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Amniotic stem cell transplantation therapy for type 2 diabetes: 3 years' follow-up report

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Abstract. – OBJECTIVE: These case series present a retrospective analysis of clinical effects that an allogeneic amniotic cell transplantation can have in treating 4 patients with type 2 diabetes.

PATIENTS AND METHODS: Eligible diabetic patients were onset within 3 years, with fasting blood glucose > 7.0 mmol/l. Stem – cells that are collected from neonatal amniotic membrane when a child is born (2 x 10^7 cells) – are used for the treatment of these patients. Measured by the flow cytometry, stem cells transfer clusters of differentiation CD113 and CD34 at a high level, and through the femoral artery on the left, they are administered into the patient's pancreatic dorsal artery.

RESULTS: The major outcome of the study is the improvement of glycemic control. After withdrawing insulin completely, 13.2 months later, there is a reincrease in the level of blood glucose of the patient. After adjusting their treatment again, no one required using insulin again; only need metformin (250-500 mg/day) to control blood glucose levels.

CONCLUSIONS: Since this therapy showed no effects on C-peptide, our results suggested one plausible therapeutic strategy improving glycemic control by increasing insulin sensitivity.

Key Words:
Amniotic stem cell, Type 2 diabetes mellitus, Transplantation, HbA1c.

Introduction

Diabetes mellitus (DM), a set of metabolic diseases in which patients defect in the action and/or secretion of insulin, are caused by the decrease in the function and mass of beta cells to some varying extent. Therapies such as replacing or regenerating beta cells are effective treatments in healing diabetes type 1 and 2 both[1,2]. Despite the better performance of islet-cell or pancreas transplantation in controlling glucose, shortage of organs from donors remains a major obstacle[3,7]. Regenerative strategies and cellular therapies in the treatment of diabetes are currently under evaluation[8,9]. Nowadays researchers are paying close attention to stem cells – the ones that can be categorized into two kinds of cells: tissue stem cells and embryonic stem cells, call can differentiate into functional β cell[10,11]. Different from the previous studies on rejuvenating functional beta cells and permanent curing diabetes mellitus, we investigate the potential of cell therapy for a better controlling of blood sugar. In our previous study[12], we reported clinical effects that allergenic amniotic cell transplants can have in treating type 1 diabetes mellitus. The case report aims at making people realize that amniotic stem cell transplantation has a potential of treating type 2 diabetes as well as reporting the clinical case series.

Patients and Methods

General Procedure of Isolating Cells and the Culture of Sdherent Cells Collected from Amniotic Membrane

After the mother has written the consent according to the Ethical Committee of Siping Hospital of China Medical University, researchers have collected five amniotic membrane samples of the similar size from her. To each sample, 0.1% collagenase I were used to treat the amniotic in 1.0 M phosphate buffered saline (pH 7.2) (PBS), and the amniotic were incubated for 20 minutes at the degree of 37°C. Low-glucose DMEM (Gibco BRL, Grand Island, NY, USA) was used to wash each amniotic membrane three times and, then, harvest the cells that are detached after gently massaging the amniotic membrane. Then there was a centrifugation of the cells for 10 minutes at a pull of 300 g at 37°C, a resuspension in Roswell Park Memorial Institute (RPMI)-1640 medium2 with 10% FBS, and then

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grow the cells in 75 cm^2 flasks at a density of 5×106 cells/ml. 24 hours of incubation later, they removed non-adherent cells. For every three days, a new culture medium would replace the old one, and the culture will continue till adherent cells reached confluence at a level of 80-90%.

Immunophenotyping

To analyze how the typical protein markers distribute, adherent cells are put into incubation at the degree of 37°C for two hours, with the rabbit anti-human primary antibodies conjugated with fluorophore diluted 1:1000 in 0.01 M PBS to directly stain them with the method of immunofluorescence. Up to 10000 labeled cells have been analyzed by the Becton Dickinson LSRFortessa™ Cell Analyzer, running Becton Dickinson FACSDiva™ software version 6.0.

Patient Information

A retrospective analysis was performed on four patients with type 2 diabetes, who were allowed to enter the Stem Cell Clinical Application Centre, Siping Hospital of China Medical University, Siping, Jilin Province, China, for stem cell transplantation from September 1, 2009 to July 1, 2010 (Table I). A case was considered when a patient with type 2 diabetes was diagnosed within 3 years with FBG > 7.0 mmol/l. The presence of renal or liver failure, presence of a chronic coronary artery disease, and clinical evidence of active infection were excluded.

As we treated a type 1 diabetic patient with a transplantation of amniotic stem cells from his son ([12]), his insulin completely withdrew in three months after the treat. The decision aims at the stem cell transplantation with the use of amniotic membrane stem cells collected from newborn babies. Before doing the transplantation, the patients' blood sugar level was under control through regularly injecting Novolin. Their fasting blood glucose was kept between 6.5-7.5 mmol/l, their 2-h postprandial blood glucose 8.0-10.0 and steady for five consecutive days to get prepared to the transplantation. The amount of insulin that are administered regularly at the time was regarded as the basic dosage.

Table I. General characteristics of type 2 diabetic patients (total number of participants=4).

Patient No.	Age	Gender	History (year)	Hypertension (year)
1	52	M	11	6
2	56	F	6	2
3	41	M	4	4
4	63	F	4	2

Amniotic Stem Cells Transplantation

15 days after purifying and amplifying the neonatal amniotic stem cells, they went into suspension in 10 ml of saline and are chosen to be used in transplantation. Doctors put the patient in a position of supine decubitus on operating table, with his left groin disinfected with iodophor. Being observed directly, the patient's left external iliac artery was inserted with a catheter, after sub-cutaneous administration of local anaesthesia. Through femoral artery on the left, amniotic stem cells are injected into the dorsal pancreatic artery after being suspended in saline with 2×107 cells in 10 ml. Then doctors withdrew the puncture and wrapped a pressure bandage that had been sterilized around the site of the puncture. The patients were sent to a ward with only one patient after laying supine for 30 more minutes on operating tables. All of the patients were allowed to enter ICU, and those who are already in ICU are taken care of after consultation and receive critical management till they are discharged from ICU. The antibiotic regimen of penicillin V sodium, 0.25 g, is administered by mouth thrice a day for a consecutive of 7 days to prevent the infections. Patients were taken great care of by the attending physicians of the ICU in accordance with practice management guidelines that is established based on evidence.

Several parameters of the patient were under observation at 0.25, 0.5, 1, 2, 3 years after the transplantation is done, with the use of standard laboratory method, and the parameters include fasting blood glucose, 2-h PG, plasma triglyceride, high-density lipoprotein cholesterol, fasting insulin, total cholesterol, glycosylated hemoglobin A1c, and low-density lipoprotein cholesterol. The follow-up of the patient lasts for 3 years after the transplantation.

Statistical Analysis

With the use of SPSS software (SPSS Inc., Chicago, IL, USA), data have been analyzed. The data have been presented as mean ± standard deviation. The paired *t*-tests have been used to measure the differences among the groups. A *p*-value less than 0.05 was considered statistically significant.

Results

Isolation and Culture of Adherent Cells from Amniotic Membrane

All the amniotic membrane sample have generated the main adherent culture, and the cells displayed a phenotype that is like mesenchymal stem cells. After 4 days in the culture, colonies were formed and after ten to fourteen days, they would reach confluence. The majority of the cells are like a spindle in shape, and resemble fibroblasts. When the 2nd passage is done, adherent cells will form layers of the homogeneous cell with a phenotype like an MSC which is similar to our previous study[12]. After the freezing and unfreezing of the adherent cells, their quantity decreased slightly, and the remained cells that are feasible could expand for consecutive days with success.

Immunophenotypic Analysis

It is extremely rare that adherent cells that are derived from amniotic membrane have hematopoietic lineage markers HLA-DR (HLA-class II) and CD45 on the surface of cells, as flow cytometry has assessed. Most of the cells have shown CD34 and CD133 proteins at a high level on the surface of the cells, similar to our previous study[5]. (Data not shown).

Insulin Usage

As table 2 has illustrated, insulin that is required had reduced in amount starting from the second or 3rd day after the transplantation, and insulin withdrew completely after 25-45 days. And 11.3-29 months later the level of blood sugar of the patient rose again. After readjusting their treatment, no one required using insulin again; only need metformin (250-500 mg/day) to control blood glucose levels[13].

Patient's Clinical Data

Both diastolic and systolic blood pressures in all patients were decreased and maintain during 36 months (Table III). As shown in Table IV-IX, the patients' FIN didn't change notably after the transplantation ($p<0.05$), but there is a significant decrease in the level of 2-h PG and FBG in contrast with the values before the transplantation ($p<0.05$ for both). The BMI stayed the same for three years following the transplantation. HbA1c, HDL-C, TG, TC, LDL-C and C-peptide did not change significantly after the transplantation.

Table II. Insulin usage and dosage of metformin before and after transplantation (N=4).

Patient No.	Pre-transplantation Insulin dosage (u/day)	Time for the start of reduced insulin usage (days)	Time when insulin use discontinued (days)	Total insulin and metformin free time (months)	Dosage of metformin after readjustment in 12 months (mg/day)	Dosage of metformin after readjustment in 24 months (mg/day)	Dosage of metformin after readjustment in 36 months (mg/day)
1	46	3	45	36	0	0	0
2	30	2	42	11.3	250	500	500
3	14	2	25	14.5	0	250	500
4	20	3	30	29	0	0	250

Table III. Patients' blood pressure before and after transplantation (N=4).

	Before transplantation		After transplantation									
			3M		6M		12M		24M		36M	
Patient No.	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)
1	155.61±11.33	95.18±9.39	120.43±9.52	80.21±8.47	115.46±13.24*	80.39±8.43*	120.35±9.51	80.23±7.01	120.45±11.22	80.19±6.08	130.09±11.53#	85.31±9.44#
2	150.46±13.22	90.47±11.22	115.28±8.56	80.39±10.25	110.21±9.53*	75.72±9.13*	115.45±8.76	80.52±8.63	115.25±9.68	80.34±7.19	125.28±11.47#	85.27±7.36#
3	160.39±9.34	105.85±9.36	120.11±9.76	85.51±6.75	120.77±10.34*	80.52±10.25*	120.15±8.55	85.29±9.48	120.33±11.02	85.35±8.11	130.36±9.63#	90.43±6.93#
4	150.26±10.45	95.57±12.19	110.66±11.53	80.46±7.65	110.33±9.08*	75.43±9.64*	110.47±10.31	80.15±10.37	110.06±9.82	80.09±7.34	115.08±10.58#	85.56±8.74#

SBP: Systolic Blood pressure; DBP: Diastolic Blood pressure. Compare to blood pressure before transplantation, *$p<0.01$; #$p<0.05$.

Table IV. Blood biochemical characteristics of type 2 diabetic patients before transplantation (N=4).

Patient No.	FIN (pmol/l)	C-peptide (pmol/l)	BMI (kg/m^2)	FPG (mmol/l)	2hPG (mmol/l)	HbA1C (%)	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
1	173.5	0.61	27.1	7.01±0.13	8.35±0.63	7.63	2.16	4.67	2.47	2.68
2	132.6	0.56	25.3	6.93±0.26	9.01±0.52	7.56	2.33	4.93	2.42	2.66
3	201.1	0.72	28.5	6.55±0.46	8.67±0.33	7.18	2.29	5.01	2.58	2.84
4	163.7	0.63	25.5	7.06±0.38	8.91±0.54	6.89	2.31	4.94	2.56	2.53

FPG: fasting plasma glucose; FIN: fasting insulin; BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; 2hPG: 2-hour postprandial blood glucose; TC: total cholesterol; TG: triglyceride; HbA1c: glycosylated hemoglobin.

Discussion

The current case series looked into the potential of amniotic stem cells that are undifferentiated in a culture in treating patients with type 2 diabetes, according to our previous results on a treatment for type 1 diabetes that is efficient and tolerated well[12]. Our results suggested one plausible therapeutic strategy to improving glycemic control.

Herein, we answered three questions. First, which kind of stem cells we use? Second, which outcome was outstanding? Third, which mechanisms are associated with the current outcome?

Firstly, we discuss the characters of the cells used in the current study. The cells isolated from amniotic membranes are heterogeneous[14-17]. There are two stem cell types on the amniotic membrane; one is an amniotic epithelial cell and the other is an amniotic mesenchymal stem cell. Base on previously reports, AMSCs positively expresses CD105, CD90, CD73 and CD44, and negatively expresses CD45, CD34, CD106 and HLA-DR[18,19]. Meanwhile, AMSCs show fibroblasts morphology. Pluripotent stem cells and embryonic stem cells have a potential in differentiating into various types of cells, and their characteristics are displayed by AECs[20,21]. Cells used in this present study expresses CD34 and CD133, which are regarded as markers on the surface of cells to indicate endothelial progenitor cell[22]; very few of the adherent cells had hematopoietic lineage markers HLA-DR and CD45 (Figure 1). Although MSCs go through changes in phenotype both in the culture and throughout the passage[23], we prefer to accept the cells we used include at least two kinds of cell lineages: AMSCs and AECs. Furthermore, we're prone to use the common method to collect activity cells, and some cells might act as an adjuvant role to promote or aid the major ones homing and differentiation. The activity of the cells was evaluated by glycemic control next.

Second, we focus our study on the outstanding outcomes. In the current study, all patients were not dependent on insulin injections or oral hypoglycemic agents for 22.7 months (from 11.3-36 months, Table II). Even when the patients required oral hypoglycemic agents therapy be brought, their daily oral hypoglycemic agents requirement decreased quite a bit (Table II), suggesting an improvement in insulin sensitivity. Unfortunately, we did not observe changes of C-peptide data before and post-transplantation, which had verified that stem cell treatment can affect positively the function of β-cells effectively. These data suggest this kind of cell therapy improves glycemic control. We were not expecting the second outstanding result. We observed both all the patients' diastolic and systolic blood pressure were decreased and maintained during 36 months (Table III). MSC co-transplantation with improved pancreatic revascularization[24] and treatment of diabetic microvascular complications with MSCs improve glycemic control[25]. In the current study, we transplanted amniotic stem cells into the dorsal pancreatic artery. Thus, we presumed the improved glycemic control might be due to the improved vascular function as a systemic response and/or pancreatic microenvironment as a local response in the current study.

Although there are many studies[26-36] on MSCs' transplantation both in animals models and in patients, the precise mechanism that form the basis of the beneficial effect is still not sure. Many re-

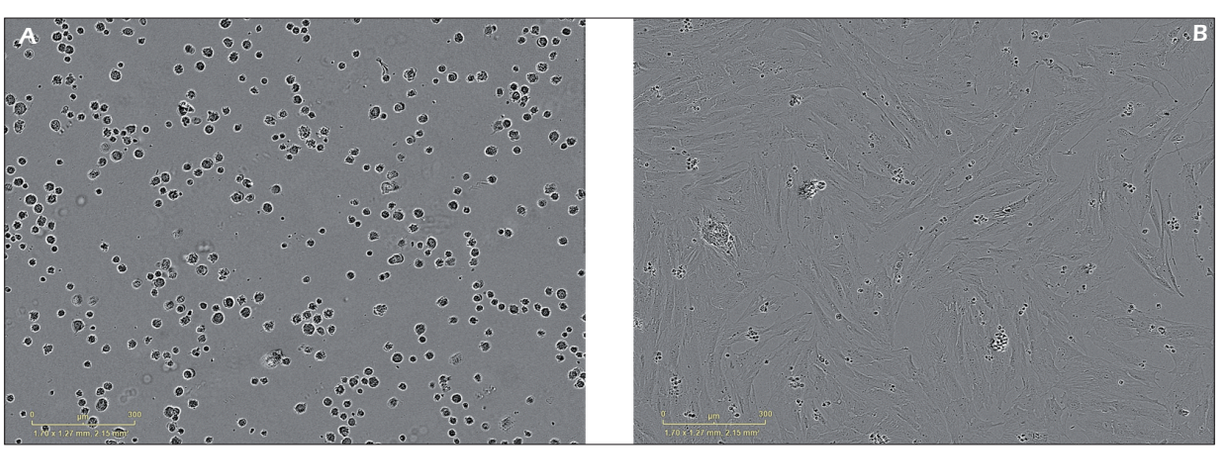

Figure 1. ***A,*** The amniotic stem cells. ***B,*** Fifth Generation (P5) of amniotic stem cells.

Table V. Blood biochemical characteristics of type 2 diabetic patients in 3 months after transplantation (N=4).

Patient No.	FIN (pmol/l)	C-peptide (pmol/l)	BMI (kg/m^2)	FPG (mmol/l)	2hPG (mmol/l)	HbA1C (%)	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
1	169.1	0.67	27.2	6.83±0.41	8.62±0.47	7.01	2.21	4.59	2.41	2.72
2	143.2	0.61	25.6	6.56±0.22	8.93±0.69	6.35	2.43	4.68	2.66	2.53
3	203.6	0.76	27.8	7.03±0.19	8.52±0.26	6.94	2.35	5.32	2.49	2.76
4	169.1	0.71	24.9	6.75±0.34	8.87±0.33	6.35	2.41	5.01	2.53	2.24

FPG: fasting plasma glucose; FIN: fasting insulin; BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; 2hPG: 2-hour postprandial blood glucose; TC: total cholesterol; TG: triglyceride; HbA1c: glycosylated hemoglobin.

Table VI. Blood biochemical characteristics of type 2 diabetic patients in 6 months after transplantation (N=4).

Patient No.	FIN (pmol/l)	C-peptide (pmol/l)	BMI (kg/m^2)	FPG (mmol/l)	2hPG (mmol/l)	HbA1C (%)	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
1	170.3	0.63	27.1	6.01±0.37	8.65±0.61	6.56	2.19	4.53	2.39	2.32
2	139.2	0.59	24.8	6.52±0.24	8.56±0.35	6.73	2.38	4.66	2.46	2.57
3	199.3	0.73	27.6	5.96±0.31	8.01±0.28	6.81	2.41	4.98	2.41	2.62
4	172.1	0.68	25.1	6.33±0.34	8.32±0.44	6.34	2.26	4.27	2.35	2.43

Table VII. Blood biochemical characteristics of type 2 diabetic patients in 12 months after transplantation (N=4).

Patient No.	FIN (pmol/l)	C-peptide (pmol/l)	BMI (kg/m^2)	FPG (mmol/l)	2hPG (mmol/l)	HbA1C (%)	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
1	168.8	0.67	26.9	6.36±0.41	8.64±0.35	6.03	2.21	4.82	2.39	2.35
2	129.1	0.52	25.4	6.58±0.25	9.25±0.61	6.58	2.03	5.06	2.05	2.59
3	189.3	0.76	27.8	6.35±0.42	8.73±0.38	6.15	2.36	5.14	2.33	2.66
4	161.9	0.69	24.9	6.62±0.25	9.03±0.32	6.36	2.18	4.86	2.19	2.58

Table VIII. Blood biochemical characteristics of Type2 diabetic patients in 24 months after transplantation (N=4).

Patient No.	FIN (pmol/l)	C-peptide (pmol/l)	BMI (kg/m^2)	FPG (mmol/l)	2hPG (mmol/l)	HbA1C (%)	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
1	170.4	0.63	27.1	6.06±0.35	8.89±0.37	6.31	2.19	4.53	2.43	2.61
2	138.6	0.61	25.9	6.25±0.61	8.92±0.56	6.55	2.28	4.98	2.39	2.46
3	191.7	0.66	28.2	6.32±0.48	8.35±0.39	6.39	2.33	4.98	2.46	2.73
4	166.8	0.63	25.2	6.29±0.27	8.21±0.13	6.25	2.26	4.59	2.58	2.49

Table IX. Blood biochemical characteristics of Type2 diabetic patients in 24 months after transplantation (N=4).

Patient No.	FIN (pmol/l)	C-peptide (pmol/l)	BMI (kg/m^2)	FPG (mmol/l)	2hPG (mmol/l)	HbA1C (%)	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
1	172.8	0.64	27.2	6.21±0.22	9.03±0.37	6.85	2.23	4.72	2.36	2.59
2	141.3	0.64	25.6	6.37±0.53	9.22±0.64	6.73	2.41	4.86	2.51	2.61
3	196.6	0.69	27.8	6.09±0.24	8.92±0.58	6.59	2.55	4.85	2.46	2.78
4	167.4	0.67	24.9	6.32±0.36	8.86±0.49	6.45	2.43	4.77	2.33	2.46

ports indicated that AMSCs and AECs support microenvironment by secreting cytokines, such as angiogenic factors and insulin-like growth factors (IGFs)[37,38]. In this study, the FIN kept unchanged before and after the transplantation. There is also the possibility that those transplanted cells-secreted IGFs, such as IGF-1 share the responsibility of angiogenesis and energy homeostasis, or other secreted cytokines help to increase the sensibility of insulin[39]. The multipotency of MSCs and their secretion will need to explore further.

There have been several studies that have made a report on stem cells transplantation in treating type 2 diabetes. Bone marrow stem cells have been put into use during phases 1 to 3 in clinical trials to treat type 2 DM already (NCT00465478, NCT00644241, NCT01142050, NCT01677013, and NCT01759823), which is on the list of Clinical Trails. gov registry[40,41]. Hyperbaric oxygen treatment and BMSCs treatment in pancreas combined are helpful in improving the control over glucose and reducing the dose of oral hypoglycemic drugs/insulin in patients suffering from type 2 diabetes, even if they can only improve the function of β-cells in the pancreas for a short time. In this study, the level of blood sugar increased again after 13.2 months of transplantation. So the results lead to another question, why the changes are so transiently? Subjective reasons might be high caloric intake; sedentary lifestyle (when the blood sugar is under control in these patients), and the objective reason is possibly related to the grafted cells died or the loss of ability for differentiation potential and functional proliferation. It is very difficult to clarify how long the cells can cause entrapment *in vivo* by now.

The case report has one defection, and that is no supervises on allograft rejections, as well as the fact that one patient successfully maintains glycemic control without any medicine regiment for 3 years. Therefore, additional suppression of the immune system would be consulted to be required in this therapeutic approach. Further randomized controlled studies, involving more patients and the safety of using undifferentiated amniotic stem cells need to be proven in clinical trials.

Conclusions

Judging from the data, amniotic membrane stem cell transplantation has improved glycemic control

in vivo, however, it worth noticing that a complete long-term recovery of the function of β-cells has not been achieved in the patients yet; there is a requirement of post-transplantation medicine treatment for several months. Because of less ethical concerns and the convenience for amniotic membrane stem cells to be isolated and then expanded *in vitro*, these cells can be appealing in search of other sources of stem/progenitor cells in doing translational or basic research to treat diabetes.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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