Original Article

The Insulin-Producing Cells Generated from Rat Adipose Tissue Mesenchymal Stem Cells via Pdx1 Overexpression Activate an Immune Response both *in Vitro* and *in Vivo*

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What's Known

- Some researchers claim that the artificial insulin-producing cells are hypo-immunogenic and lack human leukocyte antigen ABC (HLA ABC), human leukocyte antigen-DR (HLA-DR), cluster of differentiation 40 (CD40), and cluster of differentiation 80 (CD80) expression. However, some other studies show that artificial insulin-producing cell transplantation to diabetic rats results in the accumulation of immune cells and immune-reactivity.

What's New

- Our study showed that insulin-producing cells derived from adipose-derived mesenchymal stem cells expressed elevated levels of cluster of differentiation 40 (CD40), CD80, major histocompatibility complex-I (MHC-I), and MHC-II *in vitro*. The insulin-producing cells induced elevated levels of cluster of differentiation 3a (CD3a) and CD45 after transplantation to diabetic rats.

Abstract

Background: The current work investigated the immunological features of insulin-producing cells (IPCs) generated from rat adipose-derived mesenchymal stem cells (ADSCs) both *in vitro* and *in vivo*.

Methods: The research was carried out at Ahvaz Jundishapur University of Medical Sciences in 2023. ADSCs were derived from rat adipose tissues and differentiated into IPCs. The control group included undifferentiated ADSCs. The amount of secreted insulin was measured using ELISA. The expression of major histocompatibility complex-I (MHC-I) and MHC-II, cluster of differentiation 40 (CD40), and CD80 by IPCs *in vitro* was assessed using Western Blot analysis. The *in vivo* study was performed on 10 male diabetic rats. The experimental group received 10^7 IPCs in the peritoneal cavity. The control group received 10^7 undifferentiated ADSCs. After 4 hours, the expression of CD3a and CD45 by immune cells collected from the peritoneal cavity was measured using flow cytometry. All parameters were statistically analyzed using a *t* test.

Results: The differentiated cells secreted much higher amounts of insulin than the control group (P=0.04). IPCs exhibited higher expression of MHC-I and MHC-II, CD40, and CD80 (P=0.02, P=0.008, P=0.07, and P=0.02, respectively). The experimental group showed higher levels of CD3a and CD45 expression than the control group (P=0.07, P=0.04, respectively).

Conclusion: Functional IPCs generated by ADSCs differentiation exhibited immunogenic activity both *in vitro* and *in vivo*. Immune-modulating strategies are required for the effective transplantation of the differentiated IPCs generated in our study.

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Keywords ● Adipose-derived mesenchymal stem cell ● Insulin ● Cell differentiation ● Immunization

Introduction

The immunological response to transplanted insulin-producing cells (IPCs) is a significant obstacle in diabetes 1 cell therapy.[1] Identification of the major histocompatibility complex (MHC) by the immune system induces inflammation, immune cell activation,

and immune-chemokine synthesis.[1] The immune reaction eliminates foreign IPCs and leads to failure in transplantation procedures.[2] Low expression of MHC-II by mesenchymal stem cells (MSCs) inhibits antigen delivery to T cells and immunological rejection.[3] MSCs do not express co-stimulatory molecules including CD80, CD86, and CD40.[4] Some evidence demonstrates indoleamine2,3-dioxygenase(IDO),prostaglandin E2 (PGE2), and transforming growth factor beta (TGF-β) production by MSCs.[5] The cooperation between MSCs prevents natural killer (NK) cells, dendritic cells, T cells, and B cells activation.[6] Furthermore, MSCs release interleukin-10 (IL-10) and IL-4 to promote the development of regulatory T cells (Tregs).[7] MSCs suppress the production of interferon- γ (IFN-γ) and tumor necrosis factor (TNF-α).[8] Thus, under normal circumstances, MSCs are generally considered to be non-immunogenic and safe sources for cell-based therapy.[9] However, under specific experimental conditions, MSCs have been known to exhibit tumorigenic characteristics.[10] Various factors such as the cell source, donor features, culture conditions, genetic alterations, microenvironment, and animal models, can impact the tumorigenic potential of MSCs.[11] Currently, transplanting of IPCs produced from MSC differentiation is recommended.[12] Differentiated IPCs are less likely to develop into tumors because they lack telomerase activity and pluripotency.[13] However, before utilizing IPCs in clinical settings, a few significant questions need to be answered. One crucial component of a successful transplant is figuring out the immunomodulatory properties of the IPCs.[14] Successful transplantation depends on comprehensive information about the interaction between the transplanted cells and the diabetic animal's microenvironment. We need to know how long the IPCs that were transplanted to diabetic animals continue to secrete insulin. Do the transplanted IPCs develop the ability to cause immunogenicity? The published studies in this field are debatable.[14] Several factors, such as the recipient's microenvironment, the transplant site, the recipient's cell source, the differentiation procedure, and the severity of diabetes, affect the success rate of IPC transplantation.[15] Overall, autologous transplantation carries a lower probability of immunological rejection than allogeneic transplantation.[16] One autologous option involves the transplantation of induced pluripotent stem cells (iPSCs)-derived IPCs. The iPSCs are derived from the reprogramming of the somatic cells and lack immunogenicity.[17] The risk of immunological rejection may be decreased by executing the transplant in suitable organs, such as the liver or the anterior chamber of the eye.[18] Another option to prevent immunological rejection is to match MHC antigens.[19] Some researchers administered immunosuppressive drugs such as corticosteroids, calcineurin inhibitors, and mTOR inhibitors to reduce the likelihood of transplant rejection.[20] Encapsulation using biocompatible semi-permeable microcapsules is another method for shielding foreign IPCs from immunological rejection.[21] However, several studies claim that IPCs are not immunogenic at all.[22] Pancreatic and duodenal homeobox 1 (Pdx1) is a transcription factor that plays a crucial role in the development and function of the pancreas. It is specifically important for the differentiation of pancreatic beta cells, which are responsible for producing and secreting insulin. Our previous investigation in Ahvaz Jundishapur University of Medical Sciences showed that the IPCs derived from Adipose-derived mesenchymal stem cells (ADSCs) differentiation with Pdx1 overexpression, were efficient in glucose sensing and insulin secretion both *in vitro* and *in vivo*.[23] This study deals with evaluating the *in vitro* and *in vivo* immunogenicity of the differentiated cells to determine these cell's capability for probable clinical use.

Materials and Methods

An experimental study was conducted at Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, from January to July 2023.

Isolation of Rat Tissues

Five normal male Sprague-Dawley rats were chosen for tissue isolation. The protocol of pancreatic and splanchnic adipose tissue isolation was elucidated previously.[23, 24] All the animals were treated by the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences (National Institutes of Health Publication). The ethical code was IR.BPUMS.REC.1402.243 from Persian Gulf University, Bushehr, Iran.

Construction of Pdx1 pIRES-hrGFP-1a

RNA extraction and cDNA synthesis were conducted as in our previous study.[24] Pdx1 mRNA sequence was obtained from the NCBI gene bank (NM_022852.1 (1406 bp)). The primers were designed using Primer3 software version 4.1.1 (Adobe Systems, USA). The primers included recognition sites for the EcoRI and BamHI enzymes in alignment with the pIRES-hrGFP-1a vector map (Bioneer Corporation, South Korea).

The specific primers (Bioneer Corporation, South Korea) were used for amplifying the full-length Pdx1. The Pdx1 primer sequences are listed in table 1. The polymerase chain reaction PCR reaction was performed according to our previous study.[24] The Pdx1 PCR product was purified from an agarose gel using the Gel DNA Recovery Kit (GF-1 Plasmid DNA Extraction Kit (Vivantis, Malaysia) according to the manufacturer's instructions. The purified Pdx1 PCR product and the pIRES-hrGFP-1a vector (ThermoFisher Scientific, USA) were subjected to double digestion using BamHI and EcoRI (NEB, UK) restriction enzymes at 37 °C for 2 hours. The digested fragments were electrophoresed on a 0.7% agarose gel stained with Safe stain (SinaClon BioSciences, Iran) and then purified using the DNA Recovery Kit (Vivantis, Malaysia) following the manufacturer's instructions. The purified linear vector and insert underwent a ligation reaction using T4 DNA ligase (Bioneer, Korea). Two µL of the ligation product was transformed into calcium chloride-competent *Escherichia coli* Top 10F' cells (Clontech Laboratories, Japan). The transformed cells were selected on lysogeny broth (LB) medium agar (Sigma, USA) plates containing (100 µg/mL) ampicillin (Sigma, USA). Several colonies were screened by colony PCR using the universal primers T7 and BGH (Bioneer, Korea). The plasmid was purified using the AccuPrep Nano-Plus Plasmid Mini Extraction Kit (Bioneer, South Korea) and subjected to sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (USA) in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Determination of Functionality of Pdx1-pIRES-hrGFP-1a Vector

The L929 cell line (NCTC 929) was purchased from Gene Iran and cultured in Dulbecco's Modified Eagle Medium-High glucose (DMEM-HG) (Sigma, USA) contained 10% Fetal Bovine Serum (FBS) (Sigma, USA) and 1% Penicillin/Streptomycin (Pen/Strep) (Sigma, USA). A total of 10^6 L929 cells were transfected with 20 µg of the purified Pdx1-pIRES recombinant vector using an electroporator (Bio-Rad, USA). The cells received one pulse of 140 V for 15 msec. L929 cells transfected with pIRES-hrGFP-1a served as the control group. The transfected cells were transferred to cell culture flasks containing 5 mL of DMEM-HG and 10% of FBS. The transfected cells were selected using 1.5 mg/mL of ampicillin and incubated at 37 °C for 2 weeks. The cell pellet was utilized for Real-Time PCR analysis.

Isolation, Characterization, Culture, and Differentiation of ADSCs

The procedures for the isolation, characterization, culture, and differentiation of ADSCs are detailed in our earlier research.[24] On the 10th day of the differentiation process, the cells were subjected to transfection using the Pdx1-pIRES-hrGFP-1a recombinant vector.

Experimental Design

ADSCs were divided into two groups: Group 1 was named the control group and kept in DMEM-HG containing 10% FBS for 14 days; Group 2 was assigned as the differentiated group and received DMEM-F12 (Sigma, USA) containing 2% FBS and 1% insulin transferrin selenium (ITS) (Sigma, USA) for 7 days, followed by another 7 days of culture in DMEM-LG (Sigma, USA) containing 10% FBS, 1% ITS, and 1% Nicotinamide (Sigma, USA). The cells in group 2 were transfected using the recombinant Pdx1-pIRES-hrGFP-1a vector.

The animal study group consisted of 10 normal male Sprague Dawley rats at 8 weeks of age and 180-200 g weight. Rats were kept in a 12:12-hour light-dark cycle with complete access to food and water. The experimental diabetes mellitus condition was induced using 50 mg/Kg of streptozotocin (STZ) (Sigma, USA) in citrate buffer (Sigma, USA).[24] The rats with three blood glucose levels above 500 mg/ml were chosen as diabetic rats. The diabetic rats were studied in two groups. Group 1 (n=5) was injected in the tail vein with undifferentiated ADSCs, and Group 2 (n=5) received differentiated IPCs in the tail vein.

Enzyme-linked Immunoassay

The insulin concentration in the supernatant of the differentiated cells was measured using the rat-insulin ELISA kit (Monobind Inc., USA) according to the manufacturer's recommendation. The protocol for insulin secretion assay was defined previously.[24]

Real-Time PCR

Pdx1 gene expression was assessed using the qRT-PCR method. The SYBR® Green Real-Time PCR Master Kit (ParsTous, Iran) was employed for qRT-PCR analysis on the Step One Plus

Table 1: Primer sequences used for PCR

Genes	Primers	Sequence
Pdx1	F	CATGGGATCCATGAATAGTGAGGAGCAGTA
Pdx1	R	ATGAATTCTCACCGGGGTTCCTGCGGTCGC

TM Real-Time PCR Detection System (Applied Biosystems, USA). The primers were designed using Primer 3 software version 4.1.1 (Adobe, Systems, USA) (table 2). Relative quantification was performed using the comparative $2^{-\Delta\Delta Ct}$ method. To validate the assay and ensure that the target gene and housekeeping gene primers had similar amplification efficiencies, the procedure was conducted as previously described.[24] Glyceraldehyde-3-Phosphate Dehydrogenase GAPDH was used as the housekeeping gene.

Western Blotting

The protocol was performed as in our previous study.[24] The antibodies included those against CD40 (ABIN6260623), CD80 (ABIN678683), MHC-I (ABIN6263246), and MHC-II (sc-59322, Santa Cruz Biotechnology Inc., USA). Membranes were incubated with a suitable mouse anti-rabbit IgG-HRP (sc-2357) (Santa Cruz, USA). The level of protein activity was determined using an ECL detection kit. Protein loading (sc-365062, Santa Cruz, USA) was adjusted for GAPDH immunoreactivity. Optical density analysis was carried out utilizing the CLIQS 1D program from Total Lab in the UK.

Lavage Protocol

In this step, 10^7 differentiated cells were injected into the peritoneal cavity of diabetic rats. After 4 hours, the peritoneal cavity was washed with 8 mL of PBS to assess the extent of immune system stimulation and immune cell accumulation. The samples were centrifuged at 1000 g for 5 min. Then the samples were incubated with erythrocyte lysing buffer (Sigma, USA) for 10 min at room temperature. The peritoneal fluid was combined with lysis buffer at a ratio of 40 to 60. After centrifuging for 10 min at 1500 rpm, the supernatant was discarded. The samples were incubated for 5 min at room temperature. Then, the residual sediment was centrifuged once more with 1 mL of red blood cell (RBC) lysis solution. The remaining debris was washed using PBS (Sigma, USA). Following cell collection, the cells were stained with anti-rat CD3a and anti-rat CD45 and subjected to flow cytometric analysis.

Fluorescence-Based Assay

The cells were washed three times using PBS. The specific antibodies against cell surface clusters of differentiation (CDs; CD45 (30-F11): sc-53665) and CD3a ((PC3/188A): sc-20047); (Santa Cruz, USA) were dissolved in a solution of 3% BSA/PBS (Sigma, USA). The cells received a total of 1 µg of the antibody solution. The samples were incubated for 30 min in the dark at room temperature. The cells were washed three times with PBS and resuspended in 1 mL of ice-cold PBS containing 10% FBS and 1% sodium azide (Sigma, USA). The fluorescence activity of the samples was measured using a Galaxy flow cytometer (Dako, USA). The results were analyzed using the Flow Jo 8.8.7 software (Treestar, USA). Two negative controls, including an isotype control and a stainless control, were provided for each sample.

Statistical Analysis

The statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, USA). All data were reported in the form of mean±SD. All parameters were statistically analyzed using a *t* test. The statistically significant difference between distinct experimental groups was indicated as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Results

Changes in ADSC's Morphology following Differentiation into IPCs

The ADSCs exhibited an adherent spindle-like appearance in the third passage (figure 1A). The differentiated ADSCs showed a round, epithelial-like shape (figure 1B).

Validation of Transforming Process

The occurrence of the transformation process was confirmed using direct colony PCR. As shown in lane 2 in figure 2, a 1546-bp band was seen on 0.7% electrophoresis gel corresponding to the 1406-bp Pdx1 gene and 140-bp flanking regions of the Pdx1-pIRES-hrGFP-1a vector. This finding confirms the accuracy of the recombinant plasmid transformation in the Top10 F' bacteria. The sequencing of the recombinant plasmids was also performed with T7 and BGH universal primers to confirm the accuracy of the Pdx1 sequence after amplification and cloning (lane 3, figure 2). The sequence obtained was

Table 2: Primer sequences used for quantitative real-time RT-PCR

Genes	Primers	Sequence
Pdx1	F	GGAGGGTTTGGAAAACCAGT
Pdx1	R	ACAAACATAACCCGAGCACA
GAPDH	F	AGTTCAACGGCACAGTCAAG
GAPDH	R	TACTCAGCACCAGCATCACC

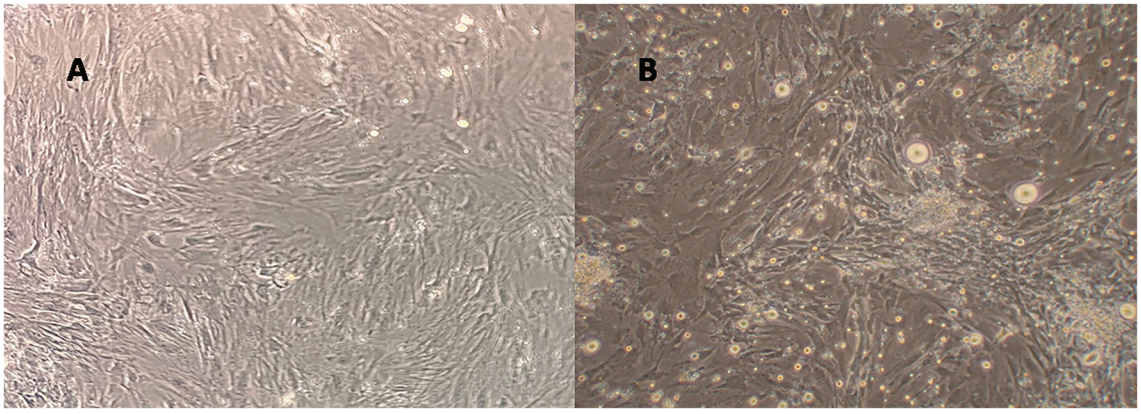

Figure 1: The morphological characteristics of ADSCs (adipose-derived mesenchymal stem cells) during differentiation into IPCs. (A) ADSCs exhibited a fibroblast-like appearance. (B) The fibroblast-like appearance of ADSCs changed to an epithelial-like shape following differentiation to IPCs. Phase contrast magnification, ×100.

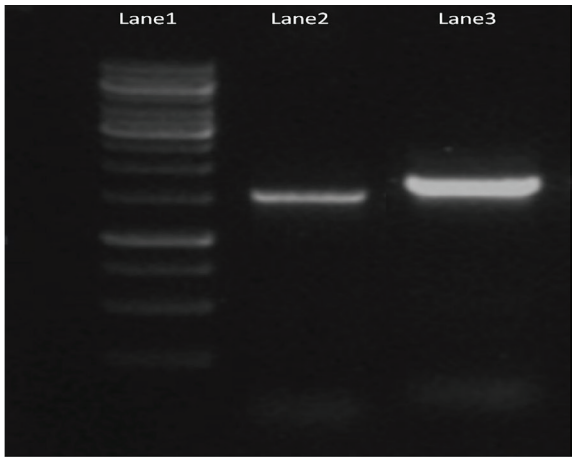

Figure 2: Validation of the transforming process by the characterization of the Pdx1-pIRES-hrGFP-1a vector. Lane 1 shows a 1 KB ladder. Lane 2 shows a 1406 bp Pdx1 gene separated from the recombinant Pdx1-pIRES-hrGFP-1a vector that was digested using EcoRI and BamHI enzymes. Lane 3 shows a 1546 bp PCR product of recombinant Pdx1-pIRES-hrGFP-1a vector.

translated using the ExPASy Translate tool (https://web.expasy.org) and then analyzed using the online tool nBLAST)https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on this finding, the cloned Pdx1 gene sequence had a 100% homology to the Pdx1 sequences submitted to GenBank (GenBank accession number: NM_022852.1).

Evaluation of Fluorescence Appearance

The L929 cells that had been transfected with the Pdx1- pIRES-hrGFP-1a recombinant vector showed a fluorescence appearance under an inverted microscope (figure 3A).

Evaluation of the transfected cells' Pdx1 Expression Potential

According to the Real-time PCR data, Pdx1 expression was much higher in transfected cells than in cells that had not been transfected with recombinant vectors (P<0.0001) (figure 3B).

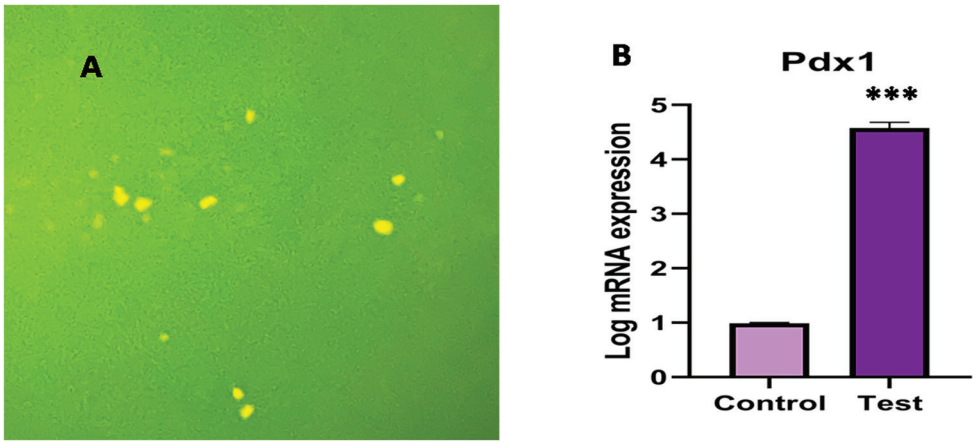

Figure 3: Validation of the transfection process by two processes: (A) Validation of the fluorescence appearance. The L929 cells transfected with Pdx1-pIRES-hrGFP-1a vector showed fluorescence appearance 24 hours following transfection under an inverted fluorescence microscope. Phase contrast magnification, ×100. (B) Validation of the potential of transfected cells to Pdx1 expression. The L929 cells transfected with Pdx1-pIRES-hrGFP-1a vector showed significantly elevated levels of Pdx1 when compared with the control group (P<0001). GAPDH was used as the calibrator for real-time PCR analysis. Data are expressed as mean±SD. ***Represents a statistically significant difference between different groups at P<0001. All experiments were carried out in triplicate.

Analyzing the Functionality of Differentiated IPCs

The ADSCs-derived IPCs demonstrated the ability to secrete insulin in response to glucose treatment. The undifferentiated ADSCs were not able to secrete insulin. Group 2 secreted significantly greater amounts of insulin compared to group 1 (P=0.04) (figure 4).

In Vitro Immunogenicity of Differentiated IPCs

Evaluation of *in vitro* immunogenicity properties of the IPCs showed that the cells were able to express MHC-I and MHC-II. Comparison between MHC expression between IPCs and undifferentiated ADSCs showed a significant increase in MHC-I and MHC-II in differentiated IPCs compared to the control (P=0.02 and P=0.008, respectively) (figure 5). Moreover, the differentiated cells showed increased levels of CD40 and CD80 in comparison with the control group (P=0.07 and P=0.02, respectively). The increase in CD80 expression was statistically significant (figure 6).

In Vivo Immunogenicity of Differentiated IPCs

Transplantation of the IPCs to the peritoneal cavity of the diabetic rats induced accumulation of immune cells. Flow cytometry analysis showed an increase in CD3a (P=0.07) (figure 7) and a significant increase in CD45 expression in the peritoneal cavity of the diabetic rats who received IPCs compared to the control rats (P=0.02) (figure 8).

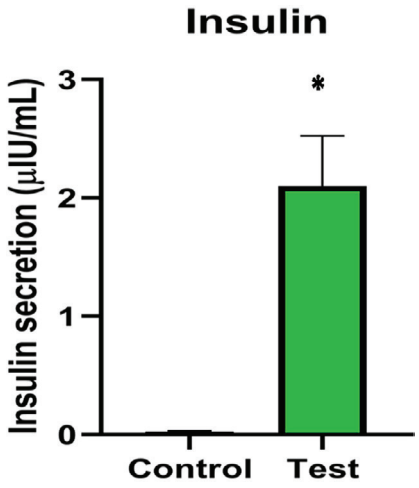

Figure 4: Analysis of the effectiveness of differentiated IPCs. The IPCs secreted significantly elevated levels of insulin in response to high concentrations of glucose (25 mg/mL) compared to the undifferentiated ADSCs (P=0.04). Data are expressed as mean±SD. *Represents statistical significance between different groups at P<0.05. All experiments were carried out in triplicate.

Discussion

Our ongoing research reveals that MSCs obtained from rat adipose tissue can effectively differentiate into IPCs. In our study, these differentiated cells exhibited the ability to produce and release insulin, and their ability to insulin secretion was notably higher than the control group. The differentiated cells showed increased levels of CD40, CD80, MHC-I, and MHC-II *in vitro*. The transplantation of differentiated

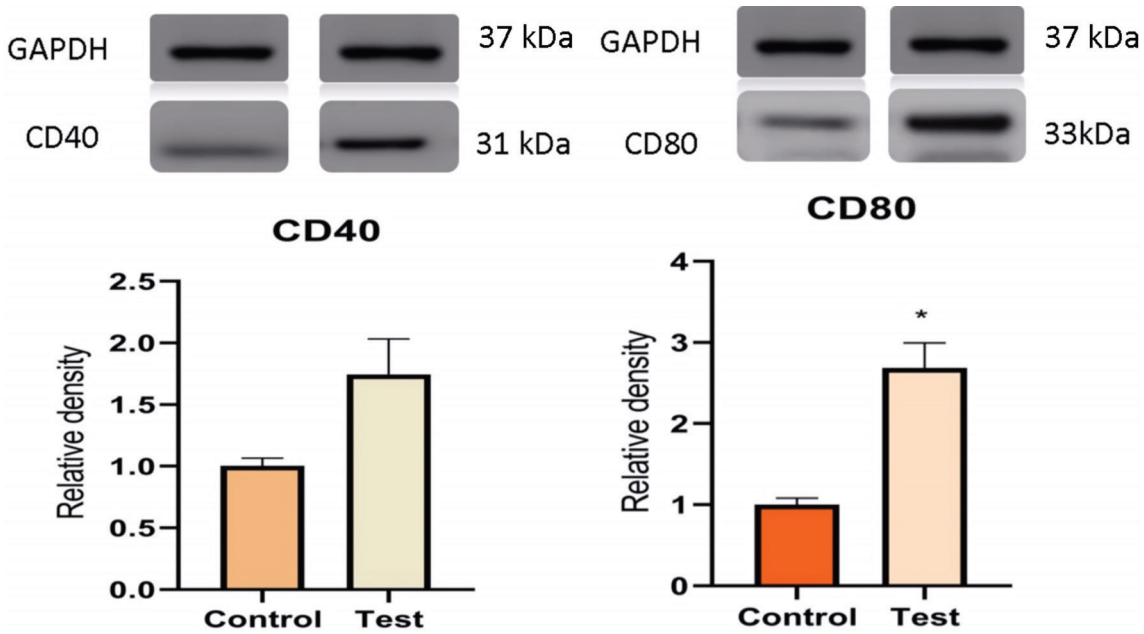

Figure 5: The *in vitro* immunogenicity of differentiated IPCs evaluated by analyzing MHC-I and MHC-II expression. The IPCs obtained from ADSCs differentiation expressed significantly elevated levels of MHC-I and MHC-II compared with the un-differentiated ADSCs (P=0.02 and P=0.008, respectively). GAPDH was used as the calibrator for Western Blot analysis. Data are expressed as mean±SD. *Represent a statistically significant difference between different groups at P<0.05. All experiments were carried out in triplicate.

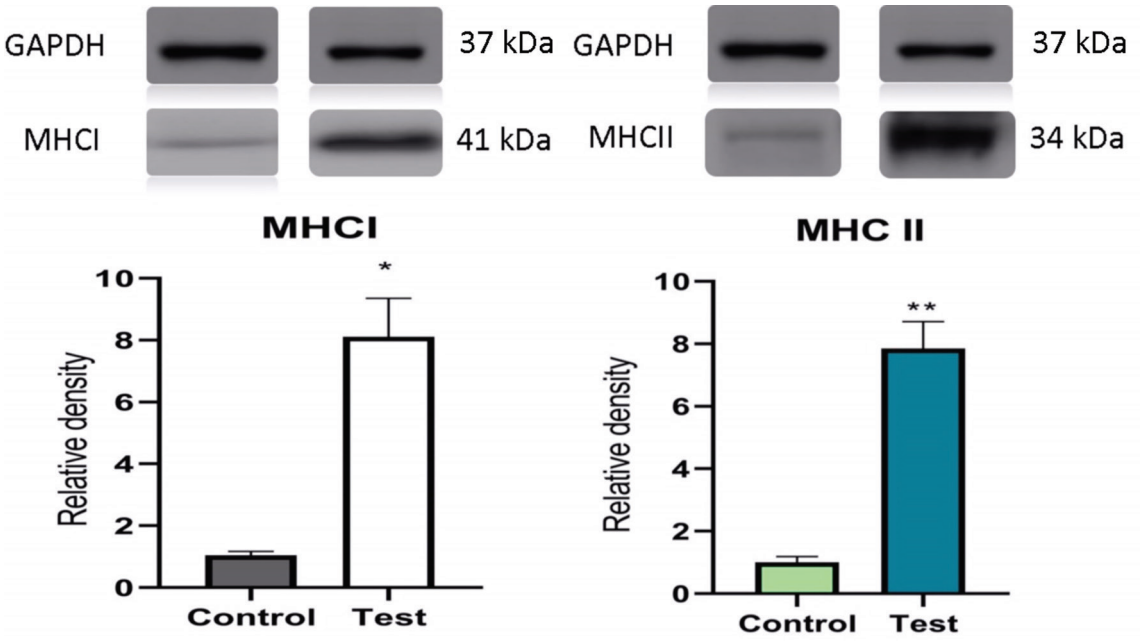

Figure 6: The *in vitro* immunogenicity of differentiated IPCs was evaluated by analyzing CD40 and CD80 expression. The IPCs obtained from ADSCs differentiation expressed elevated levels of CD40 and CD80 compared with the un-differentiated ADSCs (P=0.07 and P=0.02, respectively). GAPDH was used as the calibrator for Western Blot analysis. Data are expressed as mean±SD. * and **Represent statistically significant difference between different groups at P<0.05 and P<0.01. All experiments were carried out in triplicate.

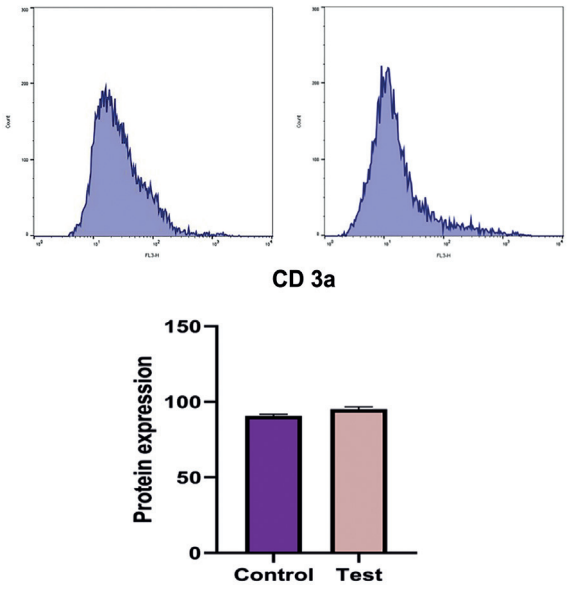

Figure 7: The *in vivo* immunogenicity of differentiated IPCs, evaluated by analyzing CD3a expression. Transplantation of obtained IPCs into the peritoneal cavity of diabetic rats induced propagation of the blood immune cells that expressed higher amounts of CD3a than control diabetic rats. However, the difference was not significant (P=0.07). Data are expressed as mean±SD. All experiments were carried out in triplicate.

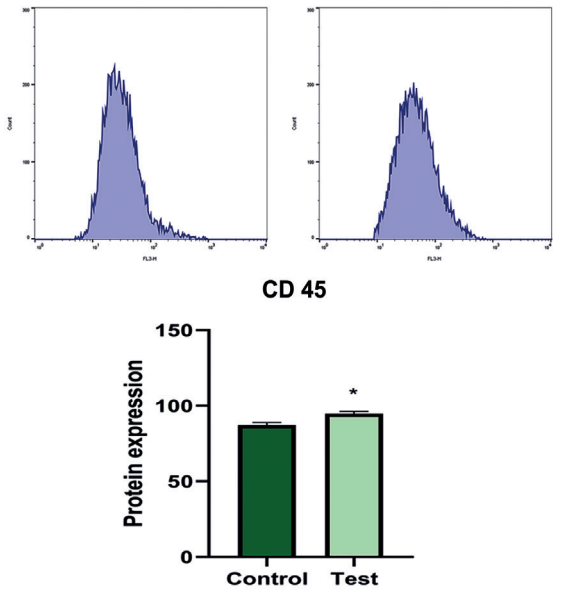

Figure 8: The *in vivo* immunogenicity of differentiated IPCs, evaluated by analyzing CD45 expression. Transplantation of obtained IPCs into the peritoneal cavity of diabetic rats induced propagation of the blood immune cells that expressed significantly higher amounts of CD45 than control diabetic rats (P=0.02). *Represents a statistically significant difference between different groups at P<0.05. Data are expressed as mean±SD. All experiments were carried out in triplicate.

cells into diabetic rats resulted in an increase in CD3a and CD45 expression as well as an accumulation of immune cells in the peritoneal cavity. Balboa and coworkers demonstrated that in laboratory settings, highly potent human stem cells can be directly converted into beta cells.[25] Tsai and colleagues reported that transplanting differentiated IPCs derived from human umbilical cord mesenchymal stem cells (hUCMSCs) can reduce hyperglycemia in NOD mice.[26] Ebrahimi and their collaborators utilized skin-derived stem cells to produce beta-like IPCs.[27] These findings present exciting possibilities for

the treatment of diabetes in the future.[28] Wu and colleagues found that co-transplanting of bone marrow-derived MSCs (BMSCs) with Langerhans islets resulted in improved blood sugar control compared to mice that received islet transplantation alone. There was also T cells generation. The researchers concluded that successful transplantation is due to reduced inflammation and immune response by Tregs.[29] Ribeiro and colleagues demonstrated that ADSCs have a more potent inhibitory effect on T cell proliferation and immune response regulation than BMSCs.[30] Zhao and colleagues indicated that hUCMSCs differentiated into hepatocyte-like cells do not express MHC-II molecules and possess immunomodulatory properties. In a study conducted by Liu and colleagues, BMSCs from rabbits did not express MHC-II molecules after undergoing osteogenic differentiation in laboratory conditions. These cells were unable to induce the proliferation of allogeneic peripheral blood mononuclear cells (PBMCs) or activate cytotoxic T cells. However, the cells showed an obvious increase in the secretion of IL-10 as an anti-inflammatory cytokine.[31] These findings suggest that the IPCs derived from MSC differentiation, despite their higher ability to MHC-I expression, cannot activate cytotoxic T cells or memory T cells.

In contrast, some evidence claims an irreversible immune response acquirement following the differentiation of MSCs to IPCs. Mohammadi and colleagues conducted a laboratory study that showed BMSCs acquire immunogenicity properties after differentiation into IPCs.[32] The results of our study revealed that ADSCs exhibited immunogenicity traits following their differentiation into IPCs. The differentiated cells exhibited higher expression of CD40 and CD80 markers than the control group. Additionally, the differentiation process toward insulin production significantly elevated the levels of MHC-I and MHC-II compared to the control group. These findings suggest that differentiation of ADSCs enhances their immunogenic properties *in vitro*. In this regard, the impact of the *in vitro* microenvironment on immunogenicity must be noted. The induction of immunological properties in the extracellular environment can be attributed to cytokine and protein interactions in the differentiation culture and their effect on the production of antigens that stimulate the immune system. FBS presents MHC-I. Hence, FBS application may lead to increased immunological responses.[33] The results of Rowland and colleagues' study indicated that autologous MSCs injection along with FBS resulted in post-injection inflammation and cellular toxicity.[33]

The present study found that the injection of differentiated cells into diabetic rats caused immunogenic reactions in the host body. When differentiated cells were injected into diabetic rats, there was an increase in CD3a and CD45 factors in the accumulated cells in the peritoneal cavity compared to the control group. Hassanin and others showed that transplantation of differentiated IPCs from human Wharton's jelly into diabetic mice triggered an immune response. They recommended assessing the immunogenicity of the disease microenvironment before conducting IPC transplantation into the recipient's body.[34] Yang and colleagues reported that IPCs derived from the human umbilical cord in laboratory conditions are hypo-immunogenic, but the allogeneic transplantation of these cells to healthy immune system mice leads to an immune response. The induced IPCs expressed low levels of HLAABC. However, they devoided HLA-DR, CD40, and CD80 expression. Induced IPCs did not induce allo-antigenic Human Peripheral Blood Mononuclear Cells (h-PBMC) proliferation after co-culturing with hPBMC cells. The researchers observed an increase in T lymphocytes and monocytes in the peritoneal cavity of diabetic rats after injecting IPCs.[35] The interpretation was that the diabetes microenvironment induces positive expression of MHC-I expression in transplanted IPCs. This finding can be explained by the diabetes microenvironment.[35] Tang and colleagues discovered that allogeneic BMSCs continue to differentiate into IPCs after transplantation into the subcapsular region of the pancreas in diabetic mice. However, along with the differentiation process, MHC-II expression on MSCs became positive, and alloantibodies were detected in the serum.[36] The site of transplantation is another important factor in regulating the immune response. For instance, the immune system is prioritized in the eye and the brain, where it responds differently than it does elsewhere in the body.[36]

Some other studies, however, demonstrate immune-suppressive behaviors of MSCs-originated IPCs. Ghoneim and colleagues reported no expression of HLA-AB and HLA-DR after IPC injection in mice.[37] In one laboratory study, the differentiation of BMSCs into muscle cells led to an increase in MHC-I and MHC-II expression and stimulated the production of immune cells. However, the effects of these cells on the survival and repair of damaged myocardium in allogeneic mice were much weaker than those of autologous cell transplantation. The researchers concluded

that the induction of muscle cells in laboratory conditions reduces immune-modulating molecule secretion and results in a decrease in the survival of differentiated cells in the host body.[38]

Changes in the microenvironment during specific diseases can enhance the differentiation of MSCs and alter their immune properties inside the body.[38] Diabetes causes oxidative stress that can be considered as an important trigger of immunogenicity. It has been found that accumulation of IFN-γ induces MHC-I and MHC-II expression by MSCs both *in vitro* and *in vivo*.[39] Therefore, the relationship between the disease microenvironment and changes in immune properties should be investigated to clarify the basic mechanism of immunological changes after IPC differentiation.[38, 39]

Boyd and others investigated the primary immune response against IPCs derived from embryonic stem cell differentiation in mice. The results of this study showed that transplantation of IPCs results in immediate accumulation of neutrophil or macrophage cells as well as a significant increase in the concentration of inflammatory cytokines at the transplant site. The researchers proposed potential techniques to prevent cell damage and assure their long-term survival and functionality *in vivo*.[40] As a result, a long-term study of the immunogenicity of transplanted IPCs is recommended.

Overall, transplantation of artificial IPCs, with continuous insulin secretion and proliferation ability, is thought to be a permanent cure for type 1 diabetes. However, the immunogenicity of IPCs generated from MSCs is controversial. The immunogenicity of IPCs varies depending on the original cell line, differentiation technique, *in vitro* micro-environment, transplantation site, host animal micro-environment, and the time after transplantation. Therefore, to avoid immunologic rejection and achieve a renewable and efficient population of IPCs, the immunogenicity properties of the cells should be investigated before transplantation. However, we are currently dealing with the issue of developing the immunogenic properties of insulin-producing cells over time following transplant.

Conclusion

Our research demonstrated that the functional IPCs produced by ADSC differentiation employing ITS and nicotinamide plus Pdx1 transfection exhibited immunogen activity both *in vitro* and *in vivo*. As a result, immune-modulating strategies must be used during the allogenic transplantation of IPCs. It is recommended to explore the encapsulation method of the artificial IPCs to limit their immunogenic effect.

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Authors' Contribution

D.D designed the study, performed the study, searched the electronic databases and extracted the data, analyzed the data, wrote the manuscript, and revised the article critically for important intellectual content; S.M and F.N.D performed the study, searched the electronic databases and extracted the data; performed the study, searched the electronic databases and extracted the data; S.J.H analyzed the data and revised the article critically for important intellectual content. All authors have reviewed and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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