

Differentiation of Adipose Tissue-derived Stem Cells into Insulin-Producing Cells for Improving the Diabetes Therapy

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Abstract: From the adipose tissue which credits as the biggest endocrine organs and significant tissue for homeostasis, people can derive a sort of mesenchymal stem cells (MSC) which is also learned as adipose tissue-derived stem cells (AD-MSC). This kind of stem cells is easier and safer for scientists to access and still have similar renewal ability and multipotent differentiation profile compared to those stem cells in bone marrow [1]. In this study, we investigate the possibility of adipose tissue-derived cells to differentiate into insulin producing cells. We cultured, characterized, and confirmed that AD-MSC can be successfully differentiated into pancreatic cell lineage which is insulin producing. Following a three-step protocol, we finally achieve insulin producing cells from AD-MSC. Through qCR analysis, marker genes of *Nkx6.1*, *Pdx1*, *Ngn3*, *Neurod1*, *insulin*, and *glucagon* are in presence in the final stage of cells. For further confirmation, we employed immunofluorescence staining on *Pdx1* and *insulin*. The outcome shows the ultimate cells can be stained by DTZ with brown coloring. Furthermore, after exposing the cells to different levels of glucose, we can see the total rise of insulin and C peptide which proves that the cells have the ability to react to the glucose stimulation. The success of the differentiation of AD-MSC to insulin producing cells probably open the gate to new kind of applicable, convenient, and undisputed treatment for diabetes.

Keywords: adipose tissue-derived Stem cells, insulin-producing cells, diabetes.

1. Introduction

Stem cells (SC) are a kind of cells with multi-directional differentiation potential that can divide and proliferate by themselves [2][3]. It can be divided into three types: totipotent stem cells, pluripotent stem cells and single stem cells. Their division and differentiation potential and direction gradually decline. With the gradual deepening of research in recent years, it has been recognized that stem cells have the following characteristics: (1) they can have multiple specific molecular markers at the same time; (2) they have cell signal pathways in different ways; (3) stem cells will enter a special division state during cell division and proliferation. Adipocytes include precursor adipocytes, mature adipocytes, fibroblasts and so on. Adipose mesenchymal cells are derived from mesenchymal cells. Their molecular expression resembles that of bone marrow cells. They can generally be extracted from human adipose tissue [4]. Adipose mesenchymal cells can be obtained by simple extraction of collagenase to obtain matrix vascular components [5][6], and then purified by means of adsorption and adhesion.

The new supersedes the old. Diabetes is a metabolic disease characterized by the acceleration of metabolism and the increase of blood sugar in the body. It can be divided into two groups according to the cause of disease, type I diabetes and type II diabetes. The cause of type I diabetes is insulin. β The disorder of insulin production caused by cell destruction often occurs in adolescents, most of which are

in acute and serious condition; Type II diabetes is mainly manifested by hyperglycemia caused by impaired insulin utilization, which occurs frequently in middle-aged and elderly patients. The serious danger of diabetes lies in its easy to cause many complications related to macrovascular and microangiopathy, including diabetic nephropathy, diabetic retinopathy, blindness, diabetic foot and so on. Grow with each passing day, the number of deaths from diabetes worldwide is about 5% of the total deaths. Meanwhile, the number of diabetic patients is increasing. At present, about 40 million of the patients are in China. If we do not control it again, we expect that the number of patients will increase to 60 million after five years. It can be said that the treatment of diabetes is urgent.

For type 2 diabetes, blood sugar can be controlled by oral hypoglycemic drugs. For type I diabetes, insulin can be used to control the disease and reduce complications. But in the long run, exogenous insulin cannot be as stable as the islet cells for long-term, precise and efficient control of blood sugar, and long-term injection of insulin not only reduces the quality of life of patients, but also causes hypoglycemia caused by excessive insulin injection. In order to overcome this problem, researchers tried to transplant islets to diabetic patients. After treatment, they found that only a small amount of insulin could be used to stabilize blood glucose after transplantation. This encouraging result has made islet transplantation a possible way to treat diabetes, but it is still restricted by many factors. Including but not limited to islet immune rejection, donor scarcity and so on. In order to solve the donor problem, researchers began to try to induce other more accessible cells into pancreatic cells. In recent years, researchers have made a series of breakthroughs in the field of cell induced differentiation. By jointly introducing a variety of genes, researchers have successfully induced and differentiated normal nerve cells [7][8]. Using the same idea, some researchers introduced some genes transcribed and translated by islet cells into mesenchymal cells [9], and found that mesenchymal cells can produce new functions similar to original islet cells. Once researchers put islets β insulin gene of the cells was introduced into mouse bone marrow mesenchymal cells, and the bone marrow mesenchymal cells successfully expressed insulin through subculture, induction and differentiation. However, after in-depth study and observation, it was found that although this kind of mesenchymal cells can effectively reduce blood glucose in mice, its response to the change of blood glucose concentration is slow and lagging, Even some cells cannot differentiate and mature in mice [10].

Compared with the above studies, adipose mesenchymal cells were selected as the induction object. Compared with bone marrow cells, adipose mesenchymal cells also have good differentiation and proliferation ability. At the same time, they can obtain [11] after liposuction in anesthetized patients. They have the advantages of less damage to the collection target, low cost, large acquisition volume, easy cold storage and not easy inactivation, weak immune rejection response of transplantation, and are more suitable for islets β Experimental materials for cell transformation research.

2. Materials and Methods

2.1 Samples

Adipose Mesenchymal Stem Cells (AD-MSCs) used in this experiment were extracted from patients conducting liposuction treatments (n=10, the extraction process has been permitted by patients). The average tissue volume is 10-15mL. The tissue extracted has been cleansed by SPSS to remove anesthetics and blood cells, and then centrifuged at 300rpm for 5 mins for twice. Experiment involved in this article was conducted using samples above and had been repeated independently to ensure the reproducibility of results.

2.2 Cell Culture

Minced pieces of adipose tissue, which was removed from patients ordering liposuction procedure, underwent digestion in the solution of collagenase, with a concentration of xxx0.04% (w/v), at 37°C, 180rpm for 30 mins. After cleansing by the SPSS by centrifuging at 1200rpm for 5 mins, the cryosupernatant was discarded. Then, the cells were added into T75 flasks with 12mL of complete medium. The planting environment of cell is humidified atmosphere with 95% air and 5% CO₂ at 37°C. The cells were gathered and collected for further developments through several replicating process. The morphology of the cells is demonstrated as below.

For biological characteristics of AD-MSCs, adipogenic differentiation of these induced cells was checked using the Oil Red staining strategy. Osteogenic differentiation was checked using the method of Alizarin Red staining strategy. The pictures of both staining methods are shown as below, and cells after

the checking process are used for further procedure: reverse transcription-polymerase chain reaction (RT-PCR).

2.3 *In vitro* Differentiation of AD-MSCs into Insulin-Producing Cells

Then, undifferentiated cells were placed into a serum-free medium (SFM)-A for resuspension and planted into a petri dish for two days. Essential components of the SFM-A include: 4 nM Activin A, 1×ITS, 1mM sodium butyrate, 50 μM β-ME, 280 μM pVc, and 1% FBS. On the fourth day, the planting medium was changed to SFM-B that contained 2 μM RA, 2 μM A83-01, 2 μM LDE225, 280 μM pVc, 1% FBS, and shifted to the medium, SFM-C, with 1 μM A83-01, 2 μM LDE225, 280 μM pVc, 1% FBS for the following three days. For the eighth to thirteenth day, cells were planted in a medium of SFM-E with 1×ITS, 3 mM taurine, 100 nM GLP-1, 1 mM nicotinamide, 1×NEAAs, and 1% FBS.

2.4 RT-PCR Analysis

Total RNA was extracted from undifferentiated adipose Tissue-derived stem cells and differentiated islet-like cells. RNA was reverse-transcribed into cDNA using Reverse Transcription kit (abmGood) according to the manufacturer's protocol. The real-time PCR was conducted on thermal cycler by using SYBR Green PCR kit (abmGood). The thermal cycling programme stopped at 40 cycles (initial denaturation at 95°C for 10min, 40 cycles of denaturation at 95°C for 15s and annealing at 60°C for 1min. The primer sequences are provided in Table 1. Comparative C_T method was used in data processing.

2.5 Immunocytochemistry

ICAs and undifferentiated AD-MSC were washed with Dulbecco's Phosphate Buffer Saline (DPBS), fixed with ice-cold paraformaldehyde for 15min and washed again. Then cells were treated by 0.1% Triton-X. 0.5% BSA was used to block and permeabilize cells for 30 min. Primary antibodies for Sox17 (mouse, Abcam), Cxcr4(goat, Abcam), Pdx1(goat, Abcam), Nkx6.1(rabbit, CST), Insulin (rabbit, CST), Glucagon (mouse, Abcam) were used to incubate cells overnight at 4°C. Subsequently cells were washed with DPBS and incubated with Alexa Fluor 647 Donkey Anti-Mouse IgG (H+L) (Abcam)/Alexa Fluor 488 Donkey anti-Goat IgG (H+L) (Abcam)/ Alexa Fluor 647 Rabbit Anti-Goat IgG (H+L) (Abcam)/Alex Fluor 488 Donkey Anti-Mouse IgG(H+L) (Abcam)/ Alex Fluor 647 Donkey anti-Goat IgG(H+L) (Abcam)/Alex Fluor 488 Donkey anti-Rabbit IgG(H+L) (Abcam)/Alex Fluor 594 goat anti-Rabbit IgG(H+L) (ZSGB-BIO) secondary antibodies at room temperature for 90min in dark. Subsequently, cells were washed, counterstained with 4'-6-diamidino-2-phenylindole (DAPI) for 5min and washed. After stain, slides were observed with fluorescence microscopes.

2.6 Dithizone staining

Ten milligrams of Dithizone (DTZ)(Sigma) was dissolved in 10 mL dimethyl sulfoxide concentration to stain ICAs.

2.7 Insulin and C-peptide Release Assay

To measure insulin release concentrations, 10 ICAs were picked out and washed with PBS for three times. Then ICAs were incubated with Krebs-Ringer bicarbonate HEPES(KRBH) buffer without glucose for about 5 hours. Subsequently ICAs were incubated with 100μL KRBH buffer containing 2.8 mM glucose for 1 hour at room temperature for 1 hour. Collect the supernatant. In addition, same ICAs were further incubated with KRBH buffer with 16.7Mm glucose for an hour in room temperature. Collect the supernatant. Insulin and C-peptide concentrations were measured with Immulite 1000 Insulin kit (Roche) and Immulite 1000 C-peptide Kit (Roche)respectively.

2.8 Statistical Analysis

All of the data were proceeded by student's t test and were expressed as mean ± standard deviation (SD). Differences were considered statistically significant when p<0.05.

Table 1 List of Primers Used in This Study.

Gene Symbol	Forward	Reverse
18sRNA	CGGCTACCATCCAAGGAA	GCTGGAATTACCGCGGCT
Sox17	CGCACGGAATTTGAACAGTA	GGATCAGGGACCTGTCACAC
Cxcr4	TGTCATCTACACAGTCAACCTC	CAACATAGACCACCTTTTCAGC
Nkx6.1	GTTCCCTCCTCCTCTTCCTC	AAGATCTGCTGTCCGGAAAAAG
Ngn3	CTCGAGGGTAGAAAGGATGACGCCTC	ACGCGTGAATGGGATTATGGGGTGGTG
Neurod1	CCTTCCTTTGATGGACCCC	GATTGATCCGTGGCTTTGG
Insulin	AGCCTTGTGAACCAACACC	GCTGGTAGAGGGAGCAGATG
PDX-1	GTCCTGGAGGAGCCCAAC	GCAGTCCTGCTCAGGCTC
Glucagon	AAGAGGTCGCCATTGTTG	TAGCAGGTGATGTTGTGAAG

3. Results

3.1 Characterization of AD-MSCs

The morphological characteristics of AD-MSCs cultured in vitro exhibited fibroblastic morphology (Fig. 1A). The growth curve of MSCs indicated that the first three days of cell growth were in the incubation phase of slow growth, and the last three days were in the exponential growth phase of rapid growth (Fig. 1B), which showed the cell viability. To further characterize the AD-MSCs, the Oil Red and the Alizarin Red staining were used to prove that AD-MSCs had potential to differentiate into adipogenic lineages and chondrogenic lineages in vitro (Fig. 1C, 1D).

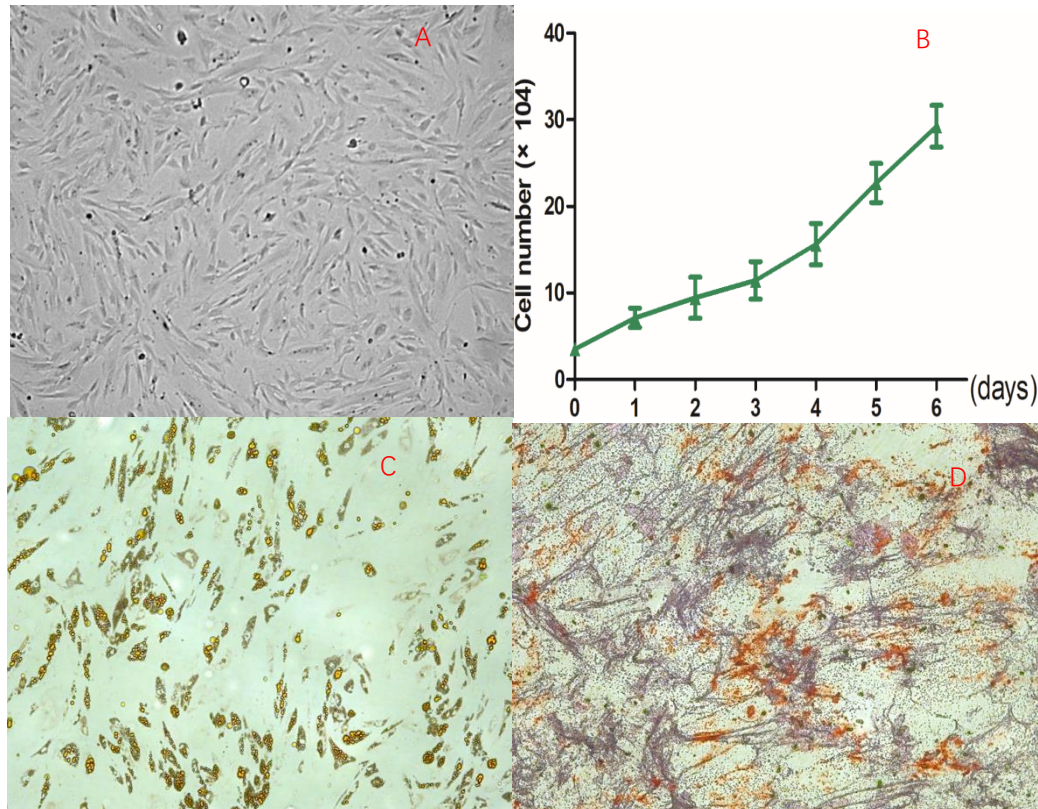


Figure 1. Biology characteristic of AD-MSCs in vitro. (A) Morphology of the cells. (B) Growth rates of Mesenchymal Stem Cells (MSCs). (C) Oil Red staining. (D) Alizarin Red staining.

3.2 Differentiation of AD-MSCs into Pancreatic Cell Lineage

In our experiment, a three-step protocol was used. In the first stage, AD-MSCs were differentiated into definitive endoderm cells. In the second stage, definitive endoderm cells were differentiated into pancreatic progenitor cells. In the last stage, pancreatic progenitor cells were differentiated into insulin producing cells. real-time PCR analysis showed that the definitive endoderm stage of cell expressed marker genes of Sox17 and Cxcr4, which had relatively high level of mRNA (Fig. 2A). Concurrently, it was also confirmed by immunofluorescence technique. As showed, the genes were evidently stained in

the new method compared the undifferentiated AD-MSCs (Fig. 2B). Thus, AD-MSCs were successfully differentiated into endoderm cells. Similarly, the presence of marker genes of Nkx6.1 and Pdx1 were verified by qPCR and immunofluorescence to prove that cells were successfully differentiated into pancreatic progenitor stage of cells (Fig. 3A,3B). Moreover, at pancreatic cell stage of cells, the qPCR analysis showed the presence of marker genes, Nkx6.1, Pdx1, Ngn3, Neurod1, insulin, and glucagon (Fig. 4A). The presence of insulin and Pdx1 was further confirmed by immunofluorescence staining, so the cells were successfully differentiated into insulin producing cells (Fig. 4B). After three steps, the insulin producing cells could be stained by DTZ, presenting brown color (Fig. 5).

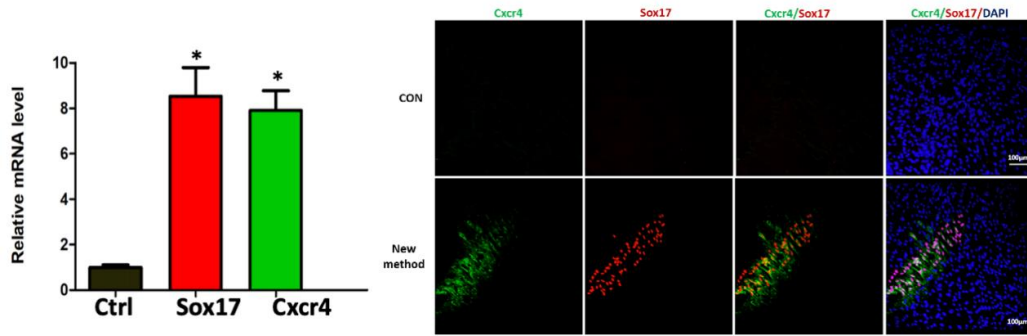


Figure 2. Expression of marker genes at definitive endoderm stage of cells.

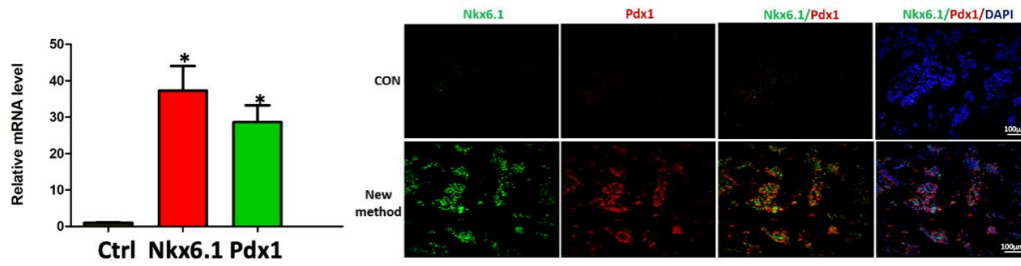


Figure 3. Expression of marker genes at pancreatic progenitor stage of cells.

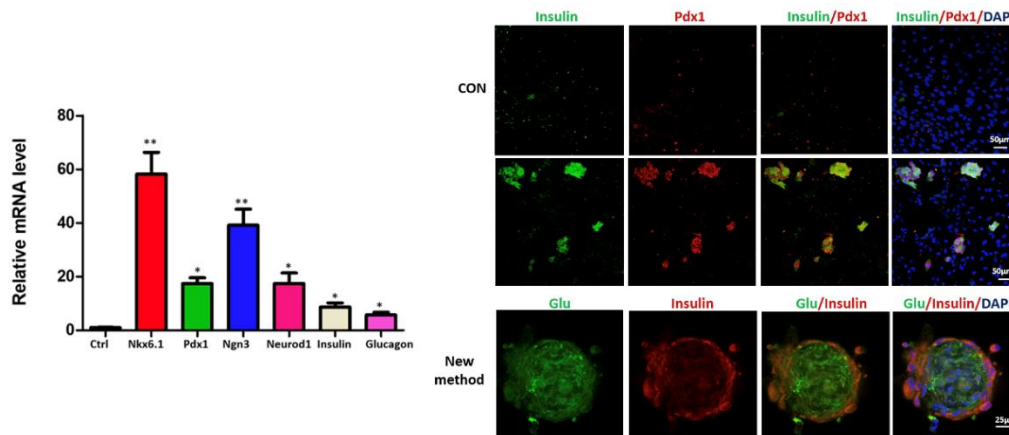


Figure 4. Expression of marker genes at pancreatic cell stage of cells.

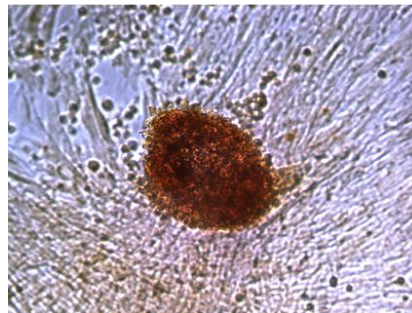


Figure 5. The stain of Dithizone (DTZ) proves the presence of insulin producing cells.

3.3 Static Stimulation and Total Insulin Content of Insulin Producing Cells

When exposed to 2.8 mM glucose, the total insulin and C-peptide content were $368.65 \pm 34.07 \mu\text{IU}/2 \times 10^5$ cells (Fig. 6A), and $81.62 \pm 11.32 \mu\text{IU}/2 \times 10^5$ cells (Fig. 6B). When stimulated by 16.7 mM glucose, the total insulin and C-peptide content were $451.89 \pm 23.352 \mu\text{IU}/2 \times 10^5$ cells (Fig. 6A), and $106.53 \pm 14.39 \mu\text{IU}/2 \times 10^5$ cells (Fig. 6B). The rise of total insulin and C-peptide indicated that the cultured insulin producing cells had ability to respond to high glucose stimulation.

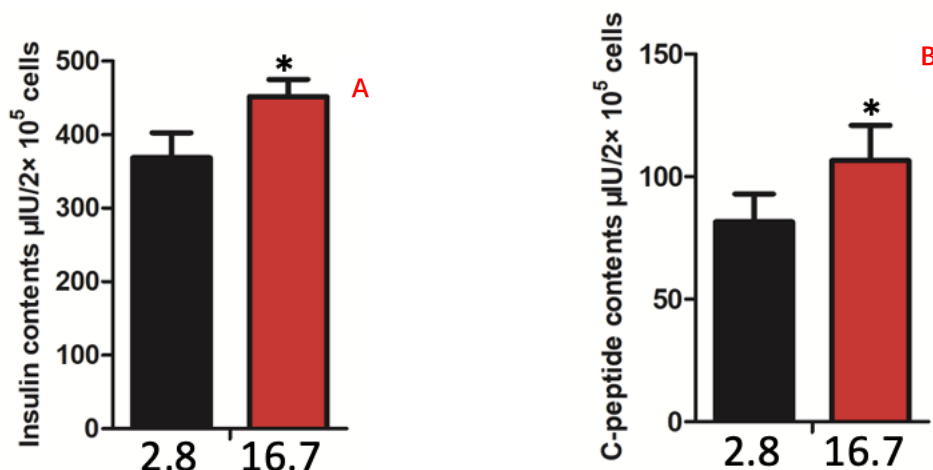


Figure 6. Insulin and C-peptide Release Assay.

4. Discussion

Diabetes mellitus as a chronic disease endangers human health, and continuous hyperglycemia could cause considerable complications. In China, the prevalence of diabetes has increases in recent decades, and the number of people suffering from diabetes has strikingly reached about 129 million in 2017 [12]. Therefore, the prevention and treatment of diabetes is extremely important.

Type 1 diabetes is a disorder that causes a severe deficiency of pancreas β -cells because of its abnormal antibodies. Thus, islet transplantation becomes a common method to cure these diseases. However, there is the possibility of immunological rejection and the lack of donor, so the transplantation method can't be widely used. In recent years, researchers have gradually turned their attention to a new method of culturing insulin producing cells with mesenchymal stem cells in vitro, which has lower cellular immunogenicity. In previous studies, bone marrow mesenchymal stem cells or BM-MSCs [13] and dental pulp stem cells or DPSCs [14] have been proved to differentiate into insulin producing cells.

Our experiment obtained AD-MSCs from fat. By inducing differentiation, AD-MSCs were respectively differentiated into endoderm cells, pancreatic progenitor cells, and finally insulin producing cells. To test the function of differentiated insulin producing cells, they were exposed to the stimulation of low glucose concentration and high glucose concentration. The results showed that insulin contents and C-peptide contents has significantly increased under high glucose condition. Hence, insulin producing cells cultured in vitro will be stimulated by high glucose stimulation and secrete insulin and C-peptide.

Compared to other stem cell sources, AD-MSCs are relatively easier to collect. For instance, DPSCs from deciduous teeth have greater differentiation potential than DPSCs from permanent teeth [14], but most people won't keep their deciduous teeth, so it increases the difficulty of obtaining mesenchymal stem cells. Moreover, AD-MSCs are relatively less painful to collect. Acquiring stem cells from bone marrow always be a painful procedure to individuals [15]. Also, with the increase of age, the number of BM-MSCs that can be extracted and the differentiation potential of BM-MSCs decrease significantly [15]. For AD-MSCs, although ADSCs from young donors showed a higher proliferation rate, overall, the differentiation ability of donors at different ages remained unchanged [15].

In conclusion, our research proposed a new method for adipose derived mesenchymal stem cells to differentiate into insulin producing cells. In the study, it was also confirmed that insulin producing cells cultured in vitro could respond to sugar stimulation. Although the experiment did not carry out further

experiments in diabetic organisms, AD-MSCs were more easily obtained, had less damage to human body, and had strong differentiation ability, so they were more suitable for clinical application than BM-MSCs and DPSCs. In the follow-up experiment, we will focus on evaluating the clinical application value of the new method.

References

- [1] Baer, Patrick. (2012). *Adipose Derived Mesenchymal Stem Cells*.
- [2] Graf T. *Differentiation plasticity of hematopoietic cells*[J]. *Blood*, 2002, 99(9):3089-101.
- [3] Watt F M and Hogan B L. *Out of Eden: stem cells and their niches*[J]. *Science*, 2000, 287(5457):1427-30.
- [4] Kern S, Eichler H, Stoeve J, et al. *Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue*[J]. *Stem Cells*, 2006, 24(5):1294-301.
- [5] Faustini M, Bucco M, Chlapanidas T, et al. *Nonexpanded mesenchymal stem cells for regenerative medicine: yield in stromal vascular fraction from adipose tissues*[J]. *Tissue Eng Part C Methods*, 2010, 16(6):1515-21.
- [6] Zuk P A, Zhu M, Mizuno H, et al. *Multilineage cells from human adipose tissue: implications for cell-based therapies*[J]. *Tissue Eng*, 2001, 7(2):211-28.
- [7] Ambasudhan R, Talantova M, Coleman R, et al. *Direct reprogramming of adult human fibroblasts to functional neurons under defined conditions*[J]. *Cell Stem Cell*, 2011, 9(2):113-8.
- [8] Kim J, Lengner C J, Kirak O, et al. *Reprogramming of postnatal neurons into induced pluripotent stem cells by defined factors*[J]. *Stem Cells*, 2011, 29(6):992-1000.
- [9] Karnieli O, Izhar-Prato Y, Bulvik S, et al. *Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation*[J]. *Stem Cells*, 2007, 25(11):2837-44.
- [10] Lu Y, Wang Z and Zhu M. *Human bone marrow mesenchymal stem cells transfected with human insulin genes can secrete insulin stably*[J]. *Ann Clin Lab Sci*, 2006, 36(2):127-36.
- [11] Cong Ran Li, Xue Fu You, Jian Dong Jiang. 2005. *Animal models of diabetes mellitus and research progress*. *Chinese Journal of Comparative Medicine*, 15:59-60
- [12] Cao J.Y, et al. (2020) *China Cardiovascular Health and Disease Report*
- [13] Yu W.H..*In vitro differentiation of rabbit bone marrow mesenchymal stem cells into pancreatic islet cells*[D]. Henan:Henan Agricultural University, 2016.
- [14] Govindasamy V, Ronald V.S., Abdullah A.N., Ganesan Nathan K.R., Ab Aziz Z.A.C., Abdullah M., Musa S., Abu Kasim N.H., Bhonde R.R. (2011) *Differentiation of Dental Pulp Stem Cells into Islet-like Aggregates*. *J DENT RES* 90(5):646-52.
- [15] Mazini L., Rochette L., Amine M., Malka I G. (2019) *Regenerative Capacity of Adipose Derived Stem Cells (ADSCs), Comparison with Mesenchymal Stem Cells (MSCs)*. *Int J Mol Sci* 20(10): 2523.