Long term effect and safety of Wharton's jelly-derived mesenchymal stem cells on type 2 diabetes

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Abstract. Cellular therapies offer novel opportunities for the treatment of type 2 diabetes mellitus (T2DM). The present study evaluated the long-term efficacy and safety of infusion of Wharton's jelly-derived mesenchymal stem cells (WJ-MSC) on T2DM. A total of 61 patients with T2DM were randomly divided into two groups on the basis of basal therapy; patients in group I were administered WJ-MSC intravenous infusion twice, with a four-week interval, and patients in group II were treated with normal saline as control. During the 36-month follow-up period, the occurrence of any adverse effects and the results of clinical and laboratory examinations were recorded and evaluated. The lack of acute or chronic adverse effects in group I was consistent with group II.. Blood glucose, glycosylated hemoglobin, C-peptide, homeostasis model assessment of pancreatic islet β-cell function and incidence of diabetic complications in group I were significantly improved, as compared with group II during the 36-month follow-up. The results of the present study demonstrated that infusion of WJ-MSC improved the function of islet β-cells and reduced the incidence of diabetic complications, although the precise mechanisms are yet to be elucidated. The infusion of WJ-MSC may be an effective option for the treatment of patients with type 2 diabetes.

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Introduction

Type 2 diabetes mellitus (T2DM) is characterized by a combination of insulin resistance and pancreatic β-cell dysfunction due to metabolic exhaustion. Sustained hyperglycemia may result in multi-system chronic complications, including micro- and macrovascular complications, which are associated with high morbidity and mortality. With current pharmacological agents, many patients find it difficult to achieve good glycemic control, and the majority of these patients will eventually require insulin therapy (1). Insulin therapy negatively impacts patients' daily lives and does not prevent the occurrence of diabetic complications (2). Therefore, it is imperative that novel strategies for optimal glycemic control or β-cell replacement are explored.

Cellular therapies offer novel opportunities for the treatment of diabetes. Previous clinical studies have demonstrated the potential of stem cells for disease treatment (3-5). Mesenchymal stem cells (MSCs) are a population of self-renewable cells that secrete various cytokines, growth factors and extracellular matrix molecules which have important roles in the regulation of hematopoiesis, angiogenesis, immune and inflammatory responses (6,7). MSCs can be easily isolated and rapidly expanded *ex vivo,* exhibit no tumor formation after long-term cultivation and express intermediate levels of major histocompatibility complex (MHC) class I molecules but not MHC class II on their cell surface, thus allowing allogeneic transplantation(8,9). Moreover, MSCs are capable of homing to injured tissues following intravenous delivery (10-12). These properties indicate that MSCs may be used as a potential therapeutic strategy for treating various diseases.

Previous studies have indicated that MSCs are capable of exerting anti-diabetic effects, resulting in the partial restoration of pancreatic islet function, increased insulin secretion and improved insulin resistance (13-16). Furthermore, it has been reported that single-dose MSC infusion may ameliorate hyperglycemia (13). Although this protocol failed to restore normoglycemia in diabetic animals, multiple infusions of MSCs may have a role in reversing hyperglycemia (13).

Jiang *et al* (14) evaluated the safety and efficacy of allogeneic human placenta-derived mesenchymal stem cells (PD-MSCs) in patients with a long history of T2DM. The results demonstrated that infusion with PD-MSCs effectively decreased plasma glucose levels, improved islet function and induced no serious adverse effects (14). Moreover, Liu *et al* (15) demonstrated that treatment with allogeneic Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) improved metabolic control and β-cell function in patients with T2DM (15). However, the follow-up time of these trials was too short to assess the long-term effect and safety of MSCs on T2DM.

In the present pilot phase I/II study, WJ-MSCs were used to explore the long-term safety and efficacy of WJ-MSCs infusion in T2DM patients with a follow-up period of 36 months.

Materials and methods

Study design. The present phase I/II, 36-month, randomized controlled study was conducted in patients diagnosed with T2DM according to the criteria outlined by the American Diabetes Association (17). The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the Affiliated Hospital of Qingdao University (Qingdao, China). Written informed consent was obtained from all patients prior to enrollment. Throughout, investigators remained blinded to the treatment administered. An independent data and safety monitoring committee monitored the safety and efficacy of the study.

Patients. Study participants were selected from patients admitted to the Affiliated Hospital of Qingdao University for the treatment of diabetes mellitus between September 2010 and December 2011. A total of 87 patients met the inclusion criteria and, following an interview, 64 patients were enrolled. Although 64 patients with T2DM were initially enrolled (Fig. 1), 2 patients in group II and one patient in group I withdrew at the start of follow-up due to immigration to other distant city and a lack of availability. The remaining 61 patients completed the entire study and their data were analyzed. Using a balanced permuted-block randomization method, participants were divided into two groups: The WJ-MSC treatment group (group I; n=31) and the control group (group II; n=30). All patients were subsequently enrolled, treated and followed-up for 36 months until April 2014 at the Stem Cell Center of the Affiliated Hospital of Qingdao University.

Inclusion criteria were as follows: Patients of either sex, aged 18-60 years, with a clinical and laboratory diagnosis of T2DM according to the criteria outlined by the American Diabetes Association (17). Exclusion criteria were: Any malignancies; pancreatic congenital anomaly; positive serology for human immunodeficiency virus (HIV), hepatitis B (HBV) or hepatitis C (HCV); underlying hematologic, nephrologic, cardiac, psychiatric, or hepatic disease; pregnancy; any acute or chronic infection; and any other endocrine and metabolic disease, including hyperthyroidism, hypercortisolism, acromegaly or chromaffin tumor.

Treatment. All patients enrolled into the present study were assessed in the diabetic out-patient clinic for a period of 3 months prior to the initiation of therapy, and were recommended a 1,500-calorie diet and exercise routine, which composed of walking or similar exercise for 1 h three times/week during the entire study and follow-up period. At the initiation of therapy, all patients had been treated with diet, exercise, oral hypoglycemic agents [1500 mg/d dimetyl biguanide (0.5 g t.i.d.) and 4 mg/d avandia] and insulin injections, which were considered baseline treatment, at stable doses for at least two months.

In addition to the baseline treatment, patients in group I were administered two WJ-MSC infusions through the veins in the back of the hand. The infusion interval was four weeks, according to previous studies (14,18). In addition to the baseline treatment, patients in group II were treated with normal saline which was administered in the same volume of parenteral solution as WJ-MSC. All patients were admitted to the hospital for infusion and, following infusion, all patients remained on the same drug therapy, and diabetic diet and exercise regimen as before.

During the 36-month follow-up, the dosages of oral hypoglycemic agents and insulin (26-48U/day, 2-4 times/day) were adjusted according to the patient's blood glucose. Dosages of insulin and oral hypoglycemic agents were increased if the patient's blood glucose had not been controlled within the normal range [fasting plasma glucose (FPG) normal range, 70-110 mg/dl; postprandial plasma glucose (PPG) normal range, ≤140 mg/dl]. Similarly, the dosages of insulin and oral hypoglycemic agents were reduced if the patient's blood glucose was successfully controlled within the normal range.

Stem cell preparation. WJ-MSCs were provided by the Human Umbilical Cord Mesenchymal Stem Cell Bank (Shandong, China). Umbilical cords were obtained from the healthy mothers of healthy full-term fetuses with no familial history of DM and no history of cancer, HBV, HCV, HIV, Epstein-Barr virus (EBV), cytomegalovirus (CMV) or syphilis detected in serum. Umbilical cord collections were approved by the Institutional Medical Research Ethics Committee of the local maternity hospitals. Written informed consent was obtained from each mother several weeks prior to delivery. WJ-MSC preparation was performed in a laminar flow laboratory, as previously reported (18,19). Briefly, umbilical cords were washed twice with phosphate-buffered saline and subsequently dissected with scissors into sections that were ~1 mm^3 in volume. Tissue sections were plated in a cell culture dish (cat no. 430597; Corning, Inc., Palo Alto, CA, USA) in serum-free NutriStem® MSC XF medium for MSCs (Biological Industries, Ltd., Kibbutz Beit-Haemek, Israel). Cell cultures were maintained in a humidified atmosphere with 5% CO_2 at 37˚C. Following 3 days of culture, the medium was replaced to remove the tissue and non-adherent cells, and was subsequently changed twice weekly thereafter. Once 80% confluence had been achieved, the adherent cells (passage 0) were detached with 0.125% trypsin and passaged in the cell culture dish. WJ-MSCs were cultured and expanded in a laminar flow laboratory, which was designed according to good manufacturing practice conditions, for four passages to prepare the final cell products. WJ-MSCs were sterile and qualified for aerobe, mycoplasma, HBV, HCV, HIV, EBV, CMV, syphilis and endotoxin testing. Subsequently, cells

were stained with CD-PE and CD-FITC (from Human MSC Analysis kit; cat no. 562245; BD Biosciences) and analyzed by flow cytometry with a FACscalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). It was determined by flow cytometry that these cells highly expressed CD90 (85.77%), CD105 (79.26%), CD73 (89.63%), and CD146 (54%), but not CD34 (0.23%), CD45 (0.02%) and HLA-DR (0.03%). The chromosomal karyotype of the UC-MSCs was determined as normal by metascan karyotyping system (IMSTAR company, France).

Clinical assessment and follow-up. Medical history was obtained from each patient at baseline, including diabetes duration, diabetes-related complications, and clinical history of hypertension, dyslipidemia and cardiovascular complications. Concomitant lipid-lowering, antihypertensive and anticoagulant/antithrombotic medications were recorded at all visits.

All patients were checked for viral infections including HCV, HBV, HIV, and urogenital infections prior to enrollment. In order to undergo MSC infusion, all patients were admitted to he Affiliated Hospital of Qingdao University. On the day of hospitalization, a primary clinical examination was performed and the following laboratory data were collected: Height; body weight; blood pressure; plasma glucose; glycosylated hemoglobin; fasting serum C-peptide; full blood count; liver and renal function tests; lipid profile tests; cardiac enzyme; cardiac troponin; serum electrolytes; blood coagulation function; microalbuminuria; and cancer screening test. These data were recollected at monthly intervals for the first 3 months and then every 3 months for the subsequent 33 months during follow-up period. Each follow-up visit included a complete physical examination and laboratory tests. In order to optimize diabetes care, each participant had 24-h access to a phone line that connected them to a physician during the follow-up period.

FPG and PPG levels were measured by an enzymatic glucose oxidase/peroxidase colorimetric method (cat no. ECS000016; OneTouch® Ultra, Johnson & Johnson, Shanghai, China). C-peptide was examined via the C-peptide response test (Roche Diagnostics GmbH, Mannheim, Germany; normal range, 1.1-4.4 ng/ml) in the fasted state and following a standardized mixed-meal test. Glycosylated hemoglobin (HbA1c) was examined using high-performance liquid chromatography (Bio-Rad D10; Bio-Rad Laboratories, Inc., Hercules, CA, USA; normal range, 3.9-6.1%). The C-peptide/glucose ratio (CPGR) was calculated to evaluate the glycemic profile of patients at various time points according to the following formula: C-peptide x 100/glucose.

Hypertension was diagnosed if the patient had a history of hypertension, was receiving medication for hypertension or had a resting recumbent blood pressure of ≥140/90 mmHg on two separate occasions. Height and weight were measured in light indoor clothing, without shoes, using a fixed rigid stadiometer and a Seca scale, respectively. Body mass index (BMI; kg/m^2) was determined by dividing the weight (kg) of each patient by their height squared (m^2).

To determine insulin sensitivity, fasting plasma C-peptide (FPC) was used instead of fasting insulin for homeostasis model assessment of insulin resistance (HOMA-IR) and pancreatic islet β-cell function (HOMA-β) analysis. HOMA-IR C-peptide was calculated using the following equation: HOMA-IR C-peptide=FPG (mmol/l) x FPC (pmol/l)/22.5, where the denominator of 22.5 is a normalizing factor. HOMA-β was calculated using the following equation: HOMA-β C-peptide=20 x FPC (pmol/l)/[FPG (mmol/l] - 3.5).

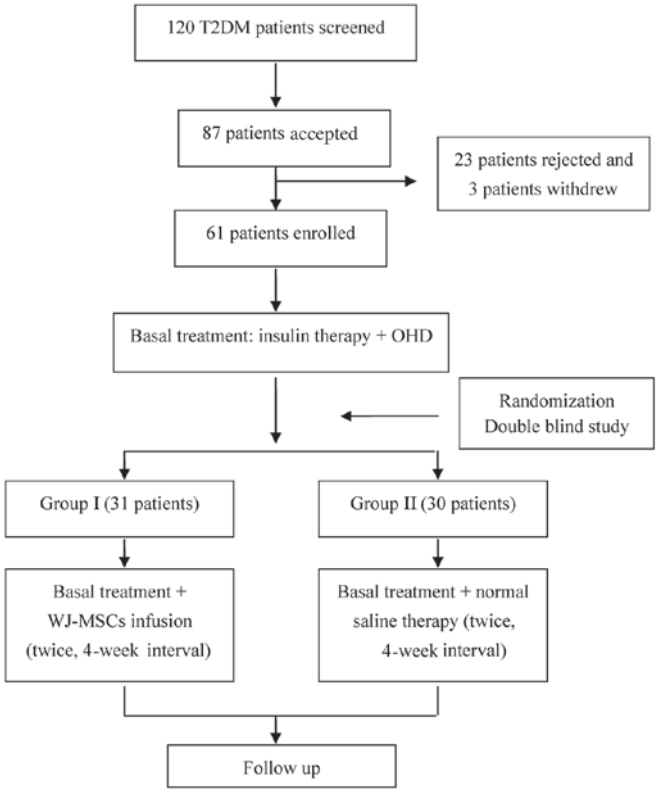

Figure 1. Treatment procedure for this study.

Diabetic complications. Diabetic nephropathy was diagnosed when the patient exhibited at least one of the following: i) Positive microalbuminuria within one year, as confirmed by elevated urine microalbumin levels in at least two of three collections; ii) positive proteinuria, which was defined as a positive urine dipstick test at least 1+ level; and iii) renal insufficiency, as defined by a serum creatinine level ≥132 μmol/l. Patients without nephropathy were defined when they had negative urine microalbumin.

Diabetic peripheral neuropathy was diagnosed when patients exhibited typical symptoms and/or signs of neuropathy, or neuropathy symptoms, as defined by a Michigan Neuropathy Score ≥3 (20), and an abnormal result on the monofilament test at the time of the follow-up visit. Information on patient awareness of diabetic peripheral neuropathy was obtained via an interview. Patient history of ocular surgery was surveyed and the presence and severity of diabetic retinopathy was assessed every 3 months by ophthalmologists. According to The International Clinical Diabetic Macular Edema Disease Severity Scale (21), the severity of diabetic retinopathy was categorized into five stages: i) no retinopathy; ii) mild non-proliferative diabetic retinopathy; iii) moderate non-proliferative diabetic retinopathy; iv) severe non-proliferative diabetic retinopathy; and v) proliferative diabetic retinopathy. 'Incidence of diabetic retinopathy' was defined

in patients with no diabetic retinopathy signs in either eye at the baseline evaluation and mild to severe non-proliferative diabetic retinopathy or proliferative diabetic retinopathy in either of the eyes at follow-up visits over two consecutive years. 'Progression of diabetic retinopathy' was defined in patients with mild non-proliferative diabetic retinopathy at the baseline evaluation, and severe non-proliferative diabetic retinopathy, proliferative diabetic retinopathy or laser photocoagulation treatment for diabetic retinopathy at follow-up visits over two consecutive years.

Study objectives and data collection. The primary objective of the present study was to evaluate the feasibility of WJ-MSC therapy and the safety of MSC infusion during the 12-month period following treatment. Secondary objectives were to assess the safety of MSC infusion over 36 months in patients treated with MSC infusion and to evaluate the therapeutic effect of MSC infusion in patients with T2DM over 36 months.

A data collection form was developed according to the objectives of the present study. Training of researchers and research assistants was performed during a pilot data collection period and a case record form was standardized. Site visits by internal and external auditors were regularly completed in order to assure the quality of the data and the study process.

Safety assessments. Safety assessments included monitoring and recording all adverse events. Potential safety concerns, including hypersensitivity, infection, hemorrhage, proteinuria, myocardial infarction, venous thromboembolic events and other arterial thromboembolic events, were recorded. Hypoglycaemia was defined in patients who exhibited symptoms that were suggestive of low blood glucose and were confirmed by self-monitored blood glucose (SMBG) measurement equivalent to <3.1 mmol/l plasma glucose. Severe hypoglycaemia was defined as any episode requiring the assistance of another party, regardless of whether or not a confirmatory SMBG measurement was available.

Statistical analysis. All statistical analyses were performed using SPSS® 15.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as the mean ± standard deviation. Between-group differences in the means of the baseline values of groups I and II were analyzed using Student's t-test. Comparisons of time-dependent changes at the time of baseline and different time points following the treatment were performed using repeated measure analysis of variance and post-hoc analysis with Bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 64 patients with T2DM were initially enrolled in the study (Fig. 1); however, two patients in group II and one patient in group I withdrew at the start of follow-up due to immigration to other distant city and a lack of availability. The remaining 61 patients completed the entire study and their data were analyzed. Overall, the present study investigated 33 men and 28 women, with a mean age of 52.7±6.3 years (range, 42-63 years). Baseline patient characteristics are shown in Table I. No significant differences in the clinical findings, laboratory examinations or diabetic complications were detected between the two groups prior to the initiation of the study. Cancer screening test confirmed no cancer in all patients. The volumes of parenteral solution of WJ-MSCs and normal saline in group I and II, respectively, were 100 ml, and the number of WJ-MSCs was determined according to the weight of patient. Mean cell number was $6.1\pm2.1\times10^7$ (1.0×10^6/kg; range, $5.3\text{-}8.9\times10^7$).

During the follow-up period, the BMI of patients in group I marginally decreased, whereas a gradual increase in the BMI of group II patients was detected throughout the follow up periods. In spite of this, the differences in BMI between the two groups were not significant. These results suggested that WJ-MSC infusion does not affect the BMI of patients with T2DM.

WJ-MSC infusion ameliorates hyperglycemia in patients with T2DM. Following WJ-MSC infusion, a gradual reduction in the FPG of patients in group I was detected during the follow-up. FPG levels were at their lowest by the third month post-therapy (baseline, 148.3±27.8 mg/dl; 3 months, 112±18.7 mg/dl) and remained stable for the following 18 months, after which, FPG moderately increased during the remaining follow-up time. FPG levels of patients in group II remained consistent for the initial 15 months then began to increase, necessitating the addition of insulin and oral hypoglycemic agents in order to maintain FPG levels within the normal range. No significant differences in FPG levels were detected between the two groups (Fig. 2). PPG levels in the patients in group I were lowest at the sixth month post-therapy and remained stable for 18 months. Compared with group II, levels of PPG in group I significantly decreased from 6-21 months post-therapy ($P<0.05$). Although PPG levels moderately increased after 24 months post-therapy, improved control was retained during follow-up, as compared with the higher and larger fluctuations of PPG detected in group II patients during the whole follow-up period (Fig. 2).

Following WJ-MSC infusion therapy, a gradual decrease in HbA1c was detected in the patients in group I and the lowest level was at the sixth month of follow-up (baseline, 7.67±1.23%; 6 months, 5.69±0.79%), after which HbA1c remained stable for 18 months, then exhibited slight fluctuations over the remaining follow-up period. In group II post-therapy, HbA1c levels remained marginally reduced for 15 months then began to fluctuate due to the addition of oral hypoglycemic agents and insulin. HbA1c was significantly decreased in group I, as compared with group II, between 6 and 24-months post-therapy and at 33-months post-therapy ($P<0.05$; Fig. 2). These results suggest that WJ-MSC infusion is able to decrease hyperglycemia in T2DM patients.

WJ-MSC infusion improves β-cell function and insulin sensitivity in patients with T2DM. Following WJ-MSC infusion, the levels of fasting serum C-peptide in patients in group I decreased at month 1, then progressively increased at month 3 and remained constant for 15 months, with a slight decrease at month 18. At the end of follow-up, the mean levels of fasting C-peptide in group I remained higher than the baseline. In group II patients, fasting C-peptide levels gradually decreased. Fasting C-peptide levels were significantly increased in group I, as compared with group II, throughout the entire follow-up period ($P<0.001$), with the exception of month 33 post-therapy

Table I. Baseline patient characteristics in the two groups.

Variable	Group I	Group II
Clinical characteristics		
Age (years)	52.43±4.88	53.21±8.22
Sex (n)		
Male	17	16
Female	14	14
Duration of T2DM (years)	8.93±5.67	8.3±6.07
Duration of insulin therapy (years)	4.28±1.64	4.14±1.23
Dose of insulin U/d (U/kg/d)	45.92±8.87 (0.79±0.23)	43.09±10.3 (0.74±0.19)
BMI (kg/m^2)	26.74±5.41	27.03±6.68
Hypertension (n)	12	11
Laboratory tests		
FPG (mg/dl)	148.27±27.81	142.31±25.88
HbA1c (%)	7.67±1.23	7.54±1.31
Fasting C-peptide (ng/ml)	1.75±0.64	1.83±0.59
Triglycerides (mg/dl)	130.57±40.22	134.23±42.76
HDL-c (mg/dl)	42.56±5.92	40.92±5.34
LDL-c (mg/dl)	74.90±29.73	75.81±31.57
Complications (n)		
Retinopathy	5	4
Neuropathy	4	3
Nephropathy	3	4

T2DM, type 2 diabetes mellitus; BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; HDL-c, high-density lipoprotein cholesterol; LCL-c, low-density lipoprotein cholesterol. Unless otherwise stated, data are presented as the mean ± standard deviation.

(Fig. 3). The CPGR gradually increased in group I during the initial 21 months of the follow-up period, followed by a gradual decline to the end of the follow-up. A gradual decrease in CPGR was detected in group II patients. CPGR values were significantly increased in group I, as compared with group II, throughout the entire follow-up period (P<0.001).

The levels of fasting glucose and C-peptide of patients in the two groups were all within HOMA limits. HOMA-β in group I patients significantly increased during the follow-up period, as compared with the baseline (P<0.05); whereas in group II patients, HOMA-β gradually decreased. There were significant differences in HOMA-β between the two groups (P<0.05; Fig. 4). HOMA-IR was also evaluated in the present study. Although a decrease in HOMA-IR was detected in group I patients between 18 and 33 months post-therapy, as compared with the group II patients, the difference in HOMA-IR between two groups was not statistically significant. HOMA-IR in group II patients gradually increase throughout the follow-up period (Fig. 4). These results suggested that WJ-MSC infusion could enhance the function of islet β-cells in T2DM patients.

WJ-MSC infusion decreases the requirement for insulin and oral hypoglycemic agents in patients with T2DM. Following WJ-MSC infusion, patients in group I receiving insulin therapy exhibited a gradual reduction in the dosage of insulin required. Insulin withdrawal was demonstrated in 32.3% (10/31) of patients in group I, ranging from 3-11 months (7.9±3.6 months) post-WJ-MSC infusion. These patients remained insulin-free for 12.5±6.8 months. In total, 58.1% (18/31) of patients in group I exhibited a ≥50% reduction in insulin requirement, in five of the remaining 13 patients, daily insulin dosage was reduced by 15-50%; whereas the insulin dosage requirements of 8 patients were maintained or reduced by <15%. In group II patients, the dose of insulin required per day gradually increased after one year. In 47% (14/30) of patients, insulin dosage increased by >50% from the baseline. In the 16 remaining patients, insulin dosage increased by 15-45%. The difference between the two groups was significant (P<0.001) throughout the follow-up period, and the serial changes in the mean doses of insulin required are presented in Fig. 5.

By the end of the follow-up period, 19% (6/31) of patients in group I who received oral anti-diabetic agents were completely drug-free 3 months post-treatment (data not shown). These patients did not relapse and exhibited good blood glucose control with only diet and exercise intervention required. A total of 16% (5/31) of patients terminated oral hypoglycemic drug (OHD) treatment at the same time as insulin treatment. The mean duration of drug discontinuance was 13.7±5.3 months. Nine of the remaining 20 patients reduced OHD by varying degrees; whereas the other 11 patients remained on the baseline dose of OHD. In group II, 77% (23/30) of patients increased the dosage of OHD by varying degrees (data not shown). This indicated that WJ-MSC infusion may decrease the dosage of insulin and oral hypoglycemic agents in T2DM patients..

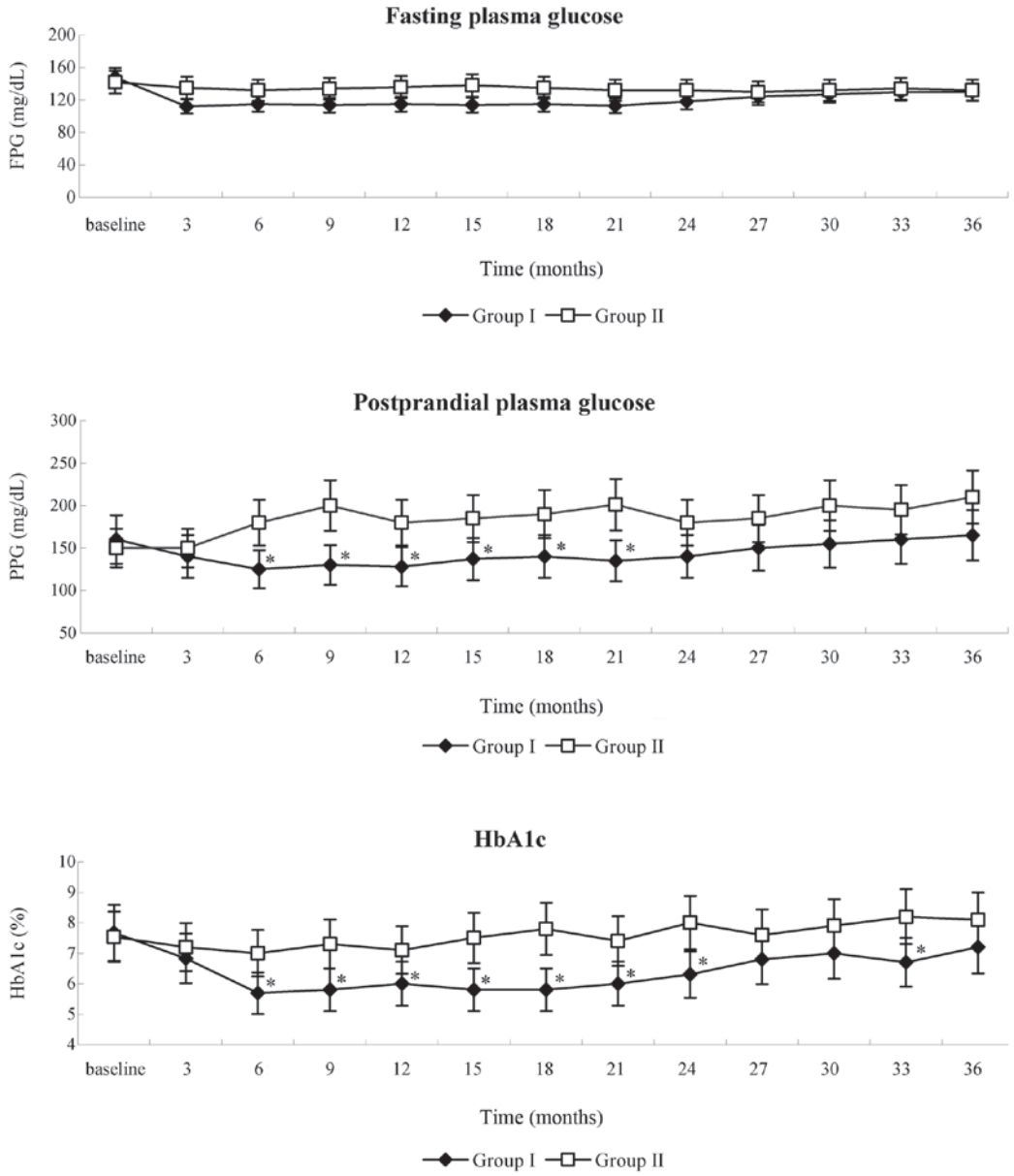

Figure 2. WJ-MSC infusion ameliorated PPG and HbA1c in patients in group I; however there was no difference in FPG between the two groups. Levels of PPG and HbA1c of patients in group I were significantly decreased, as compared with group II patients over the various time points, as detected by repeated measure analysis of variance (*P<0.05 vs.group II). Data are presented as the mean ± standard deviation. WJ-MSC, Wharton's jelly-derived mesenchymal stem cell; Group I, WJ-MSC infusion treatment group; group II, control group; PPG, postprandial plasma glucose; HbA1c, glycosylated hemoglobin; FPG, fasting plasma glucose.

WJ-MSC infusion reduces the incidence of diabetic complications. By the end of the follow-up period, in group I, there was no increase in the incidence of diabetic complications, including diabetic retinopathy (5/31; 16.1%), neuropathy (4/31; 12.9%) and nephropathy (3/31; 9.7%). In group II, the incidence of diabetic complications increased as hypothesized. Four patients were newly diagnosed with diabetic retinopathy (total, 8/30; 26.7%), three patients were newly diagnosed with diabetic neuropathy (total, 6/30; 20%) and three patients were newly diagnosed with diabetic nephropathy (total, 7/30; 23.3%). There was a statistically significant difference between the incidence of diabetic complications in the two groups (P=0.007; data not shown). This indicated that WJ-MSC infusion may reduce the incidence of diabetic complications.

Adverse events. No serious adverse reactions, including fever, chills, liver damage, hypersensitivity, infection, hemorrhage, proteinuria, myocardial infarction, venous thromboembolic events or other arterial thromboembolic events, were detected following WJ-MSC infusion in any of the patients who completed the study protocol, and no chronic side effects or lingering effects were detected during the follow-up. None of the patients enrolled in the present study developed severe hypoglycemia; whereas, 41 episodes of minor hypoglycemia were detected in 41 patients (group I, n=23; group II, n=18).

Discussion

Previous studies and clinical trials have demonstrated that MSCs are capable of reducing glucose levels in animals

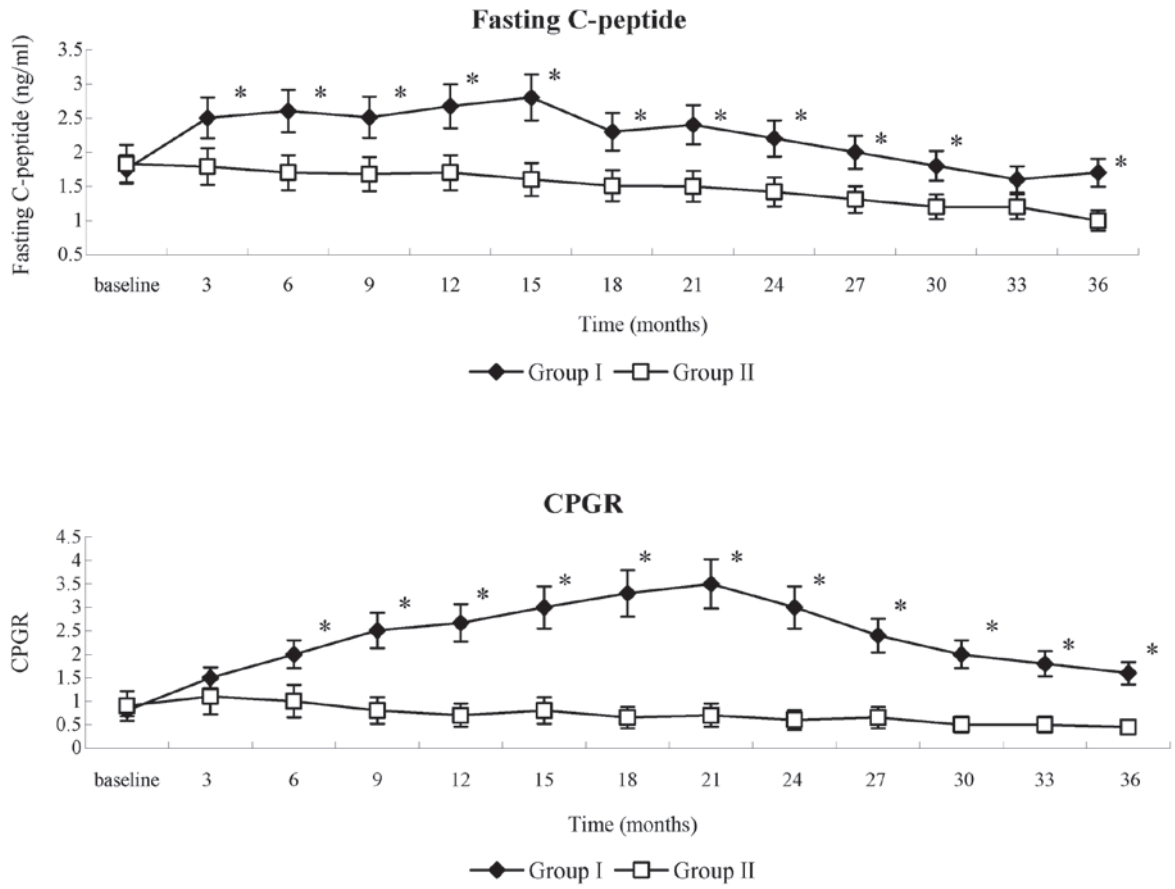

Figure 3. WJ-MSC infusion increased fasting C-peptide and CPGR in patients in group I. Levels of fasting c-peptide and CPGR of patients in group I were significantly increased, as compared with group II patients over various time points, as detected by repeated measure analysis of variance (*P<0.001 vs. group II). Data are presented as the mean ± standard deviation. WJ-MSC, Wharton's jelly-derived mesenchymal stem cell; Group I, WJ-MSC infusion treatment group; group II, control group; CPGR, C-peptide/glucose ratio.

or subjects with type 1 and type 2 diabetes (14,15,18,19). Our preliminary animal studies also suggested that the intervenous infusion of WJ-MSC promoted the increase of β-cells in the pancreatic islet of diabetic mice and rats, thus inducing an increased level of insulin and decreased blood glucose (19,22). The present study was conducted in order to explore the long-term effect and safety of WJ-MSC in patients with T2DM. The present results demonstrated that WJ-MSCs were was able to: i) Improve the function of islet β-cells, as indicated by the increase in fasting C-peptide and HOMA-β; ii) ameliorate hyperglycemia, as indicated by the decrease of FPG, PPG, HbA1c and the dosage of oral hypoglycemic agents and insulin therapy; and iii) reduce the incidence of diabetic complications, although the sample size was not large enough to assess the incidence of diabetic complications.

Accumulating evidence has indicated that paracrine signaling initiated by MSCs, which involves the secretion of various angiogenic growth factors and cytokines [such as vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor), anti-inflammatory and anti-apoptotic molecules (such as interleukin-6 and -10, and tumor necrosis factor-α), may be responsible for the therapeutic effect of MSCs (23-26). A clinical trial conducted by Jiang *et al* (14) suggested that infusion of PD-MSCs represented a simple, safe and effective therapeutic approach for T2DM patients with a six-month follow-up time. Furthermore, Liu *et al* (15) have previously demonstrated that treatment with WJ-MSC may improve metabolic control and β-cell function in patients with T2DM. These findings are consistent with the results of the present study; however, their respective follow-up periods were not adequate to demonstrate the long-term effect of MSCs on T2DM. In the present study, the follow-up period was 36 months, and the results demonstrated that ideal glycemia control due to WJ-MSC infusion was achieved at the third month post-therapy and was sustained for 18 months, as confirmed by the fasting C-peptide and HOMA-β results. These results indicated that two infusions of WJ-MSC may effectively maintain good glycemic control for ~21 months. After this point, due to the attenuation of the WJ-MSC effect and a gradual decrease in β-cell function, blood glucose levels began to rise. Repetitive WJ-MSC infusions may help to maintain good blood glucose control in the long-term; however, future studies with larger sample sizes are required in order to investigate this.

During the follow up period, a decrease in fasting C-peptide was detected in the first month post-WJ-MSC infusion. Based on the paracrine effect of WJ-MSC, this effect may be due to the factors stimulated by WJ-MSC, including pancreatic duodenal homeobox-1 and transforming growth factor-β1, which may directly or indirectly have a role in the active metabolic effect (27), and decrease hyperglycemia, blood glucose

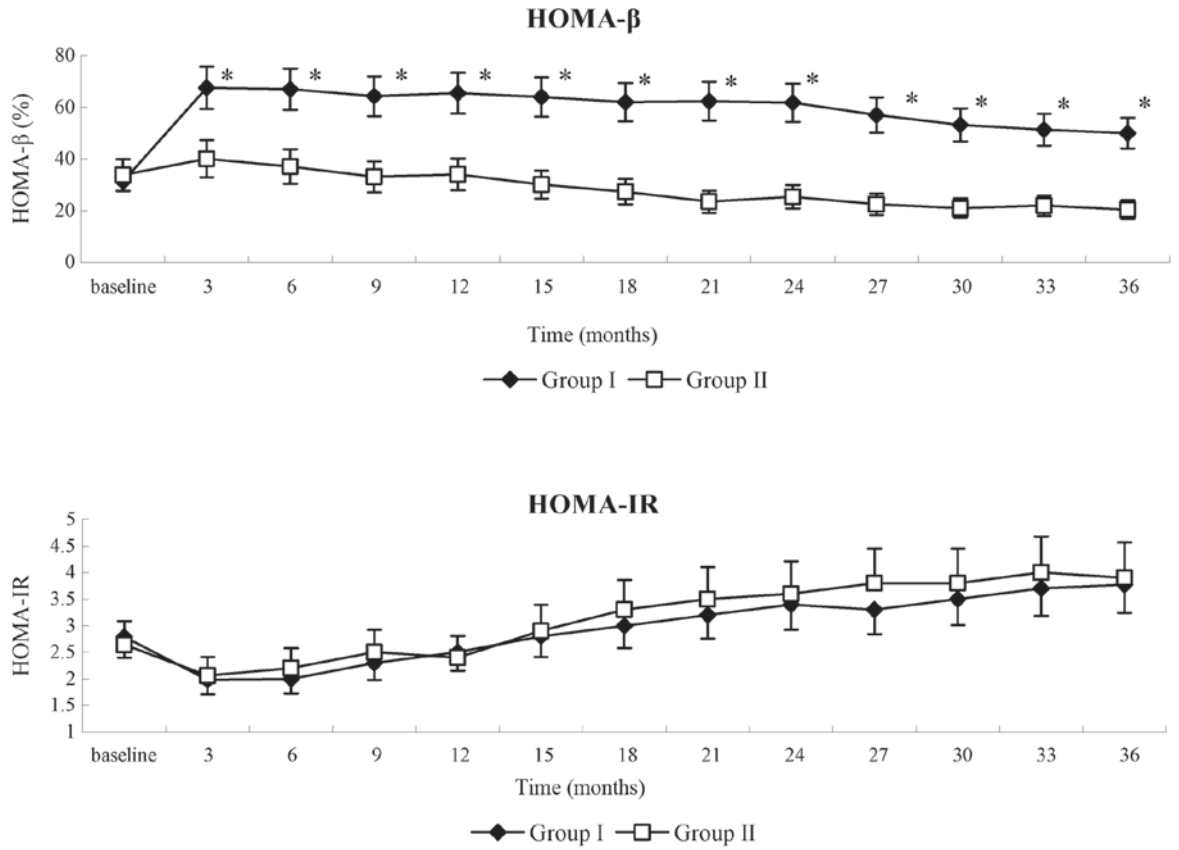

Figure 4. WJ-MSC infusion improved function of islet β cells of patients in group I. HOMA-β of patients in group I was significantly increased, as compared with group II patients over various time points, as detected by repeated measure analysis of variance (*P<0.05 vs. group II). No significant differences in HOMA-IR were between the two groups during the follow-up period. Data are presented as the mean ± standard deviation. WJ-MSC, Wharton's jelly-derived mesenchymal stem cell; Group I, WJ-MSC infusion treatment group; group II, control group; HOMA-β, homeostasis model assessment of pancreatic islet β-cell function; HOMA-IR, homeostasis model assessment of insulin resistance.

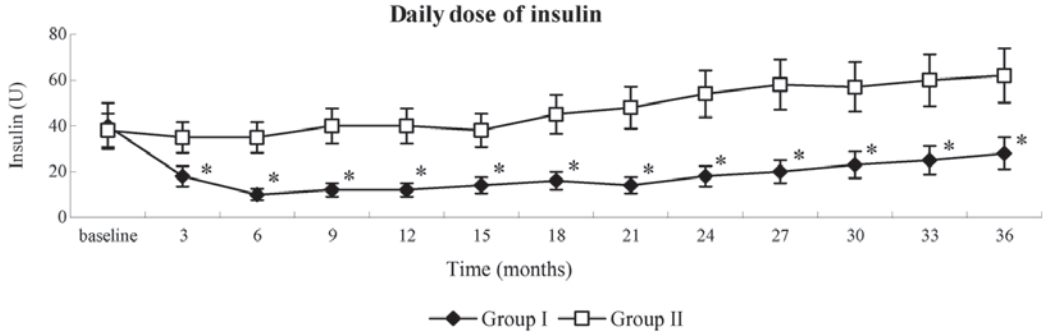

Figure 5. WJ-MSC infusion reduced the daily dosage of insulin of patients in group I. Daily dosages of insulin required by patients in group I were significantly decreased, as compared with group II patients over various time points, as detected by repeated measure analysis of variance (*P<0.001 vs. group II). Data are presented as the mean ± standard deviation. WJ-MSC, Wharton's jelly-derived mesenchymal stem cell; Group I, WJ-MSC infusion treatment group; group II, control group.

fluctuation and the need for endogenous insulin, thus inducing the decrease in fasting C-peptide.

The therapeutic effect induced by WJ-MSC infusion in the present study permitted the termination of treatment with oral hypoglycemic agents and insulin in some patients; however, some of these patients required these agents or insulin to decrease the hyperglycemia once again. The therapeutic effect induced by WJ-MSC infusion may be due to the part restoration of islet function or the increase of both islet α- and β-cells, which have previously been demonstrated in a animal model of diabetes (19). There have also been contradictory reports concerning the association between injection times and the therapeutic effect of WJ-MSC infusion on diabetes (13,28,29). Ezquer *et al* (28) demonstrated that a single-injection of MSCs into diabetic mice induced an improved therapeutic effect, as compared with multiple injections. Conversely, other studies demonstrated that multiple intravenous infusions were able to reverse hyperglycemia in experimental diabetic animals, whereas a single infusion of MSCs could not (13,29). It is believed that the therapeutic

effect of WJ-MSC infusion was associated with the injection times, cell types and number (28,29). In the present study, WJ-MSC infusion was implemented via two injections, according to our previous animal studies and clinical trials (18,22,30), and perhaps multiple injections would have been more beneficial.

The role of insulin resistance in the development of type 2 diabetes has been investigated extensively, and it has been demonstrated that glucose transporter-4 (GLUT4), insulin receptor substrate 1 (IRS-1) and Akt are crucial for glucose uptake and insulin resistance (31-33). In a previous study, Si *et al* (16) demonstrated that MSCs infusion improved insulin sensitivity by upregulating GLUT4 expression and elevating phosphorylated IRS-1 and Akt levels in tissues targeted by insulin, and concluded that infusion with MSCs was able to ameliorate insulin resistance. The results of the present study demonstrated that HOMA-IR of patients in group I decreased following WJ-MSC infusion, although this decrease was not significant when compared with patients in group II. This interesting phenomenon demonstrated that the improvement of insulin sensitivity may not be the dominant therapeutic effect induced by WJ-MSC infusion. Future studies with larger samples, multiple infusions of WJ-MSC and longer follow-up periods are required in order to investigate this.

Diabetic complications, which predominantly occur during the latter phase of diabetes due to poor glycemic control, remain severe and life-threatening. In the present study, there was no increase in the incidence of diabetic complications in the patients of group I, whereas in group II, the incidence of diabetic retinopathy, neuropathy and nephropathy increased. This indicated that infusion with WJ-MSCs may reduce the incidence of diabetic complications; this result was consistent with a previous study by Jiang *et al* (14). Although the underlying mechanisms of this therapeutic effect of WJ-MSC remain unclear, the cytokines secreted by WJ-MSC, including insulin-like growth factor, VEGF and hepatocyte growth factor, may directly or indirectly improve islet function and the associated complications (34-36).

No serious adverse reactions, including fever, chills, liver damage or immune rejection response, were observed following WJ-MSC infusion. Moreover, no positive results were detected for renal and cardiac function, blood coagulation function and tumor screening tests during the 36-month follow-up period. These results suggested that infusion with WJ-MSC may represent a safe therapeutic approach for the treatment of patients with T2DM.

In conclusion, the findings of the present study suggested that WJ-MSC infusion may effectively ameliorate hyperglycemia, improve islet β-cell function and reduce the incidence of diabetic complications over a sustained period of time. Despite the fact that WJ-MSC infusion does not appear to attenuate insulin resistance, WJ-MSC infusion may have therapeutic potential as a novel agent for the treatment of T2DM. Further follow-up and large-scale placebo-controlled clinical studies are required to fully elucidate the role of WJ-MSC in the treatment of T2DM. Their application in therapeutic regimens may be useful in treating diabetes and its complications.

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