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Long term effects of the implantation of Wharton's jelly-derived mesenchymal stem cells from the umbilical cord for newly-onset type 1 diabetes mellitus

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Abstract. T1DM is an autoimmune disorder resulted from T cell-mediated destruction of pancreatic β-cells, how to regenerate β-cells and prevent the autoimmune destruction of remnant and neogenetic β-cells is a tough problem. Immunomodulatory propertity of mesenchymal stem cell make it illuminated to overcome it. We assessed the long-term effects of the implantation of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) from the umbilical cord for Newly-onset Type 1 Diabetes Mellitus (T1DM). Twenty-nine patients with newly onset T1DM were randomly divided into two groups, patients in group *I* were treated with WJ-MSCs and patients in group *II* were treated with normal saline based on insulin intensive therapy. Patients were followed-up after the operation at monthly intervals for the first 3 months and thereafter every 3 months for the next 21 months, the occurrence of any side effects and results of laboratory examinations were evaluated. There were no reported acute or chronic side effects in group *I* compared with group *II*, both the HbA1c and C peptide in group *I* patients were significantly better than either pretherapy values or group *II* patients during the follow-up period. These data suggested that the implantation of WJ-MSCs for the treatment of newly-onset T1DM is safe and effective. This therapy can restore the function of islet β cells in a longer time, although precise mechanisms are unknown, the implantation of WJ-MSCs is expected to be an effective strategy for treatment of type1 diabetes.

Key words: Type 1 diabetes, Mesenchymal stem cell, Umbilical cord, Implantation

THE PREVALENCE of diabetes for all age-groups worldwide was estimated to be 4.4% in 2030, the total number of people with diabetes is projected to rise to 366 million in 2030 [1]. Type 1 diabetes mellitus (T1DM) comprises 5 to 10% of all causes of diabetes and is one of the most prevalent autoimmune diseases of childhood. As we all knows, T1DM is an insulin dependent, autoimmune disorder resulted from T cell-mediated destruction of insulin-producing pancreatic β-cells. At the clinical onset of Type 1 diabetes, most patients typically have 20-30% of their original β-cell mass left. However, these remaining β-cells will also be destructed along with the progression of disease, ultimately resulting in poor glucose control, ketoacidosis, infection, retinopathy with potential loss of vision, nephropathy leading to renal failure, pregnancy complications, sexual dysfunction; and many others [2-4].

The insulin injection, which is the current mainstream treatment for T1DM, can achieve adequate glycemic control, but it is inconvenient for the patient and does not completely prevent the development of diabetic complications. Other treatments include islet transplantation, have many limitations, especially due to the shortage of donors and lifetime taken of immunosuppressive agents. Hence, a therapeutic strategy aimed to regenerate insulin-producing cells and prevent the autoimmune destruction of remnant and neogenetic β-cells is highly desirable.

Stem cells, which are characterized by the potentia of self-renew and multi-directional differentiation, represent a key alternative and provide a poten-

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tially unlimited source of islet cells for transplantation. Previous researches showed that embryonic stem cells (ESCs), umbilical cord blood stem cells, induced pluripotent stem cells (iPS) and mesenchymal stem cells (MSCs) had been successfully induced to differentiate into insulin-producing cells *in vitro* [5-8].

MSCs have some advantages over other stem cells such as ESCs or stem cells from other organs. First, MSCs have been shown to have potent immunoregulatory capacity both *in vitro* and *in vivo*, as they can modulate many functions of immune cells including T cells, B cells, dendritic cells and NK [9-11]. In this regard, MSCs have been tried in preclinical animal studies and clinical trials in treatment of GVHD [12, 13] and autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus and Crohn's disease[14-16]. Recently, MSCs have been shown to be able to protect NOD mice from diabetes by induction of regulatory T cells [17-19]. Second, an important property of human MSCs, which is MSCs express intermediate levels of major histocompatibility complex (MHC) class *I* molecules but not MHC class *II* on their cell surface, allows their transplantation across MHC barriers [20]. Third, MSCs are of great multiplication potency, can be expanded in culture for more than 60 doublings [21] and histological studies on these cells have not shown any tumor formation after transplantation [22, 23]. Forth, systemic delivery of MSCs has been reported by several groups with accumulated evidence that MSCs are capable of homing to injured tissues after intravenous delivery [24, 25].

Bone-marrow-derived MSCs are used most dominantly in the market today as a stem cell source because their safety has been certified. However, there are some limitations such as invasive procedures for the donor when harvesting and cell quality and proliferation ability depending on the age and health status of the donor. In case of children and old patients, therefore, bone marrow is not appropriate stem cell source for autologous cell therapy. Wharton's jelly-derived MSCs (WJ-MSCs) are becoming a viable option because a high yield of young cells can be harvested from the umbilical cord without any additional surgery [26].

There have been some animal studies reporting that WJ-MSCs can control hyperglycemia and improve the function of pancreatic islets [27, 28], but there have been no clinical trials of long-term follow-up of WJ-MSCs therapy on T1DM. Here we report an randomized controlled trial to preliminarily evaluate the long-term effects of implantation of WJ-MSCs for newly-onset T1DM.

Materials and Methods

Patients

Between November 2009 and May 2010, 57 patients were screened for enrollment. Of those patients, 36 patients fit the inclusion criteria and were personally interviewed, 29 patients were enrolled in this study. Patients were divided into two groups by randomized blocks, fifteen patients participated in the WJ-MSCs treatment group (group *I*), while the other 14 patients participated in control group (group *II*). All patients were subsequently enrolled, treated and followed up until May 2012 in the Stem Cell Center of the Affiliated Hospital of the Medical College, Qingdao University. The study protocol was approved by the Ethical Committee of the Affiliated Hospital of the Medical School, Qingdao University. Informed consent according to the Declaration of Helsinki was provided by every patient or his/her guardian.

Inclusion criteria were: patients of both sexes, aged not exceeding 25 years, with a clinical and laboratory diagnosis of T1DM according to the criteria of the American Diabetes Association and a diabetic duration not more than 6 months, fasting C-peptide ≥ 0.3 ng/ml. Patients with malignancy; any acute or chronic infection; pregnancy; positive serology for human immunodeficiency virus, hepatitis B or C; underlying hematologic, nephrologic, cardiac, psychiatric, or hepatic disease; mental disorders; inborn or adaptive immunodeficiency and hypersensitivity were excluded.

Treatment procedure

This is a double blind study. All patients enrolled into the study were assessed in the diabetic out-patient clinic over 1 months before the start of therapy, except for patients with ketoacidosis who would be treated in hospital. After that all patients were recommended a diabetic diet and an exercise routine (walking or similar for 1 hour three times per week during the entire study and follow-up period). At the time of starting therapy, all patients had stable blood glucose levels and had been on stable doses of insulin over the previous 1 months.

All patients were treated with intensive insulin therapy by frequently injecting insulin or sc infusion of insulin for adequate glycemic control. At this basement,

patients in group *I* were treated with parenteral solution of WJ-MSCs by intravenous delivery. Patients in group *II* were treated with normal saline which is same with parenteral solution of WJ-MSCs in the appearance and volume (as shown in Fig. 1). After treatment the patients remained on the same diabetic diet, exercise regimen and insulin therapy as before.

During the follow-up, insulin were adjusted according to the patient's blood glucose. The dose of insulin would be increased if the patient's blood glucose had not been controlled to within the normal range (fasting blood glucose normal range: 70-110 mg/dL, postprandial blood glucose normal range: ≤ 140 mg/dL). Inversely, the insulin would be reduced.

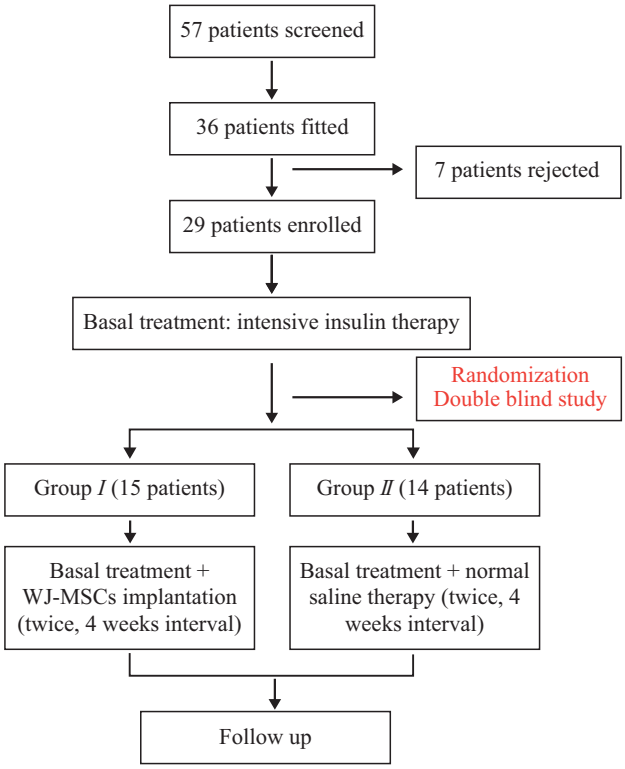

Fig.1 The treatment procedure for this trial

Stem cell preparation

WJ-MSCs were provided by Human Umbilical Cord Mesenchymal Stem Cell Bank, Shandong Province, China. Umbilical cord was obtained from a healthy mother, age 26, born a healthy term fetus and no genetic family history, no cancer history, no hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) and syphilis in serum. Umbilical cord collection was approved by the Institutional Medical Research Ethics Committee of the local maternity hospitals. Fully informed consent was obtained several weeks prior to delivery from the mother.

The preparation of WJ-MSCs were performed in the laminar flow laboratory using a method previously described [11, 32], with some modifications. Briefly, the umbilical cord was washed with phosphate buffered saline (PBS) twice and then dissected with scissors into pieces approximately 1cm^3 in volume. These tissue pieces were plated in a cell culture dish (Corning) in low-DMEM medium supplemented with 5% non-animal-derived serum. Cell cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. After 3 days of culture, the medium was replaced to remove the tissue and non-adherent cells, and changed twice weekly thereafter. Once 80% confluence had been reached, the adherent cells (passage 0) were detached with 0.125% trypsin and passaged in the cell culture dish. The WJ-MSCs were cultured and expanded in laminar flow laboratory for 4 passages to prepare final cell products which should be sterile and all qualified for the examinations including aerobe, mycoplasma, HBV, HCV, HIV, EBV, CMV, syphilis, and endotoxin testing. Also, cells were stained with a double label and then analyzed by flow cytometry with a FACS Calibur (BD). These cells expressed highly CD90, CD105, CD73 and CD146 but not CD34, CD45 and HLA-DR. Chromosomal karyotype of UC-MSC was normal.

Clinical assessment and follow-up

All patients received extensive physical and laboratory examinations, including age, gender, height, weight, diabetes duration, dose of insulin used, whole blood cell counts, liver and renal function tests, cardiac enzyme, cardiac troponin, serum electrolytes, serum lipids, blood coagulation function, microalbuminuria, cancer screening test and glutamic acid decarboxylase antibody (GADA) test, were recorded at baseline and during the follow-up period every 3 months. Plasma glucose was measured by enzymatic (glucose oxidase/peroxidase) colorimetric method. C-peptide was tested by the C-peptide response test (Roche Diagnostics, Germany; normal range: 1.1-4.4 ng/mL). After a 10-h overnight fast and acquisition of a fasting blood sample, each patient was asked to eat 100 g of steamed bread (in place of 75 g glucose for diagnosed diabetic patients), then 1 h and 2 h after ingestion, a blood sample was acquired to measure the postprandial C pep-

tide. HbA1c (Bio-Rad D10, USA; normal range: 3.9%-6.1%) was also examined, and the C-peptide/glucose ratio (CPGR) was calculated by the formula C-peptide × 100/glucose to evaluate the glycemic profile at different time points.

All patients were followed up at monthly intervals for the first 3 months and then every 3 months for the next 21 months. All adverse reactions and clinical examination findings during the whole study were carefully documented and assessed.

Study end points

The primary study end points were: 1) feasibility of the Stem Cell therapy; 2) safety of the therapy through 24 months post treatment; and 3) preliminary evaluation of the efficacy of the therapy for improving β cell function through 24 weeks. Pancreatic islet β cell function was assessed by measuring basal and postprandial C-peptide production over time. Metabolic control was monitored throughout the study. The secondary study end point was evidence of the efficacy of the therapy in modulating autoimmunity.

Statistical analysis

Analysis was performed using SPSS 13.0 software. Data are presented as mean ± SEM. Differences between the means of the baseline values of the control and experimental groups were analyzed using Student's 't' test. We estimated and compared differences across groups and between different time points (baseline vs. follow-up) using repeated measure ANOVA and/or Kruskal Wallis multiple comparisons tests with the level of significance set at a= 0.05. A two-tailed $P < 0.05$ was considered as statistically significant.

Results

A total of 29 patients with newly onset T1DM were included in the study, one patient in group *II* withdrawed the study one year later for immigration to other distant city, while other 28 patients completed this study. Their baseline data are summarized in Table 1. There were no significant differences in clinical findings, laboratory tests or diabetic complications between two groups before treatment. Cancer screening test confirmed no cancer in all patients. The volume of parenteral solution of WJ-MSCs and normal saline in two group were 50 mL, and cell number was between 1.5 and 3.2×10^7 (mean, $2.6 \pm 1.2 \times 10^7$).

Plasma glucose

Mean fasting plasma glucose (FPG) levels remained within the normal range or fluctuated a little during follow-up in group *I* patients. During this course, FPG began to decrease at the first month after stem cell therapy, necessitating the reduction of insulin in order to avoid hypoglycaemia. In group *II*, FPG remained the same as before for almost two years, but the fluctuation of FPG was very large, this led to a obvious fluctuation of insulin dose. The difference in FPG was not significant between two groups. Mean postprandial plasma glucose (PPG) levels reached the lowest level at the end of first year after therapy, then began to rise lightly, but still remained a better control during follow-up in group *I* patients. While there were higher and larger fluctuation of PPG in group *II* patients. There was statistical difference in PPG between two groups, showed in Fig. 2.

HbA1c

The mean value of HbA1c showed a gradual decrease and reached the lowest level at the sixth month of follow-up (baseline 6.8 ± 0.57%, 6 months 5.5 ± 0.67%) in group *I* patients, and demonstrated slight fluctuations over the following 18 months. In group *II*, HbA1c levels remained slightly reduced for almost six months, then began to fluctuate due to the obvious fluctuation of insulin therapy (Fig. 3). There was a significant difference in HbA1c between the two groups at the sixth month and at subsequent time points ($P < 0.05$).

C-peptide and C-peptide/glucose ratio

There was a progressive increase of mean fast-

Table 1 Patients' baseline informations in two groups

variable	group *I*	group *II*
clinical		
age (years)	17.6±8.7	18.2±7.9
sex		
male	9	8
female	6	6
BMI (kg/m^2)	20.9±3.7	21.3±4.2
Laboratory tests		
FPG (mg/dL)	102.6±30.8	97.2±29.6
HbA1c (%)	6.85±0.74	6.79±0.81
Fasting C-peptide(ng/mL)	0.85±0.47	0.89±0.39
Complication		
ketoacidosis	5	5
GADA		
Positive	11	10
Negative	4	4

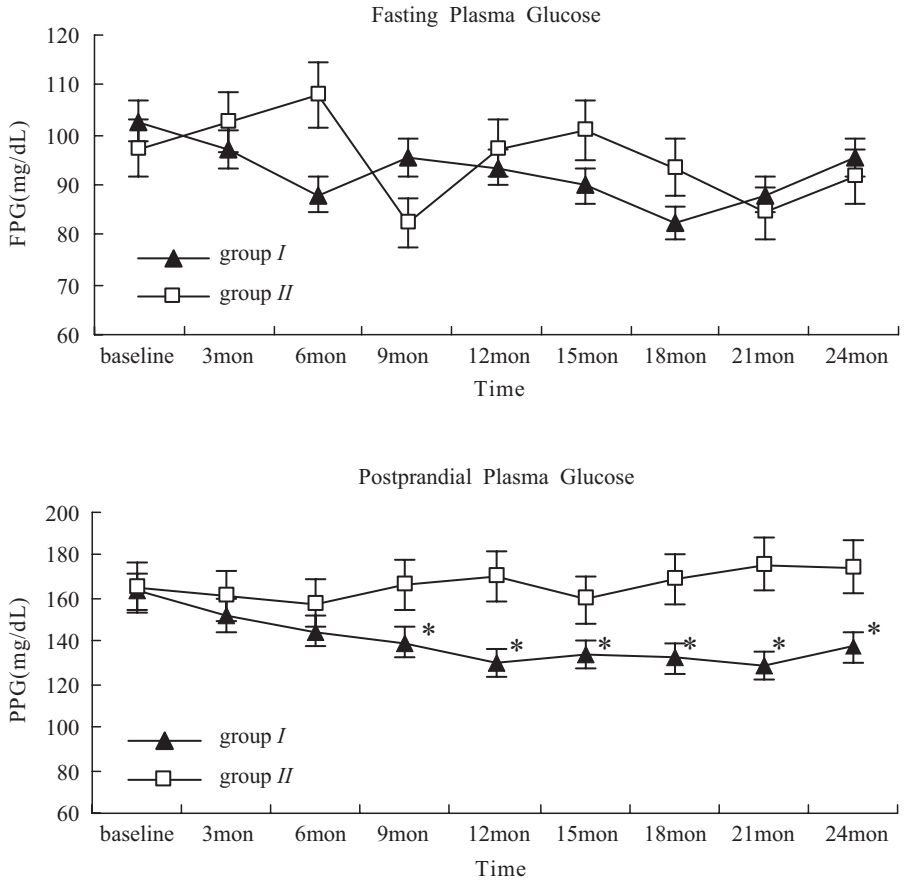

Fig. 2 Changes in FPG and PPG over time
There was no significant difference in FPG between two groups. * indicated that PPG in group *I* was significantly lower than that in group *II* over different time points using repeated measure ANOVA ($P<0.05$).

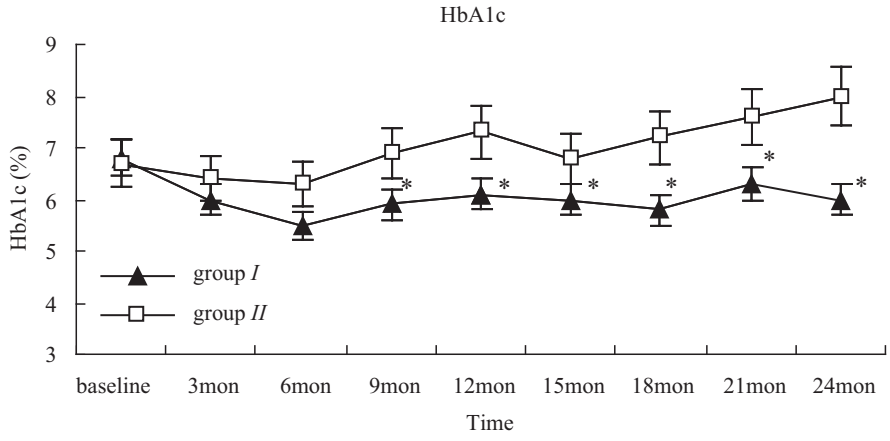

Fig. 3 Changes in HbA1c over time
* HbA1c in group *I* was significantly lower than that in group *II* over different time points using repeated measure ANOVA ($P<0.05$).

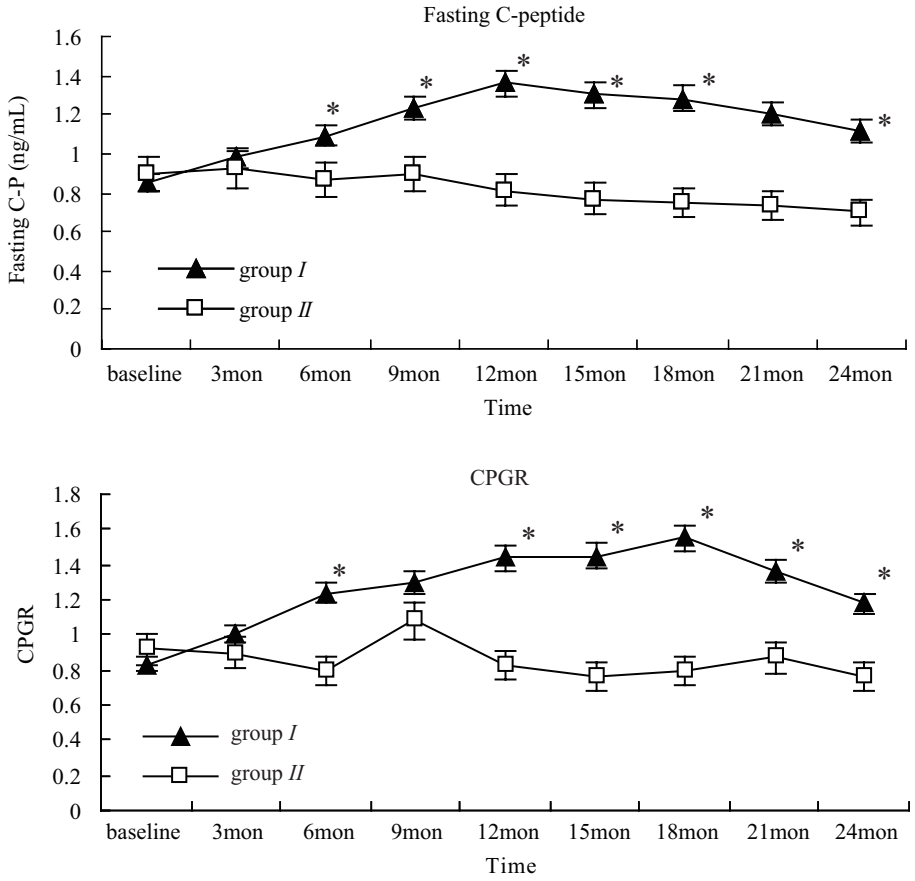

Fig. 4 Changes in fasting C peptide and CPGR over time
*Fasting C peptide and CPGR in group *I* were significantly higher than that in group *II* over different time points using repeated measure ANOVA (P<0.05).

ing C-peptide levels in group *I* patients, especially a most high level appeared at the end of first year of follow-up, then had a little decrease, but at the end of follow-up, mean fasting C-peptide levels were still better than baseline. While in group *II* patients, the mean C-peptide levels decreased gradually, as shown in Fig. 4. Mean C-peptide/glucose ratio (CPGR) levels increased progressively in group *I* patients during the entire follow-up period, but decreased gradually in group *II* patients (Fig. 4). The difference between the two groups was significant ($P < 0.05$).

Insulin requirements

In group *I* patients, the dosage of insulin per day was progressively reduced. At the end of the follow-up period, in 3/15 patients insulin was discontinued; in 8 of the remaining 12 patients, the daily insulin dosage was reduced by more than 50% of the baseline; in 1 of the remaining 3 patients, the daily insulin dosage was reduced by 15-50%; while there were 2 patients whose insulin dosage was maintained. In group *II* patients, the dose of insulin per day increased gradually. In 7/14 patients, insulin was increased by more than 50% of the baseline; in the 7 remaining patients, insulin was slightly increased by about 15-45%. The difference between the two groups was significant ($P < 0.001$), and the serial changes in the mean doses of insulin required are shown in Fig. 5.

Frequencies of ketoacidosis

During the follow-up, there was no ketoacidosis appeared in group *I* patients, while in group *II* patients, ketoacidosis appeared in three patients, one occurred after diarrhea, the other two after flu.

Characterization of GADA

At the end of the follow-up period, in group *I*, six of eleven GADA+ patients became negative, the

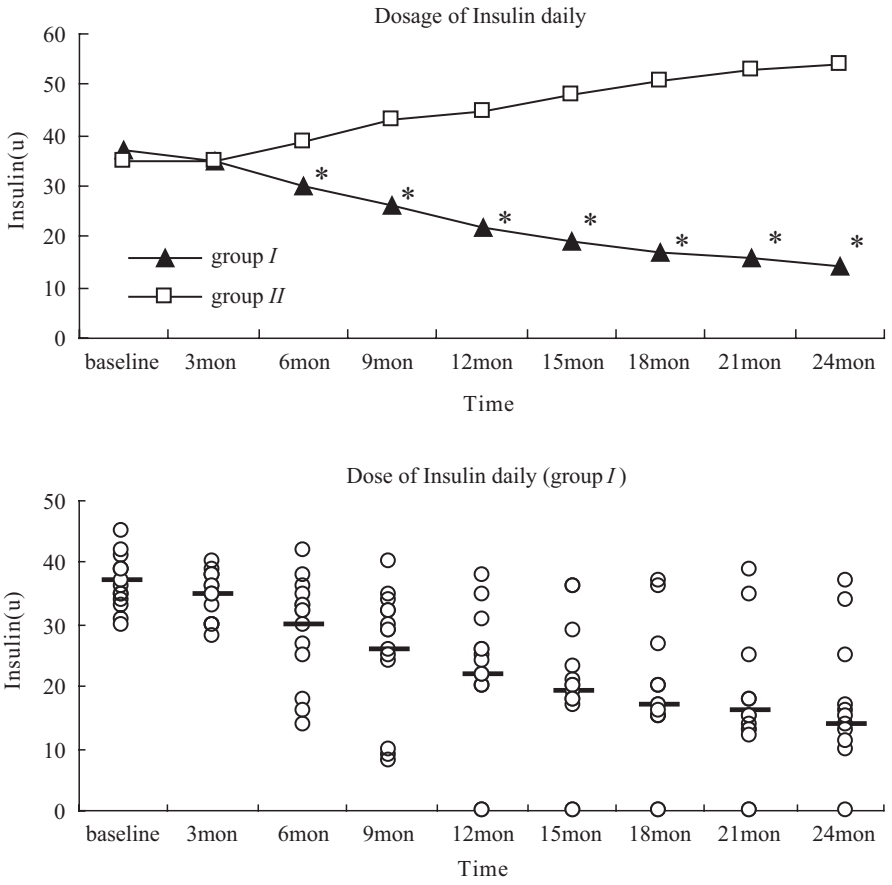

Fig. 5 Changes in the dosage of insulin daily over time
* The dose of Insulin daily in group *I* was significantly less than that in group *II* over different time points ($P<0.001$).

other five of eleven GADA+ patients remained positive and another four GADA- patients remained negative. In group *II*, three of ten GADA+ patients became negative, the other seven of eleven GADA+ patients remained positive and another four GADA- patients remained negative. There was no statistical difference between two groups.

General laboratory examinations

During the whole follow-up, except 3 patients with ketoacidosis and temporary electrolyte disturbances in group *II*, all the other patients both in group *I* and group *II* kept normal results of laboratory examinations, including liver and renal function tests, cardiac enzyme, cardiac troponin, serum electrolytes, serum lipids, whole blood cell counts, blood coagulation function, microalbuminuria and cancer screening test.

Adverse events

There were no obviously adverse reactions after stem cell therapy in any of the patients who completed the study protocol, and no chronic side effects or lingering effects appeared during the follow-up.

Discussion

In Type 1 diabetes, autoimmune destruction of pancreatic islet β cells weakens a patient's self-regulation ability of blood glucose, ultimately resulting in a high frequency of vascular complications that compromises quality and expectancy of life. Each day, millions of patients with T1DM have to receive insulin injections to maintain tight blood glucose control, but these injections do nothing to relieve the underlying cell-mediated autoimmune dysfunction. It is important to note that in recent-onset or even in preclinical phase of T1DM, immunomodulatory strategies can be done as

the unique therapeutic approach since larger residual β-cell mass is still functioning and able to be preserved. Moreover, immunomodulation secondarily facilitates endogenous mechanisms of β-cell proliferation once the pathologic process of β-cell destruction is blocked. While it is not in individuals with long-term disease [1, 29, 30]. In this setting, strategies of immunomodulation and β-cell replacement should be associated.

In 2007, Voltarelli *et al* in Brazil first reported the use of autologous nonmyeloablative haemopoietic stem cell transplantation (AHST) in newly diagnosed type 1 DM. Fifteen newly diagnosed (within 6 weeks) type 1 diabetic patients were enrolled in the study and treated with AHST after conditioning with cyclophosphamide (200 mg/kg) and rabbit antithymocyte globulin (4.5 mg/kg). After a mean time of 18.8-month follow-up, 14 patients became insulin free continuously or transiently, 1 patient was free from insulin for 35 months [31]. In 2009, the team added cases and prolonged mean follow-up time to 29.8 months. Twenty patients became insulin free for as long as 4 years with good glucose control. C-peptide levels increased significantly and were maintained for about 3 years especially in patients who were continuously free from insulin [32]. These results showed that AHST could improve the function of pancreatic β-cell and glucose control in T1DM patients, but this treatment procedure must be accompanied by conditioning with a larger dose of cyclophosphamide and rabbit antithymocyte globulin, which can cause a lot side effects such as poor appetite, nausea, vomitting even hypoleukemia on patients. A new safe, convenient and effective therapy for T1DM is urgent.

Mesenchymal stem cells (MSCs) have unique immunomodulatory effects in vitro and in vivo. First of all, MSCs express intermediate levels of MHC class *I* molecules on their cell surface but not MHC class *II* which is mainly responsible for rejection, this allows their implantation across MHC barriers. Immunosuppressant is not needed in the case of implantation of WJ-MSCs since WJ-MSCs express little MHC class *II* on their cell surface. Further in T1DM, MSCs possess specific immunomodulatory properties which are capable of disabling immune dysregulation that lead to β-cell destruction. MSCs could control the role of regulatory T cells/autoreactive T cells by secreting several negative costimulatory molecules and regulatory cytokines such as IFN-γ, TGF-β, IL-4 and IL-10, and revise the dysregulation of T cells, B cells, NK cells and dendritic cells by direct or indirect contact with these immunocytes. MSCs also exerted anti-inflammatory effects that could be important in maintaining peripheral tolerance [33, 34].

Previous studies showed MSCs can differentiate into insulin-producing cells using a specific culture medium in vitro [8, 35, 36]. In vivo upon intravenous injection, MSCs may be able to migrate into inflammatory and injured areas, where high-level chemokines are expressed, as VCAM-1, SDF, MCP-1, CX3CL1-CX3CR1 and CXCL12-CXCR4 [37-39]. Then MSCs can secrete many bioactive factors to establish a tissue microenvironment that supports β-cell activation/survival in pancreas, induce the regeneration of recipient-derived pancreatic insulin-secreting cells, and inhibit T cell- mediated immune responses against newly formed β-cells. Then new β-cells are able to survive in this altered immunological milieu [40, 41] .

The results of our trial confirmed these hypothesises. Our young T1DM patients had a better blood glucose control, C-peptide exciting reaction which indicated the endogenous insulin secretion showed an obvious recovery and regeneration of islet β-cells during a two years follow-up, as compared to controls with similar age, diabetic duration and combined therapy including intensive insulin therapy. Wharton's jelly-derived MSCs (WJ-MSCs) used in our trial are pure, high activity and proliferation cells and can be harvested from the umbilical cord without any additional surgery which is specially important for children.

Previous studies had showed that roughly 1.5% of human MSCs which were inoculated through tail vein in nude mice could be detected in the abdomen region of the mouse one week after inoculation, and persisted in a stable quantity during the remaining 31 weeks of the experiment [42]. This hinted that most MSCs survived a short time in vivo after implantation, so the oncogenicity was not a hidden danger. There was not any fore treatment such as cyclophosphamide or antithymocyte globulin before the therapy and immunosuppressive agents taken after the therapy due to the little or no expression of MHC class *II* and immunomodulatory properties of WJ-MSCs, also there was not any rejection or side effect reported during the therapy and whole follow-up in our study.

The Diabetic Control and Complications Trial demonstrated that intensive insulin therapy for patients with type 1 diabetes mellitus (T1DM) was associated with significantly improved glycemic control and decreased

rates of comorbidities such as retinopathy, nephropathy, and peripheral neuropathy. Even incremental improvements in glycemic control are now known to be associated with reductions in microvascular complications [43, 44]. MSCs therapy at the basis of intensive insulin therapy can exert better effect on T1DM petients for a longer time. As we all know, if newly diagnosed T1DM patients can be treated with adequate insulin injections to relief the hyperglycemic toxicity on islet, they might have a "honeymoons" during which they may maintain a good glycemic control with a little or no insulin injections, but this period is usually not longer than 1.5 years. After that, they would need more insulin to get glucose control. In our trial, all patients were followed up for 2 years, a longer time than honeymoons, and in 4/15 patients insulin was discontinued, the daily insulin dosage was reduced by more than 50% of the baseline in 7 of the remaining 11 patients for a time of 20-22months, this indicated that the therapeutic effect was owing to MSCs not honeymoons.

In our study, fifteen T1DM patients with fasting C-peptide ≥0.3ng/mL were enrolled in group *I* and preliminary investigated, since in these patients there was still a larger mass of islet β-cells remained. After stem cell therapy, patients were asked to be careful not to cause any infection or hypersensitiveness to avoid the aggravation of autoimmunologic derangement and continued diabetic diet and exercise regimen, however this is not easy for children. There were two patients as non-responders in this trial, one patient had a severe flu one month after therapy which might impair the therapy effect, the other had a lower level of fasting C-peptide (his C-P is 0.38 ng/mL), also individual variation might be an important reason.

Although precise mechanisms are unknown, Umbilical cord MSCs therapy shows an exciting effect on T1DM patients and is expected to be an effective strategy for treatment of type1 diabetes, it still need a larger and further investigation about precise mechanisms, the dosage, treatment juncture and frequency to consummate the therapy, since the number of children with diabetes is increasing due to population growth, aging, urbanization and increasing prevalence of obesity and physical inactivity.

There were several limitations of our study. First, the limited number of patients with WJ-MSCs therapy was due to the low incidence of T1DM, however most patients with newly diagnosed T1DM in our hospital would like to receive this new style of therapy. We would like to recruit large scale cohort of patients from multi-centers in the future study. Second, we only selected T1DM patients with fasting C-peptide ≥0.3ng/mL in our study, but for patients with fasting C-peptide <0.3ng/mL, there was no investigation reported. For these patients themselves, they would like to participate this trial, so we would start a further study to recruit these patients. Furthermore, to clarify the exact treatment mechanism, further studies that address the precise molecules and pathways involved in factors including cell homing, microenvironment improvement, interactions between WJ-MSCs and islet progenitor cells, and other types of cells which interact with WJ-MSCs during treatment, will be required.

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Disclosure Statement

No competing financial interests exist.

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