

Research Article

Insulin-producing cells from bone marrow stem cells versus injectable insulin for the treatment of rats with type I diabetes



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ABSTRACT

Recently, stem cells have been considered renewable cell sources in the treatment of diabetes and the development of insulin-producing cells. In this regard, the current study aimed to compare Insulin-producing cells from bone marrow stem cells with injectable insulin in rats with type I diabetes. For this purpose, 40 rats were divided into four groups: the control or healthy group, the diabetic control group, the group that received differentiated insulin-producing cells from bone marrow, and the group that received insulin treatment. To differentiate insulin-producing cells from bone marrow, the femoral bone marrow of rats was extracted using the flushing method. Differentiated cells were evaluated using dithizone-specific dye, anti-insulin-proinsulin antibodies, and anti-insulin beta receptors. Also, the expression of the pdx-I gene, as the specific gene of pancreatic cells, was examined by RT-PCR. The results showed that transplantation of insulin-producing cells could significantly increase blood insulin levels in diabetic rats. This increase intensified in the second stage of transplantation when more cells were injected into rats. Concerning decreasing blood sugar levels, differentiated cells were able to reduce blood sugar levels significantly. Even in the first stage of cell injection, in which the rats received a small number of cells, their blood sugar levels were controlled by these cells. As a result, the present study showed that repeated transplants of insulin-producing cells differentiated from bone marrow could decrease blood sugar and increase insulin levels.

1. Introduction

Diabetes mellitus is the most common metabolic disorder associated with high blood sugar in humans [1, 2]. Diabetes can be caused by a lack of insulin (type I diabetes) or by peripheral tissue resistance to insulin with a decrease in insulin secretion from beta cells of Langerhans Islets (type II diabetes) [3]. The disease has grown exponentially since 1985, and by 2020 the number of affected people

has reached 515 million. The number of patients in the world has increased sevenfold in the last 20 years and if the current trend continues with an annual increase of seven million people in the population of these patients, by 2030, this number will reach 585 million people [4].

Type I diabetes is an autoimmune disease that targets pancreatic insulin-producing cells,

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resulting in the activation of specific T lymphocytes against beta cells [5]. This process is mainly caused by CD4+ cells, which act on type I helper T cells, as well as proinflammatory cytokines such as interferon-gamma (IFN γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF α) [6]. It is well known that insulin therapy with exogenous insulin cannot return variable blood glucose levels to normal, accurately and severely [7]. In patients with type I diabetes, treatment with exogenous insulin is a standard and effective treatment, but with careful control of blood sugar levels and in patients with high capacity, secondary diseases such as kidney failure, retinopathy and diabetic foot are common after years of treatment [8].

Langerhans islet cell transplantation is a potential treatment for type I diabetes [9]. But this therapeutic approach is limited by the lack of suitable tissue donors for transplantation [10]. An alternative to transplantation is to implant a renewable source of insulin-producing cells [11]. In the initial trials and early stages of clinical trials, pancreatic islet transplants require repeated use of immunosuppressive drugs [12]. The use of these drugs predisposes the patient to a variety of diseases, especially infectious diseases [13]. Recent advances in genetic and immunological screening to identify pre-diabetic patients have created the opportunity to prevent or delay the progression of the disease before diagnosis [14]. Mesenchymal stem cells, in addition to their extensive ability to proliferate and differentiate into different types of mesoderm tissue, also can suppress certain subtypes of immune cells such as memoryless T cells, B cells, dendritic cells, and natural killer cells [15]. Mesenchymal stem cells can also reduce the production of inflammatory cytokines [16].

Recent studies in laboratory and animal models have shown that mesenchymal stem cells play an effective role in regulating immunity in autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and encephalitis [17, 18]. Recently, many researchers have become interested in adult stem cells because they can transform into many tissues and there is no

moral concern about using them [19, 20]. Various studies have shown that insulin-producing cells can be made from stem cells isolated from bone marrow, fetal umbilical cord, fresh or frozen cord blood, and adipose tissue [21-23].

Bone marrow-derived mesenchymal stem cells not only inhibit T cell proliferation but also inhibit the antigen-specific response in T21 cells without exposure and memory to identify the peptide in the mouse model [24]. Isolation of mesenchymal stem cells from bone marrow has been performed in many studies. Stable expression of the PDX1 gene is a key factor in the expression of beta cell genes and pancreatic evolution [25]. This genetic factor in cultured human embryonic cells can activate a phenotype similar to differentiated beta cells. Although no side effects have been reported in the treatment of mesenchymal stem cells, their long-term effects on immunogenicity and tumorigenesis need further investigation [24].

The study aimed to evaluate the therapeutic effect of bone marrow mesenchymal stem cell transplantation in the treatment of type I diabetes.

2. Materials and methods

2.1 Statistical population

The statistical population of the present study consists of 40 laboratory rats, which were divided into 4 groups of 10. The first group was the control group, the second group became diabetic by intraperitoneal injection of streptozotocin, the third group became diabetic and received bone marrow stem cells through injection, and the fourth group became diabetic and was injected with insulin.

2.2 Bone marrow preparation and Culture

Bone marrow stem cells were extracted from the femur of 40 rats by the flushing method. After isolation of bone marrow stem cells, these cells were cultured in a medium containing DMEM (Gibco, USA), 15% FBS (Sigma-Aldrich, USA), 1% non-essential amino acids (Sigma-Aldrich, USA), and penicillin/streptomycin 1% (Sigma-Aldrich, USA). After the cells filled the bottom of the

plate, the plates were washed with PBS and the cells were separated using Trypsin-EDTA (Sigma-Aldrich, USA), and finally, the cells were transferred to culture flasks by centrifugation and discarding the supernatant. The culture medium was replaced every two days with LG-DMEM containing 15% FBS serum. Cells were cultured on days 10-14 using 25% trypsin and 0.5mM ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich, USA). The resulting cell pellet with LG-DMEM medium containing 15% FBS serum was transferred to new flasks or hexahedral plates containing sterile lamellas and incubated at 37°C and 5% CO₂ pressure [26].

2.3 Differentiation of bone marrow stem cells into insulin-expressing cells

To differentiate bone marrow mesenchymal stem cells, second culture cells were cultured in a single layer with LG-DMEM medium, 3% FBS serum and 1% antibiotics in an incubator. After reaching the appropriate cell density, the cells were added to the culture medium of pancreatic extract for six to ten days and the medium containing the extract was changed every two days. Serum-free DMEM/F₁₂ medium was used for differentiation by positive Nestin cell selection method. DMEM/F₁₂ culture medium containing ITSFn was first added to the cells to isolate positive cells with positive Nestin precursor. At this stage, Nestin cells remained positive and the rest were removed. Then, in order to stimulate cell proliferation, additives such as N₂ (Gibco, USA), B₂₇ (Gibco, USA), fibroblast growth factor type b and epidermal growth factor were added to the culture medium. Finally, the pancreatic extract was added to induce differentiation [27].

Dithizone staining was used for the morphological examination of differentiated cells. 50mg of dithizone (Merck, Germany) in 5ml of dimethyl sulfoxide was stored as a stock solution at -20°C. When dithizone with a final concentration of 10µM was added to the cells and incubated in the incubator for 15 minutes. The cells that turned red as a result of this staining were insulin-producing.

After washing with Hank's balanced salt solution (HBSS) and adding the differentiated

culture medium, the cells were returned to the incubator, and a neobar slide was used to count the differentiated and undifferentiated cells, and the count was repeated five times for each sample. The stained cells were trypsinized and then centrifuged using a neobar slide and a light microscope for counting. The numbers were analyzed using the Chi-square test and the results were presented. The Immunofluorescence method was used to detect the presence of beta-cell markers in differentiated cells. After washing twice with PBS, the differentiated cells were immobilized with 4% paraformaldehyde for 20 minutes. The permeability was increased with Triton 3% X-100 and normal goat serum 10% for 30 minutes in the incubator. Then, they rinsed with PBS twice for 5 minutes each time. The cells were then incubated with the primary anti-insulin-proinsulin antibody (Sigma-Aldrich, USA) or the primary anti-insulin beta-receptor antibody (Sigma-Aldrich, USA) for two hours. Anti-mouse IgG antibody bound to fluorescence isothiocyanate (FITC) (Sigma-Aldrich, USA) produced in goats was used as a secondary antibody. The samples were then glued with 70% glycerol (Acros Organics, USA), insulated with nail polish, and examined under a fluorescence microscope.

2.4 Insulin-producing cell gene expression

RT-PCR was used to study the expression of a specific gene of insulin-producing cells, pdx-I [28]. First, all cell RNAs were extracted using an RNA extraction kit (Qiagen, Germany). One microgram of the extracted RNA was reverse transcribed using the Revert Aid™ H-Minus First Strand cDNA synthesis kit (Fermentase, USA) and Random hexamer primer and Oligo primer (Fermentase, USA). RNA was also extracted from the negative control sample. The generated cDNA was then used as a template for PCR. The sequence of primers required in this study is shown in Table 1. The PCR product was transferred to 1% agarose gel and stained with ethidium bromide. In order to ensure the accuracy of the extracted RNA and also the correctness of the steps, the Housekeeping GAPDH gene was used as an internal control.

Table 1. Primer sequence of studied genes in Real-Time PCR analysis

| Gene | | Sequence(F/R) |
|-------|---|-----------------------------|
| pdx-I | F | 5'-CCTTCCCGTGGATGAAATCC-3' |
| | R | 5'-GTCAAGTTCAACATCACTGCC-3' |
| GAPDH | F | 5'-GTTTCCACCTCCTCACCACA-3' |
| | R | 5'-GATGTCGTTGCCACCACCT-3' |

2.5 Treatment of studied rats

Mesenchymal stem cells were injected with a specified number of cells per rat in 500µl of culture medium via a tail vein into an anesthetized animal [29]. In the first step, 83,000 cells per rat in 500µl of serum-free culture medium were transplanted through the tail vein. At this stage of the study, several rats were excluded from the study due to mortality. In the second step, 250,000 cells were transplanted per tail with a serum-free culture medium via tail vein. In the fourth group of rats, instead of cells, insulin was injected into each rat.

Blood glucose levels of animals before and after transplantation were measured weekly and between 8-9 am by a glucometer (Exicheck). The level of insulin produced was also monitored by measuring the level of blood insulin by ELISA with a Mercodia kit made in Sweden.

2.6 Statistical analysis

The obtained results were analyzed by SPSS 23 software and descriptive statistics, t-test and analysis of variance. All experiments were repeated three times. The significance level ($P \leq 0.05$) was considered.

3. Results

3.1 Differentiation of bone marrow stem cells into insulin-expressing cells

Bone marrow mesenchymal stem cells were obtained from adult male rats. These cells had the property of adhering to plastic containers and the ability to form colonies *in vitro*. At the beginning of extraction, these cells were spherical and after a week, they became spindle-shaped and elongated. The appearance of these cells is similar to fibroblast cells. The difference is that fibroblast cells are not able to tolerate successive passages and are not able to differentiate into different cell lineages.

Insulin-producing cells derived from bone marrow mesenchymal stem cells were reddened using dithizone staining. There were also a small number of red cells in the negative control group, indicating spontaneous differentiation of some cells into pancreatic beta cells. In vitro, these cells were able to differentiate spontaneously into insulin-producing cells. But the number of red cells in the treatment group (81.3 ± 6.9) was significantly higher than the control group (14.7 ± 7.3) ($P \leq 0.05$). In the immunofluorescence method, in order to study the expression of specific factors of insulin-producing cells, anti-insulin-pro-insulin primary antibodies and insulin-beta receptor anti-receptor antibodies were used separately. Insulin-producing cells were able to express insulin-pro-insulin protein and insulin beta receptors.

3.2 Insulin-producing cell gene expression

To study the expression of pdx-I gene, RNA of differentiated cells, undifferentiated cells (as a negative control sample), and pancreatic tissue (as a positive control sample) were extracted and converted to cDNA. Then, the PCR product was applied on an agarose gel. The results showed that differentiated cells could induce pdx-I gene expression. No expression was observed for pdx-I gene expression in mesenchymal stem cells (Fig. 1).

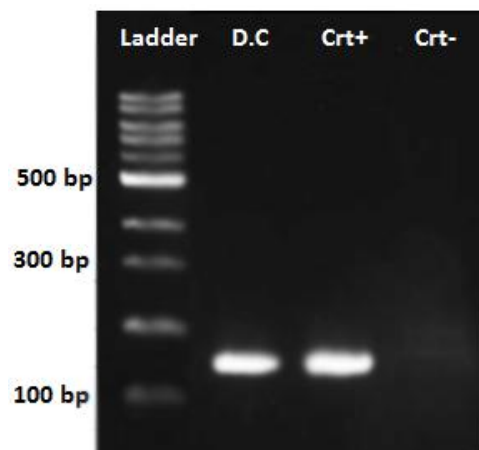


Fig. 1. Expression of pdx-I gene in differentiated cells by RT-PCR method; D.C= Differentiated Cells, Crt+= Pancreatic Tissue, Crt-= Undifferentiated Cells

3.3 Evaluation of insulin and blood sugar levels in the experimental groups

Figure 2 shows the mean insulin one week after the first stage of transplantation on the studied groups, which there was a significant difference between them ($P < 0.001$). Although there was a significant difference between the third and fourth groups, the results showed that cell transplantation was able to increase blood insulin levels in diabetic rats more than the control group (healthy rats) ($P < 0.05$).

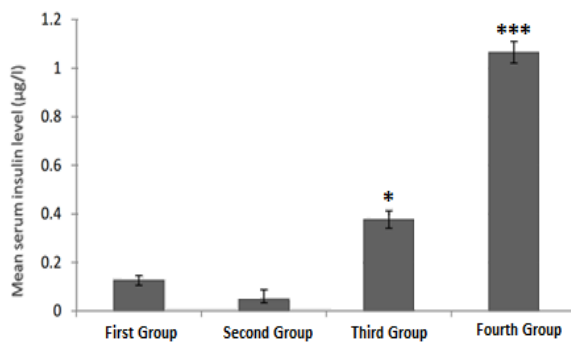


Fig. 2. Mean serum insulin level after one week from the first stage of stem cell use in the study groups; First group: control group, Second group: diabetic rats, Third group: diabetic and received bone marrow stem cells rats through injection, Fourth group: diabetic and injected with insulin rats

Figure 3 shows the mean insulin one week after the second stage of transplantation in the study groups. The results showed that with increasing the number of transplanted cells, the level of insulin in the third group increased as much as the fourth group, that received direct insulin injection, and there was no significant difference between the groups 3 and 4, but there was a very significant difference between groups 3 and 4 with groups 1 and 2 ($P < 0.001$).

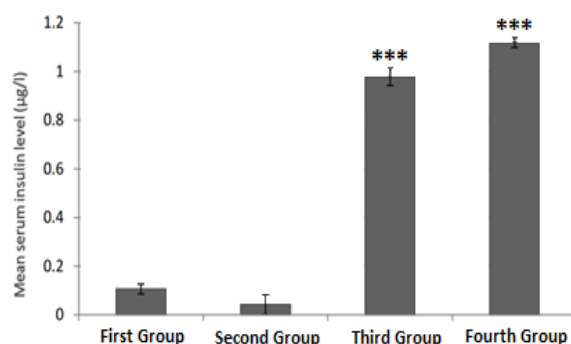


Fig. 3. Mean serum insulin level after one week from the second stage of stem cell use in the study groups; First group: control group, Second group: diabetic rats, Third group: diabetic and received bone marrow stem cells rats through injection, Fourth group: diabetic and injected with insulin rats

Figure 4 shows the mean blood sugar in different groups before and one week after the first stage of cell transplantation. In the insulin group, the mean blood glucose before injection was 901 mg/dl and after injection was 325 mg/dl, which was a significant difference. This mean was also seen in the cell recipient group, with mean blood sugar averaging 821 mg/dl before transplantation and 463 mg/dl after transplantation. By comparing the mean changes between groups, there was a decrease in mean blood glucose of 576 units in the insulin group and a decrease in mean of 358 units in the cell recipient group versus increasing changes in the diabetic group. The results showed a significant difference between all groups.

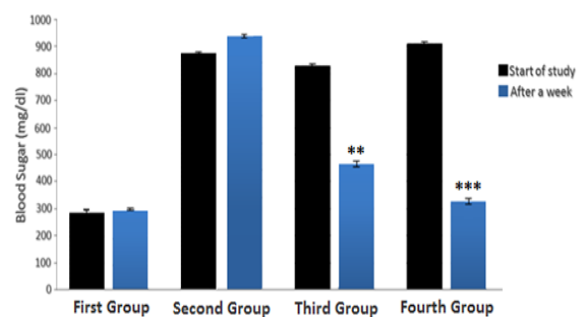


Fig. 4. Mean blood sugar level after one week from the first stage of stem cell use in the study groups; First group: control group, Second group: diabetic rats, Third group: diabetic and received bone marrow stem cells rats through injection, Fourth group: diabetic and injected with insulin rats

Figure 5 shows the mean blood sugar, in the different study groups, before and one week after the second stage of cell transplantation. The mean blood glucose of the insulin group was 311 mg/dl before transplantation and 298 mg/dl after injection, which was not a significant difference. But there was a significant difference in the group that received the cells, with an average of 450 mg/dl before transplantation and 320 mg/dl after transplantation. Comparing the mean changes between groups, the decrease in mean blood glucose of 87 units in the insulin group and the decrease in changes of 130 units in the cell group, it can be concluded that cell transplantation was able to reduce blood sugar levels well.

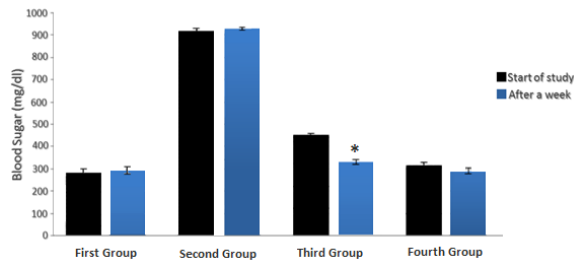


Fig. 5. Mean blood sugar level after one week from the second stage of stem cell use in the study groups; First group: control group, Second group: diabetic rats, Third group: diabetic and received bone marrow stem cells rats through injection, Fourth group: diabetic and injected with insulin rats

4. Discussion

Type I diabetes results from the destruction of beta cells by killer cells derived from T lymphocytes. However, there is no definitive treatment for this disease, and insulin injections or oral medications are common to treat diabetes [30]. Therefore, in this study, we tried to evaluate the effect of insulin-producing cells derived from bone marrow stem cells on the treatment of rats with type I diabetes. For this purpose, stem cells prepared from bone marrow were first differentiated into insulin-producing cells. The expression of the pdx-I gene was used to evaluate differentiated cells.

The pdx-I is one of the major genes in pancreatic formation [31]. In the current study, RT-PCR showed that the pdx-I gene transcript was visible in the differentiated cells. Choi *et al.* [32] showed that bone marrow mesenchymal stem cells treated with rat pancreatic extract could express specific genes for the development of beta cells such as pdx-I, so in this study, the pancreatic extract was used to differentiate.

After ensuring the differentiation of insulin-producing cells, these cells were injected into a group of rats. In the present study, fewer cells were first transplanted for immunomodulatory effects. In the next step, more cells were transplanted in order to create cell differentiation in the living environment. The results showed that transplantation of insulin-producing cells could significantly increase blood insulin levels in diabetic rats. This increase intensified in the second stage of

transplantation when more cells were injected into rats. Therefore, insulin-producing cells were able to increase insulin levels to the level of insulin injection and there was no significant difference between them. In relation to decreasing blood sugar levels, differentiated cells were able to significantly reduce blood sugar levels. Even in the first stage of cell injection, in which the rats received a small number of cells, their blood sugar levels were controlled by these cells [33, 34].

The study conducted by Aali *et al.* [35] was similar to the present results. They also showed that blood sugar decreased after each stage of differentiated cell transplantation. In addition to differentiated cells, they injected undifferentiated stem cells into mice. These undifferentiated cells were also able to cause a significant reduction in blood sugar. This could raise the possibility that undifferentiated cells in the body differentiated into pancreatic cells to reduce the blood sugar of the diabetic model. In another study conducted by Urban *et al.* [36], Their results were different from the results of the present study, and there was no significant difference between the cell-receiving group and the control group in reducing blood sugar levels, which could be due to differences in the number of transplanted cells per case and difference in the type of tested animal. The animal was the mouse and one million cells were used in this study.

A study by Ezquer *et al.* [37] on six mice found that mice that received 500,000 bone marrow-derived mesenchymal stem cells had hyperglycemia controlled and increased insulin production. Also in a study by Li *et al.* [38] in diabetic mice treated with mesenchymal stem cells, they showed that intravenously injected mesenchymal stem cells could prevent the destruction of residual beta cells and differentiate them into insulin-producing cells, and able to reduce blood glucose levels.

5. Conclusion

Generally, the results of the present study showed that the treatment of type I diabetes using insulin-producing cells derived from

bone marrow stem cells has the ability to significantly reduce blood sugar and control hyperglycemia through possible mechanisms such as immune system modulation and differentiation. It has insulin-producing cells, which paves the way for further studies in the future. What can be deduced from the present study is the use of stem cells to treat diabetes. Stem cells are effective in treating diseases such as pancreatic-related diabetes due to their ability to replace defective and damaged cells. In diabetes, due to damage to the pancreas, it is possible to control blood sugar levels by replacing stem cells and treat diabetes. According to the data of this study and the existence of a significant relationship between blood sugar levels of the studied animals in the stem cell recipient group, it is suggested that similar studies be performed on other animals with larger sample sizes to ensure the results. These studies can help effectively treat diabetes and identify more ways to control it. It should be noted that after confirming the results on other animals, this kind of researches could be done on humans, for treating diabetes.

Conflict of interest

None of the authors have any conflict of interest to declare.

Consent for publications

All authors approved the final manuscript for publication.

Availability of data and material

The authors have embedded all data in the manuscript.

Authors' contributions

S.H.A. helped to do, helped in data analysis, and article drafting, **S.N.J.** helped for doing and helped in sampling and data collection, **Z.A.** helped in study design, data collection, genetic evaluation, and writing Manuscript, **L.F.** helped in sampling and data collection, and writing Manuscript, **A.H.M.** helped in doing, and **M.F.E.** helped in study design, sampling, data collection, doing, and article drafting.

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Ethics approval and consent to participate

This study design was approved by ethical code IR.AKMU.AHA.REC.1398.564 in the research unit.

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