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Original Article

Administration of Autologous Mesenchymal Stem Cell Transplantation for Treatment of Type 1 Diabetes Mellitus

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Abstract

Background: The aim of the present clinical trial was to investigate the efficacy of autologous bone marrow mesenchymal stem cells (BM-MSCs) in glycemic control of diabetic patients without using any immunosuppressive drugs over a nine-month period.

Method: Twenty-three patients with T1DM, at 5 to 30 years of age and in both sexes, participated in this study. This trial consisted of two phases; in the end of the first phase (three month after the transplantation), if the patient still needed exogenous insulin to control his/her glycemic state, the second phase of study was performed. In both phases, 100 milliliter of mixed mesenchymal stem cells and normal saline containing 2×10^6 autologous cells/kg for each patient was delivered to patients through cubital vein. All patients were evaluated at 1, 3, 6 and 9 months after the procedure.

Result: Twenty-one patients underwent a second injection. Nine patients (39%) responded positively and 14 patients (61%) responded negatively based on their HbA1c levels and insulin requirements in both injections. Two patients became insulin-free during two rounds of injections. In responder patients, mean levels of C-peptide and HbA1c as well as prescribed insulin dosage significantly decreased compared to baseline measures (P=0.002, P=0.007 and P<0.001). In the second phase, responder patients did not show significant reduction in C-peptide levels compared to the baseline of the second phase. Mean levels of HbA1c and prescribed insulin dosage significantly decreased in comparison to the beginning of the study (P<0.05).

Conclusion: Transplantation of BM-MSC can be viewed as a promising, simple, safe, and efficient therapeutic modality for T1DM.

Keywords: Hematopoietic stem cell transplantation, Diabetes mellitus type 1, Autologous

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder that results in beta cell destruction and impaired insulin production (1). Insulin administration is now the only standard manage-

ment of T1DM to maintain glucose levels as near to normal as safely possible to decrease the rate of complications. However, insulin administration is associated with severe episodes of hypoglycaemia and fails to maintain sufficient metabolic control or prevent diabetes complications (2). Therefore, alternative methods for management of T1DM, which confer better glycemic control and fewer complications such as pancreas and islet cells transplantations have always been sought to replace the lost pool of β cells (3, 4). However, inadequate number of donors, life-long immunosuppressive therapy and immune rejection do not allow widespread use of these approaches and limit them to patients with labile diabetes (5).

Many clinical trials have used immunosuppressive drugs to prevent residual beta cell loss. Although these therapies have declined the rate of beta cell destruction, their associated complications have restricted their administration (6-8).

Recent advances in biomedical science have made it possible to work on new strategies to decrease autoimmune destruction and promote regeneration of pancreatic β cells. For instance, some clinical studies using hematopoietic stem cell in combination with immunosuppressive protocols revealed decreased destruction of β cells in patients with T1DM. However, complications associated with this combination therapy limited their success (9, 10).

Human mesenchymal stem cells (also known as multipotent stromal stem cells) (hMSC) are multipotent adult stem cells that were first isolated from bone marrow (BM) (11). These clonogenic cells have self-renewal and multilineage differentiation capacities and can give rise to multiple cell lineages such as osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes and neural cells (12).

MSCs can be differentiated into insulin-producing cells (IPCs) spontaneously or under special culture medium conditions (13, 14). Rodent and human BM-MSCs have been differentiated to IPCs with specific inductive protocols without transfection (14, 15).Several studies have demonstrated that transplantation of these IPCs in experimental diabetic models could ameliorate hyperglycemia and even keep the blood glucose levels within the normal ranges (14, 16).

In addition, undifferentiated MSCs have the ability to control glycemic status in diabetic animals due to their immunomodulatory and regenerative properties (17-20). Multiple in vivo studies have shown that MSCs can regenerate impaired beta cell mass (17, 21). Thus, MSCs can also modulate the function of various immune cells (20, 22, 23). With respect to these properties, MSCs have been used in multiple clinical trials on patients with autoimmune diseases (20, 24, 25). However, there are still limited data on the efficacy of human BM-MSCs in treatment of patients with T1DM.

Accordingly, we have conducted a clinical trial to investigate the efficacy of autologous human BM-MSCs in glycemic control of 23 patients with T1DM without using any immunosuppressive drugs and evaluated changes in their daily doses of insulin administration, as well as any improvements in their clinical conditions and laboratory tests over a six-month period.

Methods

Patients and study design

In this clinical trial, 23 patients, at 5 to 30 years of age and in both sexes, were selected from the patients referred to the diabetes clinic at Shariati Hospital, Tehran, Iran. All patients were diagnosed to have T1DM over the past 20 weeks before the study. The diagnosis was confirmed by the measurement of serum levels of anti–glutamic acid decarboxylase (anti-GAD) antibodies.

The protocol of this study was approved by the Ethics Committees of Endocrinology and Metabolism Research Institute at Tehran University of Medical Sciences and the Iranian Ministry of Health (Ethic code: E-00182, IRCT13881027-1414N8). The informed consents were signed by the patients (or their parents in cases younger than 18 years of age) to participate in the study.

Patients were selected through the following exclusion criteria as follows: any history of diabetes acute complications (e.g. diabetes ketoacidosis (DKA), positive serology for hepatitis B or C, and human immunodeficiency virus (HIV), history of

cardiac, nephrologic, hematologic, psychiatric, hepatic or any chronic disease as well as pregnancy in female patients.

This study was designed to have two phases; each phase last for 3 months and in the end of the first phase, if the patient still needed exogenous insulin to control his/her glycemic state, the second phase of study (second set of MSCs injection) was performed. All patients were monitored for 6 months. HbA1c levels were measured before the study and in the first and the third month of each phase. Moreover, blood samples were collected to measure fasting blood sugar before and every month after transplantation. In addition, daily FBS levels reported by patients themselves via self-monitoring blood glucose assessment using glucometer devices were also recorded. C-peptide levels, also were measured through mixed-meal tolerance test before and every month after transplantation.

MSCs preparation

50 ml of bone marrow was aspirated from the posterior iliac crest of the patients under local anesthesia and sterile conditions. Stem cell isolation and expansion was performed in a clean room (FS 209 E & ISO 14 644). Bone marrow mononuclear cells were isolated by density gradient centrifugations as described previously with some modification (26).

Briefly, heparinized bone marrow was washed with phosphate-buffered saline (PBS) and centrifuged at 900×g for 10min. Washed cells were resuspended in PBS and layered over a ficoll solution and centrifuged at 1200 × g for 20 min. Mononuclear cells were separated from the interface, and resuspended in low glucose Dulbecco's modified Eagle's medium (L-DMEM; Sigma) and 10% fetal bovine serum. Then, the cells were centrifuged and counted. Afterwards, mononuclear cells were cultured with DMEM-LG containing 10% FBS supplemented with antibiotic-antimycotic solution (Penicillin G sodium: 100U/ml, Streptomycin sulfate: 100Ìg/ml, Amphotericin B: 0.25Ìg/ml; Gibco/BRL) at a density of $2.8\times10^7/175$ mm^2 culture flask and incubated at 37°C in a humidified atmosphere with 5% CO2. Medium was exchanged every 3 days. When these primary MSCs reached 80–90% of confluency, they were detached with trypsin and EDTA, counted and passaged at a density of $0.5–0.7\times10^6$ in 75 mm^2 culture flasks and were referred as the first passage MSCs. The MSCs were expanded for three passages in vitro. After 4 weeks, the cell number reached 2×10^6 / kg for each patient. After trypsinization, MSCs were washed repeatedly and resuspended in normal saline to a final concentration of 2×10^6 cells per ml. Thereafter, normal saline was added again to raise the final volume of transplantation suspension to 100 ml. In other words, the transplantation suspension contained 2×10^6 cells/kg for each patient and had the total volume of 100 ml. MSCs viability, tested via trypan blue assay, was greater than 95% for every patient. For any possible re-transplantation in the second phase of the study, an approximate number of 4×10^6 MSCs at passage 1 was frozen and preserved in liquid nitrogen.

Microbiological analysis was done for MSC cultures weekly and was negative for bacterial, fungal, viral, or mycoplasmal contamination. In addition, superficial markers checked through flow cytometry was proved to be positive for CD73, CD90, CD105 and negative for CD11b, CD14, CD19, CD29, CD34, CD45, and HLA-DR. Furthermore, karyotyping analysis was performed to prove genomic stability of MSCs.

The first phase of mesenchymal stem cells transplantation

To get patients prepared before transplantation - over the period harvested cells reached adequate numbers – the patients underwent nutritional care to better control their blood glucose levels. Moreover, their daily doses of insulin were adjusted every week. After the cells had been prepared for transplantation, patients were admitted to Hematology, Oncology and Stem Cell Transplantation Research Center at ShariatiHospital. Then each patient's blood was sampled to measure anti Glaydin, HbA1C, FBS and C-Peptide. Afterwards, the pre-prepared transplantation solution (100 milliliter of mixed mesenchymal stem cells and

normal saline) was injected to patients through the cubital vein over 30 minutes. The recipients were closely monitored for the whole period of the intervention and their blood glucose levels were measured every hour by commercially available glucometer to keep their blood glucose levels less than 200 mg/dl. Patients were discharged on the same day in case of no adverse reactions or symptoms post-transplantation.

Patients recorded their blood glucose levels by self-monitoring every three-hour in the first week, and then four times a day to maintain their average blood glucose levels below 200 mg/dl, through sliding scale insulin therapy. Moreover, the patients underwent standard diabetic diet over the three-month follow up period.

The second phase of mesenchymal stem cells transplantation

Re-injection was performed for 21 patients who still needed exogenous insulin in the last month of the first phase. The patients were admitted to Hematology, Oncology and Stem Cell Transplantation Research Center at Shariati Hospital and blood samples were taken to measure HbA1C, FBS and C-Peptide**.** After thawing the cryopreserved MSCs, the cells were expanded for three passages and reached 2×10^6 / kg for each patient. Preparation of the transplantation solution was the same as the method explained in the first phase. Finally, the pre-prepared transplantation solution (100 milliliter of mixed mesenchymal stem cells and normal saline containing 2 $\times10^6$cells/ kg for each patient) was injected to the patients through their cubital vein over 30 minutes. The recipients were taken under close observation and discharged on the same day if they showed no adverse reactions or symptoms following transplantation. Insulin titration was based on sliding scale method to control their blood glucose levels under 200 mg/dl. Blood glucose levels over the next three months post-transplantation were reported similar to the first phase. In both phases of transplantation, all patients were evaluated at 1, 3 and 6 months after the procedure .Their visits included a thorough physical examination, adjustment of insulin doses according to patient's blood glucose levels as well as taking blood samples to measure FBS, HbA1C and C-Peptide levels.

Statistical analysis

All data analysis was done using the statistical program for the Social Sciences (Release 16.0, PC Windows; SPSS Inc., Chicago, IL). In order to compare trend of HbA1c and C-peptide, Spearman correlation coefficient was calculated. To assess the change of mean HbA1C, C-peptide, and Insulin over follow-up time separate mixed effect models with random intercept with time as the only predictor in the models were fitted. A *P* value less than 0.05 was considered statistically significant.

Results

Twenty-three patients with T1DM, aged 5 to 31 years (mean: 18.4 years), participated in the study. Patient 'demographic data and follow-up variables are shown in Table 1. All participants had a short duration of disease (maximum duration of 20 weeks) and reported no history of previous diabetic ketoacidosis. All patients presented clinical symptoms of hyperglycemia at diagnosis. Anti–glutamic acid decarboxylase antibody was positive in all patients. Mean body mass index (calculated as weight in kilograms divided by height in meters squared) was 19.7 (range, 16.6-23.4) at diagnosis.

The number of infused mesenchymal stem cells in each phase was 2×10^6/kg. No adverse effects related to transplantation such as fever, chills, liver damage or immune rejection were observed after transplantation.

The patients were categorized in two groups. If patient's insulin requirement and HbA1c decreased, they considered that as responder to treatment (responder) but if, in spite of treatment, patient's insulin requirement or HbA1c remained constant or increased, the case was considered as nonresponder to treatment (nonresponder).

Twenty-three patients with type1 diabetes were enrolled in the study, of whom 14(70%) were female and 9 (40%) were male.

Table 1: Demographic Characteristic of Patients at the First Injection

	Responder(n=9)		Non-Responder(n=14)		*P* value
	Mean	SD	Mean	SD	
Age (yr)	13.2	8.44	12.15	3.8	0.678
Weight	41.33	23.33	41.21	15.86	0.988
Sex (M/F)	4/5		5/9		0.657

Mean age of participants was 12.56(±5.83) ranging between 5-33 years. Patients were followed 3 months for the first Injection and 6 months for the second injection.

Eight patients responded positively and 13 patients responded negatively in both injections, while two patients, cases number 6 and 20, responded differently in both injections. Case 6, a 14 years old boy, was a responder in the first injection and a non-responder in the second injection and case 20, a 15-year-old girl, did not respond in the first injection but considered as a responder in the second injection. Patients' age, weight and sex were not different between responder and non-responder groups (*P* values>0.05) (Table1).

All the Measurements of HbA1c, C-peptide, and prescribed dosage of insulin are shown for each person under study through Tables 2-3. Results are shown based on patient's response to injections.

Table 2: C-peptide and HbA1c Follow-up in Responder Group

Patient ID		1st Injection				2nd Injection			
		Pre-Injection	1st Month	2nd Month	3rdMonth	1st Month	2nd Month	3rdMonth	6thMonth
6	HbA1C	8.4	6.6	-	6.8	-	-	-	-
	C-Peptide	1.5	1.5	-	0.99	-	-	-	-
	Insulin	24	7.2	6	6	-	-	-	-
7	HbA1C	12	8.6	-	8.8	10	-	10.4	10.4
	C-Peptide	0.8	1.5	-	0.01	0.1	-	0.05	0.5
	Insulin	28	16.1	22	19	23	22	22	22
8	HbA1C	10.5	6.1	-	7.7	8.2	-	8.4	8.4
	C-Peptide	1.1	0.87	-	0.44	0.3	-	0.08	0.8
	Insulin	22	12.8	16	9	7	11	15	15
12	HbA1C	6.3	5.3	-	5.9	6.3	-	7.7	6.9
	C-Peptide	1.3	2	-	1.08	0.5	-	0.6	0.3
	Insulin	9	2.9	8	10	8.5	14	16	18
14	HbA1C	9.8	6.2	-	6	6	-	7.5	7.5
	C-Peptide	2.8	3.2	-	2	0.7	-	0.51	0.51
	Insulin	15	1.9	8	9	4.4	16	19	19
15	HbA1C	6.7	6.1	-	6.3	6.3	-	6.4	5.3
	C-Peptide	0.8	0.5	-	0.5	0.5	-	0.5	0.05
	Insulin	11	9.8	8	8	10.5	16	18	18
16	HbA1C	7.2	6.5		6	6.3	-	6.6	6.7
	C-Peptide	2.4	1.99		1	1	-	0.85	0.58
	Insulin	14	7.8	11	12	0	0.5	10	12
19	HbA1C	6.6	6.5		7.2	7.2	-	7.2	7.2
	C-Peptide	1.4	1.82		1.6	1.6	-	1.6	1.6
	Insulin	**20**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
20	HbA1C	-	-	-	-	8.5	-	8.6	8.6
	C-Peptide	-	-	-	-	1	-	0.8	0.8
	Insulin	-	-	-	-	1.8	4	20	20
23	HbA1C	5.9	6.2	-	5.1	7.2	-	7.2	6.5
	C-Peptide	3.7	3.96	-	3.2	3.2	-	3.2	1.3
	Insulin	**18**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Mean	HbA1C	8.16(2.15)	6.46(0.89)	-	6.64(1.12)	7.33(1.33)	-	7.78(1.22)	7.5(1.47)
	C-Peptide	1.76(0.99)	1.92(1.08)	-	1.20(0.96)	0.99(0.94)	-	0.91(0.97)	0.64(0.52)
	Insulin	7.79 (6.77)	6.47(6.45)	8.78 (7.03)	8.11 (5.86)	6(7.58)	9.28(8.32)	13.33(8.29)	13.78 (8.32)

Cases 6 and 20 were non-responder for the 2nd and 1st injection, respectively

Responder Group

Mean of C-peptide levels in the responder group showed a slight increase one month after the first injection, but it decreased after the first month (Fig. 1) and this reduction was statistically significant from baseline (baseline, 1.76±0.99; 1st month, 1.92±1.08 (β=0.17, P=0.243); 3rd month, 1.20±0.96 (β=-0.55, P=0.002). After the second injection, mean level of C-peptide showed a steady decline from pre-injection level with the least of 0.64±0.52 six month after the injection. This reduction in C-peptide levels was not significantly different from baseline for months1, 3 and 6. after the second injection (1st month, 0.99±0.94 (β=-0.24, P=0.124); 3rd month, 0.91±0.97 (β=-0.32, P=0.062); 6th month, 0.64±0.52 (β=-0.51, P=0.064) but these reduction in C-peptide levels were statistically significant compared to the mean C-peptide level of the patients before cell-therapy (P=0.003 for the 5th month, P<0.001 for the 7thand 10th month).

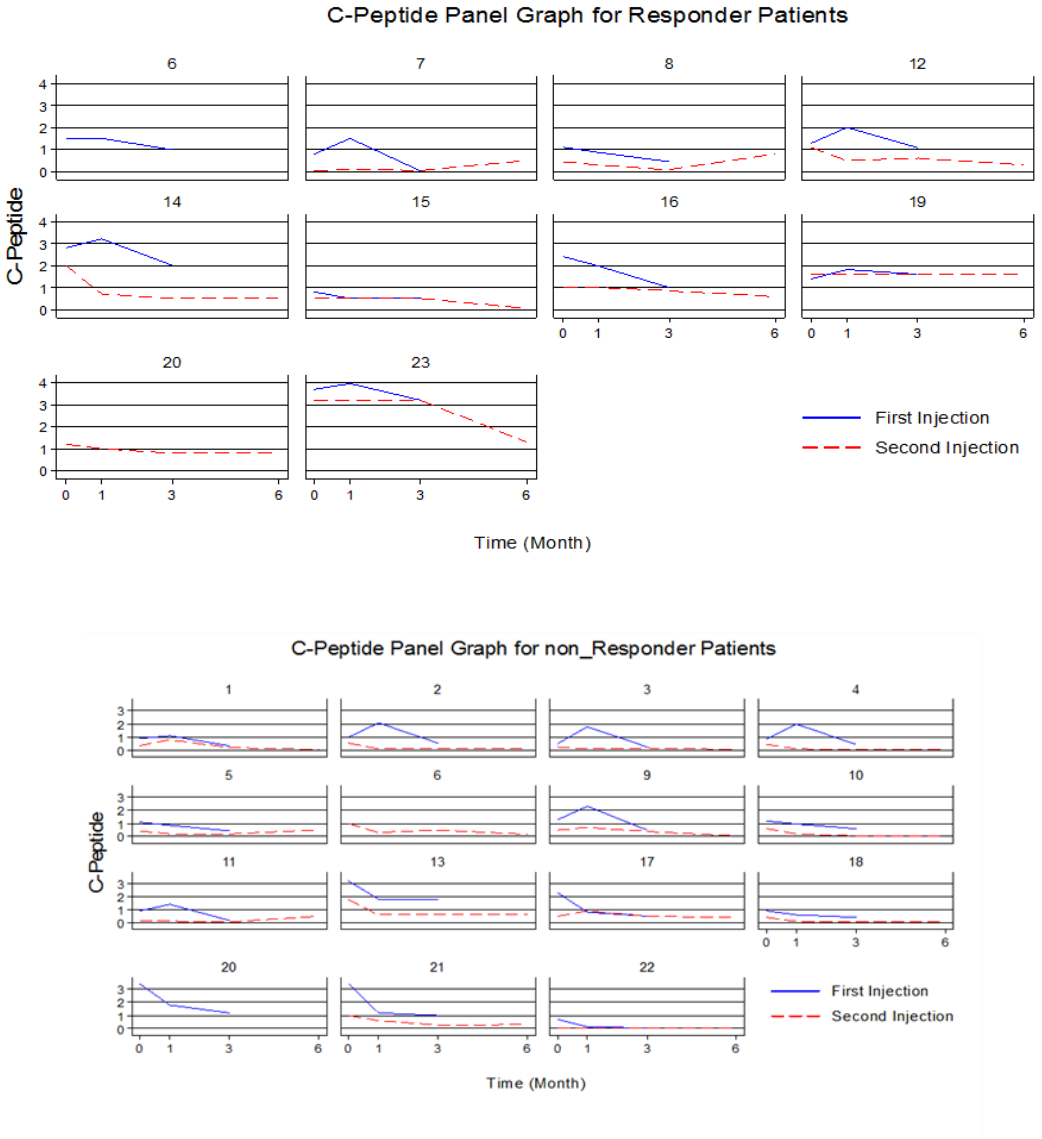

Fig.1: Individual Trajectories of C-Peptide Level of Studied Patients by id (a).responders, (b).non-Responders

In the responder group, after the first injection, mean of HbA1C levels significantly decreased compared to baseline measures (baseline, 8.16±2.15; 1st month, 6.46±0.89 (β=-1.7, P=0.008); 3rd month, 6.64±1.12 (β=-1.51, P=0.007). After the second injection, although mean of HbA1c levels increased until the 3rd month and then it slightly decreased, this increase from the baseline was statistically significant for months three and six (1st month, 7.33±1.3 (β=0.49, P=0.055); 3rd month, 7.78±1.22 (β=0.93, P=0.003); 6th month, 7.5±1.47 (β=0.66, P=0.031), but compared to the HbA1c level of these patients at the beginning of the study significant reduction was observed $\beta = -1, P = 0.008$ for the 5th month, $\beta = -0.56, P = 0.131$ for the 7th month, and $\beta = -0.83, P = 0.026$ for the 10th month.

The average FBS levels decreased with a mean of 10 percent over time compared to the baseline measures (134.33±25.02) after the first injection (Table 3) while this decreasing trend was not significant (β=-0.69, P=0.360). Mean of the pre-injection FBS level for the second injection was 134.33±25.02 and the average of FBS levels over the follow-up periods was 30 percent greater than this value. Compared with baseline, this increase was significant; 1st week, 169.56±68.26 (β=52.3, P<0.001); 2nd week, 160.56±43.34 (β=43.3, P=0.004); 3rd week, 148.89±33.87 (β=31.7, P=0.029); 4th week, 153.33±32.15 (β=36.11, P<0.014); 2nd month, 145.56±25.21 (β=28.3, P<0.051); 3rd month, 140.44±28.92 (β=23.2, P=0.108); 6th month, 149.11±41.35 (β=31.9, P<0.029).

While two patients, case 19 (a thirteen-year-old female) and case 23(a seventeen-year-old male), became insulin-free during two rounds of injections, in other responder patients, mean levels of prescribed insulin dosage decreased after the first injection till first month and then slightly increased. Compared to baseline mean values (17.89±6.23), a significant reduction in insulin dosage was observed for each follow-up intervals; 1st week, 7.79±6.77 (β=-10.10, P<0.001); 2nd week, 7.08±6.93 (β=-10.81, P<0.001); 3rd week, 5.82±6.01 (β=-12.07, P<0.001); 4th week, 5.2±6.09 (β=-12.69, P<0.001); 2nd month, 8.78±7.03 (β=-9.11, P<0.001); 3rd month, 8.11±5.86 (β=-9.78, P<0.001). A slight decrease was observed till one month after the second injection, but insulin dosage significantly increased after the third month of injection; pre-injection 7.56±6.31, 1st week, 4.09±6.67 (β=-3.5, P=0.057); 2nd week, 5.23±7.18 (β=-2.3, P=0.198); 3rd week, 6.89 ±8.78 (β=-0.7, P=0.709); 4th week, 7.93±7.7 (β=0.4, P=0.833); 2nd month, 9.28±8.32 (β=1.7, P=0.338); 3rd month, 13.33±8.29 (β=5.78, P=0.002); 6th month, 13.78±8.32 (β=6.22, P<0.001). Finally, after the second injection lower dosage of insulin was used in comparison to the start of the study ($\beta = 10.17, P < 0.001$ for the 5th month, $\beta = -8.83, P < 0.001$ for the 6th month, $\beta = -4.78, P = 0.037$ for the 7th month, and $\beta = -4.33, P = 0.058$ for the 10th month).

Non-responder Group

Mean of HbA1c levels increased after both injections compared to the pre-injection measures. Although a gentle rise from the baseline was not significant after one month, it was significant after three months for the first injection (baseline, 6.83±1.13; 1st month, 8±2.86 (β=1.17, P=0.11); 3rd month, 8.47±1.82 (β=1.64, P=0.004). For the second injection, mean of HbA1c levels increased with the largest value after the third month and then slightly decreased for month six (pre-injection, 8.34±1.88; 1st month, 9.16±2.51 (β=0.82, P=0.096); 3rd month, 10.13±2.42 (β=1.79, P=0.002); 6th month, 9.58±2.53 (β=1.24, P=0.076), all P values obtained from comparison with pre-injection mean values), also compared to the start of the study mean of the HbA1c level were significantly increased after the second injection (P<0.001 for all the periods).

Mean of C-peptide levels decreased over the follow-up period, this reduction was significant after month three for both injections. The average of C-peptide means over the follow-up period had a 37 percent reduction compared to baseline for the first injection, while it was about half of the pre-

injection value for the second injection (for the first injection: baseline, 1.54±1.05; 1st month, 1.35±0.64 (β=-0.2, P=0.52); 3rd month, 0.59±0.45 (β=-0.96, P<0.001), and for the second injection: baseline, 0.57±1.47; 1st month, 0.35±0.3 (β=-0.22, P=0.057); 3rd month, 0.23±0.19 (β=-0.34, P=0.003); 6th month, 0.18±0.19 (β=-0.36, P=0.001), also compared to the C-peptide level of the patients at the beginning of the study, mean of the C-peptide was significantly decreased after the second injection (P<0.001 for all the periods).

Table 3: C-peptide and HbA1c Follow-up in non-Responder Group

			1st Injection			2nd Injection			
Patient ID		**Pre-Injection**	**1st Month**	**3rdMonth**	**3rdMonth**	**1st Month**	**2nd Month**	**3rdMonth**	**6thMonth**
1	HbA1C	6.3	5.4	-	6.6	7.20	-	9.6	12.4
	C-Peptide	0.9	1.1	-	0.35	0.8	-	0.21	0.05
	Insulin	24	12.3	20	16	6	24	22	22
2	HbA1C	7.4	8.5	-	8.6	7.5	-	9.6	7.20
	C-Peptide	1	2.1	-	0.55	0.1	-	0.1	0.1
	Insulin	17	135	28	25	7.8	14	20	16
3	HbA1C	5.9	6.3	-	6.6	7.1	-	6.9	8.8
	C-Peptide	0.5	1.8	-	0.24	0.1	-	0.16	0.05
	Insulin	20	12.8	24	19	7.8	19	18	22
4	HbA1C	6.5	4	-	6.5	7.1	-	6.9	8.8
	C-Peptide	0.88	2	-	0.45	0.1	-	0.05	0.05
	Insulin	20	6.2	27	40	9.4	22	20	25
5	HbA1C	6.9	8.5	-	9.5	12	-	13.6	10.2
	C-Peptide	1.1	0.87	-	0.43	0.2	-	0.2	0.5
	Insulin	62	71.5	72	72	30	36	41	56
6	HbA1C	-	-	-	-	7.8	-	12.4	10.9
	C-Peptide	-	-	-	-	0.3	-	0.49	0.17
	Insulin	-	-	-	-	20.8	35	100	92
9	HbA1C	6.2	8.8	-	8.7	12.5	-	11.4	13.6
	C-Peptide	1.3	2.3	-	0.49	0.7	-	0.38	0.05
	Insulin	16	15	30	35	25.8	75	70	78
10	HbA1C	5.6	5	-	7.7	11.7	-	10.3	9.9
	C-Peptide	1.2	0.96	-	0.61	0.2	-	0.05	0.05
	Insulin	42	35.8	22	22	24	44	46	46
11	HbA1C	5.4	5.5	-	7.1	6.3	-	7.3	7.3
	C-Peptide	0.9	1.4	-	0.17	0.11	-	0.05	0.05
	Insulin	12	9	12	12	10	12	14	23
13	HbA1C	7.3	7.2	-	7.7	5.4	-	5.5	5.6
	C-Peptide	3.2	1.8	-	1.76	0.6	-	0.6	0.6
	Insulin	7	5.5	8	14	0	0	3	3
17	HbA1C	7.4	12.7	-	10.1	10.9	-	11.3	8
	C-Peptide	2.3	0.81	-	0.5	0.9	-	0.51	0.39
	Insulin	48	11.4	24	28	24.8	40	38	52
18	HbA1C	7.3	8.6	-	8.5	8.8	-	12.2	11.5
	C-Peptide	0.9	0.62	-	0.4	0.1	-	0.08	0.05
	Insulin	44	24	36	38	30.5	36	38	45
20	HbA1C	6.6	6.5	-	7.2	-	-	-	-
	C-Peptide	1.4	1.82	-	1.6	-		-	-
	Insulin	26	0.3	1	1	-	-	-	-
21	HbA1C	6.9	8.1	-	8.9	10.7	-	9.6	9.1
	C-Peptide	3.4	1.19	-	1	0.6	-	0.25	0.3
	Insulin	24	0.3	6	10	7.5	26	30	32
22	HbA1C	6.5	14.3	-	13.5	12.8	-	13.2	13.2
	C-Peptide	0.7	0.13	-	0.05	0.05	-	0.05	0.05
	Insulin	55	20.3	26	28	13	28	32	32
	HbA1C	1.54(1.05)	1.35(0.64)	-	0.59(0.45)	0.35(0.30)	-	0.23(0.19)	0.18(0.19)
Mean	C-Peptide	6.83(1.13)	8 (2.86)	-	8.47(1.82)	9.16(2.51)	-	10.13(2.42)	9.58(2.53)
	Insulin	29.79(17.14)	17(18.54)	24(17.07)	25.71(17.39)	15.5(11.31)	29.36(17.83)	35.14(24.86)	38.86(24.53)

Cases 6 and 20 were non-responder for the1st and 2nd injection, respectively.

Insulin mean levels decreased with the maximum reduction two weeks after the first injection and one week after the second injection, and then started to increase. For the first Injection with baseline, 29.79±17.14; 1st week, 16.51±18.17 (β=-13.3, P=0.003); 2nd week, 15.89±18.7 (β=-13.9, P=0.002); 3rd week, 17.57±18.8 (β=-12.2, P=0.007); 4th week, 17.96±18.35 (β=-11.8, P=0.008); 2nd month, 24±17.07 (β=-5.8, P=0.176); 3rd month, 25.7±17.4 (β=-4.1, P=0.337). Compared to mean insulin requirement before the injection 26.07 ± 16.89, there was a significant reduction in insulin dosage mean till three weeks after the second injection (1st week, 8.64±8.78 (β=-17.4, P<0.001); 2nd week, 13.64±10.25 (β=-12.4, P=0.006); 3rd week, 17.5±12.16 (β=-8.6, P=0.58), and after the second month of the injection there was a rise in insulin dosage (2nd month, 35.1±24.86 (β=9.1, P=0.045); 3rd month, 38.86±24.53 (β=12.8, P=0.005), but in comparison to the beginning of the study significant changes in the insulin dosages were not observed ($\beta = -7.35, P = 0.169$ for the 5th month, $\beta = -0.28, P = 0.958$ for the 6th month, $\beta = 5.50, P = 0.304$ for the 7th month, and $\beta = 9.21, P = 0.086$ for the 10th month).

The mean levels of FBS increased from baseline measures (141.29±40.19) during the follow-up period with fifteen percent increase in the average of mean FBS levels for the first injection, but this increasing trend was not significant (β=2.19, P=0.085). For the second injection, mean of the FBS level increased one week after injection and remained at the stable level for three months, this rise was not significant (β=0.69, P=0.489) during follow-up.

Despite moderate negative correlation between mean level of HbA1c and C-peptide the follow-up period in responder patients (r=-0.414, P=0.355), there was a strong negative correlation between these quantities in non-responder patients (r=-0.929, P=0.003).

In responder patients, insulin injection was stopped when the blood glucose levels were below 200 mg/dl in order to investigate infused MSCs effect.

Mean fasting blood glucose levels (FBS) before transplantation were controlled with insulin injections (mean 132mg/dl). After transplantation, despite significant decrease in exogenous insulin requirements, FBS levels showed no increase (mean 118 mg/dl) (P>0.05 between pre-transplantation and all the follow up values).

No episodes of ketoacidosis occurred during this study in patients.

Discussion

There are only a few clinical studies on the efficacy of stem cells in treatment of diabetes. In two studies using hematopoietic transplantation in diabetic patients, the therapeutic effects were accompanied with the complications of immunosuppressive drugs (9, 10).

Our study evaluates the safety and efficacy of autologous BM-MSC in patients with T1DM. Anti-GAD antibody was positive in all patients at different levels. Transplantation of BM-MSCs reduced the daily insulin requirement in 39 percent of patients and two patients did not need to inject insulin any longer.

In the first phase of this study, significant exogenous insulin reduction was observed in 9 patients and 2 patients did not administered insulin for 1 month and 2 other patients got free from insulin injections for 9 months. In responder group, HbA1c decreased from 8.16% to 6.64% on average, and reached nearly normal levels. This further supports the concept that autologous transplantation of BM-MSC is effective in treating type 1 diabetes.

We evaluated the efficacy of BM-MSC mainly according to the values of C-peptide to reflect the function of islet β cells.

After BM-MSC transplantation, in 2 patients, C peptide levels increased or sustained the same over the whole study duration, and in other responder patients, although C peptide levels reduction was statistically significant but this reduction occurred with slow rates during first phase period in comparison to non-responder patients, indicating that in responder group β-cell destruction was decreased and insulin secretion was therefore increased in regards to other group.

However, in two patients who did not need to inject insulin for nine months, honeymoon period must be considered. For better clarification of this issue, more studies must be designed to track transplanted cells in recipients to identify the probable homing sites. In other responder patients, significant reduction in daily insulin requirement was observed during the follow-up period compared to baseline insulin dosage.

In the second phase of our study, after BM-MSC transplantation in 21 of 23 patients, although exogenous insulin requirements were reduced compared with the insulin dosage prior to the study, the insulin requirements in the second phase were increased in comparison to that of the first phase. Moreover, there was a slight increase in HbA1C levels compared with the first phase. However, there was a reduction in HbA1C levels compared with the levels before transplantation.

After the second phase of injections, C-peptide levels decreased more slowly compared with the changes in the first phase, which is indicative of decrease in β -cell destruction and the continuity in endogenous insulin production as a result.

The underlying mechanism of BM-MSC effects on β-cell function improvement after transplantation remains unclear at present. Several studies indicate that BM-MSC can differentiate into islet β cells and then secrete insulin (13-15, 27). BM-MSCs-derived beta cells can improve hyperglycemia, and even lead to euglycemia (14, 16, 27).

Variety of soluble factors secreted by MSCs much more resulted in tissue repair than cell differentiation (28-30). BM-MSC can secrete a number of growth factors and cytokines, like vascular endothelial growth factor (VEGF), insulin-like growth factor (IFG) and hepatocyte growth factor (HGF), which may be able to affect islet function and their regeneration (28, 31, 32).

In addition, transplanted BM-MSC can differentiate into endothelial cells, induce neo-angiogenesis and increase blood supply of pancreas tissue to improve β cell function (33-37).

Finally, MSCs have potential immunomudulatory properties. Many experimental and clinical trial studies have demonstrated that MSCs can modulate immunologic responses. MSCs can impair proliferation and function of CD4+ T cells, CD8+ T cells, and B cells by releasing soluble factors and direct cell contact mechanism (23, 38-41). MSCs increase the number of CD4+ and CD25+ regulatory T cells, favored Foxp3 and CTLA4 expression, suppress function of other T cells subpopulations, and help to promote self-tolerance (23, 42-44).

MSCs suppress monocyte-derived dendritic cells (DCs) differentiation (45, 46). Zhang et al revealed that MSCs and MSCs supernatant could suppress endocytosis of monocyte-derived DCs and the ability to stimulate T lymphocyte proliferation (45). Allogeneic or syngeneic BM- MSCs could prevent or revert autoimmune diabetes in diabetic animals (17-19, 21, 22, 47, 48).

In patients who were responsive to the first phase of BM-MSCs transplantation, the role of diet and life style modifications in achieving such results should also be taken into consideration. Furthermore, poor attachment to dietary intake after the promising results in the first phase might lead to the increase in blood glucose levels. Indeed, more investigations are necessary to rule out these factors.

In patients who were unresponsive to both phases of BM-MSCs transplantation, the failure in maintaining blood glucose levels in normal ranges might have several underlying reasons as if the number of transplanted cells was not sufficient or the cells homing to the pancreas of these patients were not adequate to make any changes to metabolic profiles. Moreover, harvesting BM-MSCs in FBS containing medium might have limited the growth, differentiation capacities and the immunomodulatory functions of the transplanted cells (49-52). Further studies using human serum obtained from the patients themselves or human platelet lysate (HPL) (53-55) are crucial to shed more light on these issues.

Conclusion

Although it is not revealed to be much effective in glycemic control of patients with T1DM for long periods, MSCs transplantation can still be considered a promising approach for treatment of T1DM as the results obtained from the initial ten

patients with T1DM suggest that transplantation of BM-MSC represents a simple, safe, and efficient therapeutic modality for T1DM. However, more clinical trials with larger populations, longer follow-up periods, different routes of transplantation, different sources of MSCs and even more advanced harvesting and transplantation techniques are essential to support this idea.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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