blotting (Figure 5). In Figure 5, the band in lane 1 is related to positive control (human insulin protein as control) and the band in lane 2 is associated with extraction of rabbit thigh muscle transfected by pBINDMTChIns plasmid, thus it confirms the protein expression.

5. Discussion

Diabetes mellitus type 1 is one of the metabolic diseases caused by autoimmune destruction of islet β cells (22). Although, some developments have been made in improving pathologies of diabetes and the quality of patients' life, no treatment has been effective in reducing clinical complications or reversing progression of the disease (23). Until now, many studies have been carried out about type 1 diabetes gene therapy, but none of

them have been done on larger animals like rabbits. In this study, non-viral vector was made differently from other vectors and used in NIH3T3 cell line and rabbit muscle tissues, which its protein expression was shown for the first time. Here we also assessed whether production of insulin in NIH3T3 cell line and rabbit muscle cell can be stimulated by glucose. So far, various therapeutic strategies have been used for improving type 1 diabetes, including functional pancreatic tissue or β cells transplantation in type 1 diabetes patients and conversion of mesenchymal stem cells into pancreatic islet cells (24). Islet β cells transplantation as a promising therapy for type 1 diabetes patients can control blood glucose level and accomplish insulin; however, insulin therapy is still a major approach, because the pancreas donors are limited, and long-term immune system suppression is needed too (22, 25, 26). Nevertheless, there are many major problems about islet transplantation such as adverse immune responses induced by the islet transplantation process, host autoimmunity recurrence, allorejection and shortage of organ donors (27).

Therefore, researchers have been encouraged to use other approaches like unlimited expression vectors. Glucose responsive insulin production is a principle regulatory component. Many attempts have been carried out for constructing insulin gene vector to response to glucose that was led to use the promoters containing the glucose-responsive elements. This is not perfect; however, it looks to be efficient (28). Many efforts have been made to prevent graft rejection such as constructing retroviral vector and double-stranded adeno-associated virus serotype 8 (dsAAV8) containing IL-4 gene in combination with β cell growth factors by Gaddy and Kapturczak (23, 29). The Kapturczak`s designed vector had a desirable rate of protein expression, but there were some restrictions in carrying out and biosafety due to vector expression rates (29). On the other hand, Gaddy claimed that aDsAAV8 vectors containing IL-4 gene along with hepatocyte growth factor/NK1 or GLP-1 under the control of the insulin promoter would increase islet β cell proliferation rate and survival, in NOD mice, and reversed diabetes progression in approximately 10% of these mice (23).

But one of the important differences of the mentioned vectors compared to our designed vector is that their structures were viral, thus their safety is disputed in the animal body. In comparison with designed non-viral vector expressing preproinsulin in this study, which can compensate the weakness in insulin production; designed viral vectors by Gaddy and other researchers targeted the immune response to improve T1DM disease, although many clinical trials have been performed using antigen-specific techniques and immune correcting drugs, they often have demonstrated too toxic or have been unable to prepare long-time islet β cell protection (30).

For this reason, multiple therapeutic approaches should be used for the generation of new islet β cells. Typically, two types of non-viral and viral constructs have been designed to target cells for type 1 diabetes treatment. Some researchers like Zipris designed retroviral vector of IL-4 gene for the correction of autoimmune T1DM (31), though there were still former problems as mentioned above about Gaddy and Kapturczak reports (23, 29). Using the recombinant viral vectors has been created numerous problems such as frequent administration, unstable and variable expression, induction of immune response and hepatic inflammation, in spite of positive performance and high transfer efficiency (32), while in this study, we tried to provide a new efficacious non-viral construct and consider the mentioned disadvantages about viral vectors immune response. Dong and colleagues used recombinant adeno-viral construct against experimental induced T1DM, and their results indicated that the viral vectors have immunogenic effects and increase the inflammatory factors in CD-1 mice tissues such as hepatic tissue (33). In addition, a remarkable and contradictory result was also achieved in their report. They expected that insulin synthesis in liver increases the glycogen, but the results indicated that insulin production inhibited the glycogenesis in liver. It may be relevant to the cytokines. The same findings were also obtained by the hepatic insulin gene therapy (HIGT) demonstrating prevention of glycogen biosynthesis in hepatic cell culture medium by high concentrations of insulin and glucose (34).

Regarding so many drawbacks in the application of viral vector and use of hepatic tissues as target recipient tissue for gene delivery, we were encouraged to make non-viral vectors. However, we had previously developed a gene construct containing the insulin gene and MT2A promoter based on pcDNA3.1(-), but it lacked the GFP for gene expression detection (35). The designed vector in this study was made based on pBIND eukaryotic plasmid and contains bifunctional metallothionein2A promoter (MT2A) along with the carbohydrates response element (ChoRE) as multimer; basic metallothionein is expressed in the liver as metal cis-acting response element. The ChoREcis-acting element involved in glucose induced transcription, which contains two E-box and CACGTG motifs separated by 5 bp. The metallothionein2A is the main promoter sequence and helps glucose-induced transcription. On the other hand, due to capability of glucose responsive multimeric ChoRE, it was used as an auxiliary DNA element. The pBINDMTChIns is the first gene construct based on pBIND eukaryotic expression vector. It contained luciferase gene for easy detection of recombinant plasmid containing the insulin gene in cells and tissues as an advantage to other non-viral vectors. Testing pBINDMTChIns into NIH3T3 cell culture in the presence and absence of glucose as a stimulus showed the capability of inducing insulin production. The semi-quantitative proinsulin gene expression was detected by western blotting with satisfactory results, although for more accuracy, more advanced techniques such as Real-Time PCR are needed.

This study revealed that pBINDMTChIns can produce the preproinsulin in the NIH3T3 cell line so the glucose can act as a stimulant in this process. MT2A promoter in pBINDMTChIns vector has low ability to react to glucose stimulation, thus, adding ChoRE to MT2A promoter could enhance its ability. Concerning the existence of ChoRE as multiple copies following MT2A, this promoter can be an appropriate candidate for viral and non-viral vectors and even it can be used in liver tissues compared to L-pyruvate kinase, glucose transporter-2 (GLUT-2), glucose 6-phosphatase (14, 15), and glucagon-like peptide-1 (GLP-1) promoters (36). However, using GLP-1 as a cell therapy-based treatment may regulate blood sugar properly (28), but it may have immunogenic property that has not been known yet. Administration of naked recombinant plasmid into NIH3T3 cell indicates that the transfection has been carried out properly, and the non-viral gene construct can be expressed and stimulated by glucose.

These results could provide some information for subsequent success in the progress of the non-viral gene constructs as new gene therapy approach. Since first observational studies have been accomplished by all kinds of vectors on various cell lines, the assessment of vectors is a substantial subject for passing from cell lines to animal models (33). Most viral and non-viral constructs were tested on small animals such as mice and rats, although the evolution of gene constructs are very important. On the other hand, it should be noted that achieving the ultimate goal of gene therapy for use in human eventually leads to the transition from small animals to larger animals. Therefore, in this study the rabbit was used as a host for non-viral construct for the first time, because of the similarity of its sugar metabolism to that of human (37). Also, there are a large number of similarities in terms of metabolism between humans and rabbits (20, 38). Niessen performed a similar study on canine muscle cell line, whose results showed that muscle cell line could be induced to secrete canine insulin when transfected with non-viral vector containing a mutant canine preproinsulin gene, which is able to produce furin enzyme cleavable preproinsulin in host cells (39).

Thus, observing the preproinsulin gene expression in pBINDMTChIns plasmid in NIH3T3 cell line and rabbit muscle tissue for the first time may be the basis for the subsequent investigations related to T1DM gene therapy on the large animals. Therefore, we recommend subsequent investigations study non-viral constructs on larger animal models and also the use of quantitative Real-Time PCR for evaluation of gene expression in cell lines and animal tissues.

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Author's Contributions

Study concept and design: Hossein Piri, Mohammad Taghi Goodarzi, Bahram Kazemi and Iraj Khodadadi; acquisition of data: Hossein Piri, Mojgan Bandehpour, Jamshid Karimi, Amaneh Koochaki, Ali Torabi and Maryam Javadi; analysis and interpretation of data: Bahram Kazemi and Mojgan Bandehpour; drafting of the manuscript: Hossein Piri, Bahram Kazemi and Mohammad Taghi Goodarzi; critical revision of the manuscript for important intellectual content: Mohammad Taghi Goodarzi and Bahram Kazemi; statistical analysis: Maryam Javadi and Amir Ziaee; administrative, technical, and material support: Amir Ziaee and Ali Torabi; and Study supervision: Bahram Kazemi, Iraj Khodadadi and Mohammad Taghi Goodarzi.

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