Mesenchymal Stem Cell Transplantation in Newly Diagnosed Type-1 Diabetes Patients: A Phase I/II Randomized Placebo Controlled Clinical Trial

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Research

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**Mesenchymal Stem Cell Transplantation in Newly Diagnosed Type-1 Diabetes Patients:
A Phase I/II Randomized Placebo Controlled Clinical Trial**

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Abstract

Background:

Type-1 diabetes (T1D) occurs following autoimmune-induced pancreatic beta cells death. Among several treatment modalities, Mesenchymal Stem Cells (MSCs) transplantation is promising for autoimmune disorders due to immunomodulation, regeneration, and migration to damaged tissue upon systemic injection. This study assessed the safety and efficacy of

intravenous injection of autologous bone marrow-derived MSCs in newly diagnosed T1D patients.

Methods:

After receiving informed consent, 21 patients who met the study criteria were enrolled and randomly assigned to receive either MSCs or placebo. Each patient in the experimental group received two doses of MSCs and followed for at least one year post-transplantation.

Results:

The results have shown that this transplantation is safe and significantly reduces the number of hypoglycemic episodes. MSCs transplantation improved glycated hemoglobin (HbA1c), shifted serum cytokine patterns from pro-inflammatory to anti-inflammatory, increased the number of regulatory T-cells in the peripheral blood, and improved quality of life. Early transplantation of MSCs significantly improved HbA1c and C-peptide levels and shifted pro-inflammatory cytokines to anti-inflammatory cytokines. Also, exercise combined with MSCs transplantation improved glycemic and immunologic indices.

Conclusions:

In conclusion, autologous MSC transplantation is safe and effective, and its early transplantation is a promising treatment in newly-diagnosed T1D children suffering from hypoglycemic episodes.

Trial registration:

This clinical trial was registered at the Iranian Registry of Clinical Trials (IRCT) with the identifier IRCT ID: IRCT2016070428786N1 registered on August 20, 2016 (Retrospectively registered) (<https://en.irct.ir/trial/23256>) and at the U.S. National Institutes of Health (ClinicalTrials.gov) with the related identifier NCT04078308 registered on September 6, 2019 (Retrospectively registered). (<https://clinicaltrials.gov/ct2/show/NCT04078308>)

Keywords: Type 1 Diabetes, Mesenchymal Stem Cells, Cell Therapy, Immunomodulation, Randomized Controlled Trial, Regulatory T Cells

Introduction

Type-1 diabetes (T1D) is a metabolic disease characterized by the autoimmune destruction of insulin-secreting pancreatic beta (β) cells. Hyperglycemia is a common problem of diabetics due to a lack of or low insulin secretion (1-7).

The International Diabetes Federation (IDF) introduces diabetes as one of the fastest-growing global health emergencies of this century and estimates that in 2019, 463 million people worldwide had diabetes. This number is expected to increase to 578 million by 2030 and 700 million by 2045 (8). Among adults diagnosed with diabetes, about 5.2%-5.6% have T1D (9, 10). The American Diabetes Association (ADA) reported an increased number of affected children with T1D and an increase in prevalence in most sex, age, and ethnic subgroups, in addition to an increased incidence in almost all age groups (5).

In 2019, the IDF reported that 7808 children and adolescents between zero and 19 years old had T1D in Iran (8). The innate and adaptive immune systems are deregulated in T1D, resulting in pancreatic islet β -cell destruction through CD4 and CD8 T-lymphocytes, natural killer (NK) cells, and B-lymphocyte infiltration following the invasion of dendritic cells and macrophages (11-13). Immune cells and their secretory factors, such as pro-inflammatory cytokines, make an inflammatory environment that gradually mediates β -cell death. As a result, patients' physiological blood glucose regulation fails, and diabetes complications endanger their life expectancy and quality of life (QOL) (11, 13, 14).

Diabetes causes severe chronic complications with irreversible multi-organ damage in patients. These complications include diabetic nephropathy, neuropathy, retinopathy, and cardiovascular diseases (1, 15-17).

T1D Patients' survival depends on daily doses of exogenous insulin (EI) to balance their blood glucose levels. Although this is the standard treatment, it does not provide an adequate physiological response and cannot prevent the progressive degeneration of islet β -cells; consequently, these patients might experience hypoglycemic episodes (1, 2, 18-20).

Current therapies for T1D have significant limitations. Insulin injections are the first-line treatment with different challenges: long-term dependency, difficulty with EI level management, and potential insulin resistance with long-term usage. Second-line treatment, pancreas transplantation has faced multiple obstacles such as donor shortages, the possibility of transplant rejection, usage of immunosuppressive drugs, and high risks with an organ transplantation surgery (4, 21). Thus, improved diabetes treatment methods that can address these limitations will be applicable in diabetes research and future management (21).

Cell-based therapy could ameliorate diabetes complications by providing better glycemic control (22). Different cell therapy strategies for T1D include cell replacement, cell regeneration, and cell reprogramming. Various stem cells that have been tested for cellular therapy include hematopoietic stem cells (HSCs), induced pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs) (2, 23, 24). Other potential cell-based treatments for T1D include islet transplantation, transplantation of encapsulation devices, polyclonal regulatory T (T reg) cell transplantation, and alterations to the immune system response (19, 25-29).

To date, MSCs are the most studied cell population in clinical trials. The therapeutic capacity of MSCs is based on their ease of isolation, ability to differentiate into multiple cell types, low immunogenicity, abundant source, minimal ethical concerns, and, most notably, their release of biological factors that can alleviate impaired tissues (30). The findings of a preclinical study showed that MSCs could be effective as a distant immunomodulator in addition to their homing to injured sites (31).

MSCs have a mesodermal origin and are defined as multipotent cells that can adhere to plastic; self-renew; express specific surface antigen markers (CD73, CD90, CD105); lack hematopoietic antigen expressions (CD45, CD34, CD14 or CD11b, CD79 α or CD19, HLA-DR); and can differentiate into osteocytes, adipocytes, or chondrocytes (30, 32). MSCs are derived from many sources such as the bone marrow, umbilical cord, umbilical cord blood, adipose tissue, and dental pulp (33-35).

Immunomodulatory therapeutic approaches can be used to preserve residual β -cells in recent-onset T1D patients with a greater amount of functional β -cell mass (11, 36).

MSCs have a superior biosafety profile and negligible risk of tumorigenicity compared with induced pluripotent or embryonic stem cells (37). Researchers have proposed using MSCs as an attractive therapy to ameliorate or reverse diabetes because of their systemic immunomodulatory and regenerative properties, no immunogenicity due to a lack of major histocompatibility complex (MHC) class II, and ability to home to damaged pancreatic islets and local lymph nodes (11, 13, 38-45).

We took into consideration the promising features of MSCs and designed a randomized placebo-controlled phase I/II clinical trial. In this trial, we sought to examine the safety and efficacy of autologous bone marrow-derived MSCs as a treatment for newly diagnosed patients with T1D.

Materials and Methods

Study design

A triple-blinded parallel randomized placebo-controlled trial was conducted between July 2015 and January 2020 at Royan Institute, Tehran, Iran, in collaboration with the Growth and Development Research Center of Tehran University of Medical Sciences, Tehran, Iran. The

Research Ethics Committee at Royan Institute approved this study (approval code: IR.ACECR.ROYAN.REC.1394.50), and the study was conducted under the Declaration of Helsinki. The trial was registered at the Iranian Registry of Clinical Trials (IRCT) with the identifier IRCT ID: IRCT2016070428786N1 and at the U.S. National Institutes of Health (ClinicalTrials.gov) with the related identifier NCT04078308. Of note, we originally designed this study as a crossover study; however, our data analysis showed that the residual effects of the MSC transplantation were present after the 12-month follow-up. Therefore, the MSC versus placebo comparison was compared during the initial 12-month follow-up, and early versus late transplantation was compared in the second-year follow-up. Figure 1b provides a brief description of the trial steps and timeline.

Study population

A total of 21 patients (age: 8 to 40 years) enrolled in this study. The patients were diagnosed with T1D according to diagnostic criteria and the ADA guidelines (5) within six weeks before enrollment and were already under classic insulin therapy. Patients were enrolled between July 2015 and January 2018 if they met the following inclusion criteria: evaluated fasting C-peptide level ≥ 0.3 nmol/L and presence of at least one of three autoantibodies against pancreatic β cells (islet cell antibody [ICA], glutamic acid decarboxylase antibody [GADA], or insulinoma associated-2 antibody [IA-2A]). The exclusion criteria consisted of pregnancy or breastfeeding; cancer; any acute or severe diseases (cardiac, pulmonary, hepatic, kidney, mental, or other diseases); positive test results for human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV), hepatitis B virus (HBV), hepatitis C virus (HCV), or cytomegalovirus (CMV); other immune-deficiencies; hyperesthesia; or history of severe ketoacidosis. Figure 1a presents the CONSORT flow diagram of the study population and their respective group assignment. All participants or their parents or legal guardians (when required

for pediatric participants) received comprehensive oral and written information about the nature and possible consequences of the study. They signed a written informed consent form before participating in this clinical trial.

Randomization and masking

Patients were randomly assigned to either the MSC or the placebo group by block randomization. A block size of four was used for randomization, and the sequences were calculated by Random allocation software v. 2.0. The research coordinator had exclusive access to the numbered, sealed, opaque envelopes that indicated each patient's randomized treatment assignment.

Blinding

In this triple-blinded study, the participants, care providers, and outcomes assessors were blinded to the treatment assignments. To protect the blinding, two different physicians conducted the intervention, patient assessments, and follow-ups. The vials that contained either the MSCs or the placebo were similar. Only the specific randomization code assigned to an individual patient was visible on the vial. The person who prepared the cells and the research coordinator were aware of the vials' exact contents.

Study interventions

Participants were randomly assigned to receive either two doses of 1×10^6 autologous MSCs or a placebo per kilogram of the patient's body weight at weeks 0 and 3. Besides, we compared the effects of early versus late transplantation of MSCs. Newly diagnosed T1D patients who received the MSCs transplantation during the first year were considered the early transplantation group (Early Tx). The late transplantation group (Late Tx) were patients who received the placebo during the first year of the study and were then assigned to receive the MSCs transplantation at least one year after their diabetes diagnosis. According to the eligibility

criteria, all patients underwent baseline laboratory blood tests (Table S8), EKG, and chest X-ray assessments to confirm their general health. After randomization, bone marrow was aspirated from all the participants' iliac crests while under general anesthesia.

Mesenchymal stem cell (MSC) preparation and transplantation

Clinical-grade MSCs were isolated from bone marrow, expanded in passages 2-3, and cryopreserved under good manufacturing practice (GMP) conditions at Royan Institute, as previously described (46). The release criteria for the therapeutic use of isolated MSCs included the absence of viral, microbial, fungal, and mycoplasma contamination; a less than acceptable level of endotoxin; normal karyotype; and cell viability of more than 95%. The MSCs were assessed by flow cytometry for their ability to express CD105, CD90, CD44, CD73, and lack of CD34 and CD45 expression.

Three to four weeks after bone marrow aspiration, 1×10^6 autologous MSCs in normal saline per kilogram of the patient's body weight were administered for each dose of two intravenous infusions at weeks 0 and 3. The placebo group received a similar amount of normal saline. The first transplantation was regarded as day zero in this study.

Patient follow-up

The patients were followed for one year after the first infusion to assess safety and efficacy parameters. During this time, the patients were seen at weeks 1, 2, and 4, and months 2, 3, 6, 9, and 12 for physician visits and laboratory blood analyses. Furthermore, the patients recorded blood glucose self-monitoring (at least two or three times per day) during this period to evaluate treatment safety and efficacy.

Study outcomes

Primary outcome: Safety

219 The patients provided medical histories and underwent physical examinations at each outpatient
220 visit to monitor for treatment-related adverse events and hypoglycemic episodes. Safety was
221 assessed based on the Common Terminology Criteria for Adverse Events version 5 (CTCAE
222 v.5). The number of hypoglycemic episodes was assessed by evaluating patients' blood glucose
223 monitoring sheets. Hypoglycemia was defined as each blood glucose recording below 70 mg/dl
224 according to CTCAE v.5.

225 *Secondary outcome: Efficacy*

226 Efficacy was defined as changes from baseline in fasting blood sugar (FBS), two-hour
227 postprandial glucose test (2hpp), C-peptide, and serum levels of Glycated hemoglobin
228 (HbA1c), daily dose of injected EI, and lability index (LI).

229 FBS and 2hpp were evaluated using GLUC3 kit (04404483190, Roche Diagnostics GmbH), C-
230 peptide using Elecsys C-peptide kit (03184897, Roche Diagnostics GmbH), HbA1c using Tina-
231 quant® HbA1c Gen. 3 (05336163190, Roche Diagnostics GmbH). These parameters were
232 measured by COBAS® 6000 analyzer and COBAS INTEGRA® 400 plus analyzer Roche
233 Diagnostics.

234 Serum levels of interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF- α), interleukin 4 (IL-4),
235 interleukin 10 (IL-10), and transforming growth factor-beta 1 (TGF- β 1) were evaluated using
236 Enzyme-linked immunosorbent assay (ELISA) utilizing the following kits: Human IL-6 ELISA
237 Kit(E0090Hu, Bioassay Technology Laboratory),; Human TNF- α ELISA Kit (E0082Hu,
238 Bioassay Technology Laboratory), Human IL-4 Quantikine ELISA Kit (D4050, R&D
239 Systems), Human IL-10 Quantikine ELISA Kit (D100B, R&D Systems), and Human TGF- β 1
240 Quantikine ELISA Kit (DB100B, R&D Systems).

241 Patients' QOL was assessed by the 36-item Short Form Survey (SF-36) and Diabetes QOL
242 (DQOL) questionnaires. The effects of physical exercise on metabolic and immunologic indices
243 between the MSC transplantation and placebo groups were compared in some patients.

The percentages of CD4⁺ CD25⁺ Foxp3⁺ T reg cells were also assessed in patients' peripheral blood before the MSCs transplantation and 48 hours after transplantation, as described before (47). Briefly, Ficoll density gradient (Cat#: P04-60100, PAN biotech) was used to separate peripheral blood mononuclear cells (PBMCs) from collected blood in EDTA-containing tubes. The PBMCs were incubated with FITC conjugated anti-CD4 (Cat#: 550628, BD Biosciences) and PE-conjugated anti-CD25 (Cat#: 557138, BD Biosciences) antibodies to stain the surface markers. Then, the PBMCs were incubated with fixation and permeabilization buffer BD Cytofix/CytopermTM (Cat#: 554722, BD Biosciences), washed with BD Perm/Wash buffer (Cat#: 554723, BD Biosciences), and stained with PerCP anti-Foxp3 antibody (Cat#: NBP2-34432PCP, Novus Biologicals). The stained PBMCs were characterized by a flow cytometer (BD FACSCaliburTM, BD Biosciences, San Jose, CA, USA) equipped with BD CellQuest Pro software. The acquired data were analyzed by FlowJo software (Version: 7.6.1, BD Biosciences). Isotype controls were utilized to assist precise compensation and evaluate antibody specificity.

Sample size

The calculated sample size was based on HbA1c improvement over 12 months in the MSC group versus the placebo group. We assumed the anticipated effect size of $f = 0.52$, type I error of 0.05, test power of 80%, and correlation coefficient of 0.5 for repeated measurements based on Hu et al.'s study (11). A total sample size of $n=20$ was calculated with G*Power 3.1 (University of Kiel, Germany) using repeated measures analysis of variances (ANOVA).

Statistical analysis

All data were collected and stored at Royan Institute. Primary and secondary outcome analyses were performed on all randomized, eligible patients. All analyses were based on a statistical analysis plan prepared by a statistician blinded to the study assignments, and all analytic conclusions were based on masked data reviews.

We used the modified intention to treatment (MITT) method for the standard primary analysis. Patients were analyzed according to their randomized arm. Missing data were excluded at each time point, and the analysis was based on the available cases at each time point.

Analyses were performed using Stata software (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP.), and the graphs were generated with GraphPad Prism version 9.1.1 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. The significance level for all analyses was set at $\alpha = 0.05$. Baseline continuous characteristics are reported as mean $\pm$ standard deviation, and categorical variables are reported as N.

Interventional effects on primary and secondary outcomes were evaluated using a generalized estimating equation (GEE) for longitudinal data with an identity link and an exchangeable correlation matrix to give population-averaged estimates. The placebo group was considered the reference group to evaluate the effects of the intervention. The Early Tx group was used as the reference group to evaluate the effects of new-onset on intervention. Also, we assessed the residual data from the GEE model. GEE models were used to evaluate changes from baseline to 3, 6, 9, and 12 months for primary and secondary continuous outcomes measured longitudinally. Groups were included in the models as the covariate, and visit time was the categorical measure. The interaction between groups and time was considered in the model, and confidence intervals (CI) and P-values were adjusted by the confounders of body mass index (BMI), age, and sex.

Results

Study population

A total of 212 patients were screened for eligibility from July 2015 to January 2018, in which 21 met the eligibility criteria and were randomly assigned to either the MSC transplantation or the placebo group (Figure 1a). The patients were followed up for a year after they received their first injection. Figure 1b provides a brief description of the trial steps and timeline.

a

b

Figure 1. a. CONSORT flow diagram. A total of 212 patients were assessed for eligibility, of which 43 were selected for further verification of the eligibility criteria. Finally, 21 participants were selected and randomly assigned to either the mesenchymal stem cell (MSC) or placebo group. The participants were followed for one year and then analyzed for study outcomes. **b. Schematic description of the study.** The process of this clinical trial is generally divided into three main steps, including Patient allocation, MSC preparation, and Monitoring and Follow up. Briefly, selected patients gave informed consent, and after checking full eligibility criteria, they were randomly allocated to either receive MSC or Placebo. Then patients who underwent the first evaluation were subjected to bone marrow aspiration. MSCs were isolated from harvested bone marrow and expanded via culturing in a cleanroom facility. The cells that passed quality-control tests were intravenously injected into group A (early transplantation), while patients in group B received normal saline as a placebo. All patients were followed up for one year, and then the patients in group A received a placebo, whereas patients in group B received MSC (late transplantation). Data were analyzed after 12 and 24 months of follow-up.

Baseline demographic and clinical characteristics were similar in both groups (Table 1).

Table 1. Baseline demographic characteristics of the study participants (n=21)

	Placebo ^a (n=10)	MSC ^a (n=11)
General characteristics		
Demographics		
Age (years)	11.50 ± 2.63	10.27 ± 1.67

BMI (kg/m ²)	18.91 ± 3.41	16.75 ± 2.57
Male / Female	5/5	6/5
Residence		
Local (Tehran Province)/other	3/7	6/5
Auto-antibodies (AA) (negative/positive)		
ICA	6/4	6/5
GADA	0/10	3/8
IA-2A*	5/3	3/7
Metabolic indices		
FBS (mg/dl)	149.4 ± 51.97	165.27 ± 97.49
2hpp (mg/dl)	234.7 ± 80.39	269.00 ± 173.29
HbA1c (%)	7.85 ± 1.45	8.63 ± 2.19
C-peptide (ng/ml)	0.92 ± 0.57	0.72 ± 0.38
EI (IU/kg/day)	0.71 ± 0.30	0.78 ± 0.44
LI (mmol/l2/h.week-1)	42.09 ± 44.31	29.44 ± 35.50
Cytokines		
<i>Pro-inflammatory</i>		
IL-6 (ng/l)	86.66 ± 30.11	106.96 ± 57.67
TNF-α (pg/ml)	68.29 ± 34.49	94.30 ± 55.55
<i>Anti-inflammatory</i>		
IL-4 (pg/ml)	325.02 ± 179.45	249.84 ± 208.04
IL-10 (pg/ml)	412.38 ± 336.84	236.22 ± 221.86
TGF-β1 (pg/ml)	802.58 ± 386.44	539.96 ± 529.99
Quality of life questionnaires		
DQOL	75.97 ± 11.16	75.91 ± 9.69
SF-36	77.09 ± 13.46	74.43 ± 17.98
^a Variables are expressed as mean ± standard deviation and count BMI: Body mass index ICA: Islet cell antibody		
EI: Exogenous insulin LI: Lability index IL-6: Interleukin 6 TNF-α: Tumor necrosis factor-alpha		

GADA: Glutamic acid decarboxylase antibody	IL-4: Interleukin 4
IA-2A: Insulinoma associated-2 antibody	IL-10: Interleukin 10
* Other missing	TGF-β1: Transforming growth factor-beta 1
FBS: Fasting blood sugar	DQOL: Diabetes Quality of Life
2hpp: Two-hour postprandial	SF-36: 36-Item Short Form Survey
HbA1c: Glycated hemoglobin	QOL: Quality of life
MSC: Mesenchymal stem cell	

303

304 **Major complications and death**

305 In this study, we did not find any major complications or deaths among participants.

306 **Losses and exclusions**

307 Two patients, who received the allocated treatment in the first year and completed the 12
308 months of follow-up, declined to participate in the second course of treatment due to personal
309 issues unrelated to treatment.

310 **Primary outcomes: Safety**

311 *Transplantation-associated adverse events*

312 There were no major transplantation-related adverse events observed in either group. One
313 adverse event potentially unrelated to the MSC transplantation was a mild injection site reaction
314 observed in two patients (one in each group). A grade 3 urticaria was observed in P-1 during
315 the first injection. Symptoms were relieved with the administration of a mild anti-inflammatory
316 medication. A mild increase in lymphocytes occurred in P-2 and mild hyperkalemia in P-4 from
317 the MSC group, both of which occurred 12 months after transplantation and appeared unrelated
318 to the treatment procedure.

319 Other adverse events reported in the MSC and placebo groups were negligible or reported only
320 in the placebo group, mainly without any medical intervention.

321 Table 2 lists the observed adverse events in this trial. The observed adverse events were not
322 serious, and, when necessary, the required medical interventions were applied.
323

Table 2. List of observed adverse events. Observed adverse events were not serious, and the required medical considerations were applied in case they needed

No.	MedDRA	CTCAE term	Observed	Placebo (n=10)		MSC (n=11)	
	Code			grade	N	Patient ID /time	N
INVESTIGATIONS							
1	10001551	Alanine aminotransferase (ALT) increased	1	1	P-11/M6		
2	10001675	Alkaline phosphatase (ALP) increased	1	4	P-3/M6		P-2/M6, 9, 12
					P-8/M6, 12	5	P-6/M6
					P-9/M12		P-10/M6
3	10003481	Aspartate aminotransferase (AST) increased	1	2	P-9/M12 P-11/M6		
4	10025258	Lymphocyte count increased	2			1	P-2/M12
5	10047900	Weight loss	1	1	P-18/M6		
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS							
6	10016558	Fever	1	1	P-18/M1		
7	10022095	Injection site reaction	1	1	P-3/M1	1	P-6/W1
			2	1	P-5/M1	1	P-6/M1
8	10033371	Pain (epigastric region)	1	1	P-12/M3	1	P-13/M2
METABOLISM AND NUTRITION DISORDERS							
9	10020587	Hypercalcemia	1	1	P-7/M12		
10	10020647	Hyperkalemia	4			1	P-4/M12
11	10020907	Hyperuricemia	1	1	P-18/M12		
12	10020949	Hypocalcemia	2	1	P-12/M12		
14	10021018	Hypokalemia	1	1	P-14/M6		
15	10021038	Hyponatremia	1	2	P-3/M12		
					P-9/M12		
SKIN AND SUBCUTANEOUS TISSUE DISORDER							
16	10046735	Urticaria	3			1	P-1/First Injection
ENDOCRINE							
17	10021114	Hypothyroidism	1	1	P-8/M9		

Table 2. List of observed adverse events. Observed adverse events were not serious, and the required medical considerations were applied in case they needed

No.	MedDRA	CTCAE term	Observed	Placebo (n=10)		MSC (n=11)	
	Code		grade	N	Patient ID /time	N	Patient ID /time
NEUROLOGY							
18	10037175	Psychiatric disorders	1	1	P-8/M6		
MSC: Mesenchymal stem cell; M: Month; W: Week; N: Number of events							

A complete list of the assessed adverse events is found in table S1.

Hypoglycemia

Hypoglycemic events are defined as the observation of blood glucose levels lower than 70 mg/dl. The number of hypoglycemic events was considered not only a safety parameter but also an efficacy outcome. These events are graded from I to V according to the severity in the CTCAE v.5, as follows: grade I (< lower limit of normal [LLN] - 55 mg/dL); grade II (<55-40 mg/dL); grade III: (<40-30 mg/dL); grade IV (<30 mg/dL, life-threatening consequences, seizures); and grade V (Death). The MSC transplantation group had significantly lower numbers of grade I (P=0.0457) and II (P<0.001) hypoglycemic episodes and a total number of hypoglycemic events (P=0.0164) compared to the placebo group (Figure 2a). Early transplantation of MSCs significantly reduced the number of grade II hypoglycemic events compared to late transplantation of MSCs (P=0.008, Figure 2b).

Figure 2. Hypoglycemic rates (events per patient - year) a. Mesenchymal stem cell (MSC) transplantation versus placebo, **b.** Early versus late transplantation (Grade I: <lower limit of normal (LLN) - 55 mg/dL; Grade II: <55-40 mg/dL; Grade III: <40-30 mg/dL; Grade IV: <30 mg/dL, life-threatening consequences, seizures; Grade V: Death. Error bars represent the standard deviation. *P<0.05, **P<0.01, ***P<0.001)

Secondary outcome: Efficacy

Mesenchymal stem cells (MSCs) versus placebo

Metabolic indices

There were no significant differences in FBS and 2hpp between the MSC and placebo groups (Table S2, Figure 3a, b). As shown in table S2, MSCs significantly reduced the percent of HbA1c 12 months after transplantation (P=0.043). There were considerable mean changes in HbA1c at month 9 (P=0.061, Figure 3c). The mean differences in C-peptide levels between the two groups showed higher but insignificant changes that favored MSC transplantation (Figure 3d). No significant changes in the amount of EI and LI were reported (Figure 3e, f). However, although the 6-month follow-up showed a reduction in EI, there was a slight increase at the 12-month follow-up (Figure 3e). The LI decreased at the 12-month follow-up in patients who received MSCs (Figure 3f).

Figure 3. Comparison of metabolic indices between the mesenchymal stem cell (MSC) and placebo groups. The left Y-axis represents the mean in each group drawn by column charts, and the right Y-axis belongs to the mean differences compared to the baseline drawn by scatter plots. **a.** Fasting blood sugar (FBS), **b.** Two-hour postprandial (2hpp), **c.** Glycated hemoglobin (HbA1c), **d.** C-peptide, **e.** Exogenous insulin (EI), **f.** Lability

index (LI). Error bars represent the standard deviation for the bar charts and the standard error of the mean for scatter plots. Chart legend for the entire picture is shown in part **a**. *P<0.05

350

351 *Immunologic indices*

352 According to previous studies (42, 48), IL-6 and TNF- α have pro-inflammatory effects, whereas
353 IL-4, IL-10, and TGF- β 1 show anti-inflammatory effects. Transplantation of MSCs, as an
354 immunomodulator, significantly increased the anti-inflammatory cytokines IL-4 (P=0.032) and
355 IL-10 (P=0.30). This effect surprisingly persisted after 12 months (Figure 4c, d; Table S3).
356 TNF- α levels significantly reduced after six months (P=0.027) and showed a considerable
357 decrease after 12 months (Figure 4b, Table S3).

358 T reg cells are a group of immune cells that play an essential role in peripheral tolerance
359 induction and maintenance; therefore, they modulate the immune response and prevent
360 autoimmunity. Flow cytometry analysis of their antigens showed significant increases in T reg
361 cell levels (P=0.0088) and Forkhead box P3 (FOXP3) expression (P=0.0004) 48 hours after
362 transplantation of the MSCs (Figure 4f).

Figure 4. Comparison of cytokine levels between mesenchymal stem cell (MSC) and placebo groups. The left Y-axis represents the mean in each group drawn by column charts, and the right Y-axis belongs to the mean differences compared to the baseline drawn by scatter plots. **a.** Interleukin 6 (IL-6), **b.** Tumor necrosis factor- α (TNF- α), **c.** Interleukin 4 (IL-4), **d.** Interleukin 10 (IL-10), **e.** Transforming growth factor-beta 1 (TGF-

β1), **f**. Regulatory T cell (T reg) population and Forkhead box P3 (FOXP3) expression before and 48 hours after transplantation. Tx: Transplantation. Error bars represent the standard deviation for bar charts and the standard error of the mean for scatter plots. Chart legend for the entire picture is shown in part **a** unless otherwise indicated. *P<0.05, **P<0.01, ***P<0.001

Quality of Life (QOL)

DQOL questions are categorized into three subsections: satisfaction, impact, and worry. The SF-36 questions are categorized into eight subsections: physical functioning (PF), role - physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role - emotional (RE), and mental health (MH). Two main sections of the SF-36 consist of these eight subsections. Section A: Physical Health includes the PF, RP, BP, GH, and VT subsections, whereas section B: Mental Health includes the GH, VT, SF, RE, and MH subsections.

After six months, patients reported a significant reduction in worry related to DQOL in the MSC group (P=0.012). The MSC group reported a significant improvement in RP (P=0.020) at the 6-month follow-up and a significant improvement in GH (P=0.037) at the 12-month follow-up (Table S4, Figure 5a).

Figure 5. Quality of life (QOL) **a**. Comparison of patients' QOL between the mesenchymal stem cell (MSC) and placebo groups based on the Diabetes specific QOL (DQOL) and 36-Item Short Form Survey (SF-36)

questionnaires. The left Y-axis represents the mean in each group drawn by column charts, and the right Y-axis belongs to the mean differences compared to the baseline drawn by scatter plots. There were no significant changes reported. Error bars represent the standard deviation for bar charts and the standard error of the mean for scatter plots. **b.** Comparison of patients' QOL between early (Early Tx) and late (Late Tx) transplantation of MSCs based on the DQOL and SF-36 questionnaires. The left Y-axis represents the mean in each group drawn by column charts, and the right Y-axis belongs to the mean differences compared to the baseline drawn by scatter plots. Error bars represent the standard deviation for bar charts and the standard error of the mean for scatter plots. *P<0.05

375

376 *Early versus late transplantation*

377 *Quality of life (QOL)*

378 Table S5 shows that the total DQOL (P=0.026) and social functioning subsection (P=0.025) of
379 the SF-36 questionnaire showed significant improvements in the Late Tx group at 12 months
380 follow-up (Figure 5b).

381

382 *Metabolic indices*

383 We compared the new-onset group that received MSC transplantation in the first year when
384 they were newly diagnosed with T1D (Early Tx) and the patients who received the
385 transplantation at least one year after their diagnosis (Late Tx). Table S6 shows that early
386 transplantation of MSCs can significantly decrease the percentages of HbA1c at 3 (P<0.001), 6
387 (P=0.015), and 12 (P=0.041) months after transplantation compared to the Late Tx group
388 (Figure 6c). Serum C-peptide levels in the Early Tx group were significantly higher than the
389 Late Tx group at 3 (P=0.003), 6 (P=0.023), and 9 (P=0.016) months of follow-up (Figure 6d).

Figure 6. Comparison of metabolic indices between early transplantation (Early Tx) and late transplantation (Late Tx) groups. The left Y-axis represents the mean in each group drawn by column charts, and the right Y-axis belongs to the mean differences compared to the baseline drawn by scatter plots. **a.** Fasting blood sugar (FBS), **b.** Two-hour postprandial (2hpp), **c.** Glycated hemoglobin (HbA1c), **d.** C-peptide, **e.**

Exogenous insulin (EI), **f.** Lability index (LI). Error bars represent the standard deviation for bar charts and the standard error of the mean for scatter plots. Chart legend for the entire picture is shown in part **a.** *P<0.05, **P<0.01, ***P<0.001

390

391 *Immunologic indices*

392 TNF- α levels were significantly lower in the Early Tx group than the Late Tx group at the 6-
393 (P=0.002) and 12- (P=0.028) month follow-ups. IL-6 levels were not significantly different
394 between the groups (P=0.877) (Table S7, Figure 7a, b). IL-4 and IL-10 levels were significantly
395 higher in the Early Tx group compared to the Late Tx group at the 6- and 12-month follow-ups
396 (P₆=0.025, P₁₂=0.001, and P₆<0.001, P₁₂<0.001, respectively) (Table S7, Figure 7c, d). TGF-
397 β 1 levels were significantly higher in the Early Tx group than the Late Tx group at the 6-month
398 (P=0.038) and 12-month (P=0.016) follow-ups (Table S7, Figure 7e), which indicated a
399 successful shift from pro-inflammatory to anti-inflammatory cytokines following MSC
400 transplantation in newly diagnosed T1D patients.

a

b

c

d

e

Figure 7. Comparison of cytokine levels between the early transplantation (Early Tx) and late transplantation (Late Tx) groups. The left Y-axis represents the mean in each group drawn by column charts, and the right Y-axis belongs to the mean differences compared to the baseline drawn by scatter plots. **a.** Interleukin 6 (IL-6), **b.** Tumor necrosis factor-alpha (TNF- α), **c.** Interleukin 4 (IL-4), **d.** Interleukin 10 (IL-10), **e.** Transforming growth factor-beta 1 (TGF- β 1). Error bars represent the standard deviation for bar charts and the standard error of the mean for scatter plots. Chart legend for the entire picture is shown in part **a**. *P<0.05, **P<0.01, ***P<0.001

401

402 *Exercise*

403 ADA guidelines recommend that children 5 to 18 years of age exercise at least three days each

404 week, with more than 60 minutes of daily activity and 150 minutes of weekly exercise for adults

405 (5, 49). This study measured the metabolic and immunologic indices by considering the 3.5

406 hours per week exercise cut-off for some patients.

407 The percentage of HbA1c in patients who received MSCs and exercised more than the cut-off

408 mentioned above (in figures shown by “Yes”) was significantly lower than those who received

409 MSCs or placebo and did not exercise (in figures shown by “No”) based on ADA

410 recommendations (P=0.0035, P=0.0033, respectively, Figure 8c). HbA1c levels in patients who

411 received MSCs and who had adequate exercise levels were significantly lower than the placebo

412 group, who had adequate levels of exercise (P=0.0117, Figure 8c).

413 As shown in Figure 8b, exercise significantly increased the serum C-peptide level of patients

414 who received the placebo and had adequate levels of exercise (Yes, Placebo) compared to the

415 patients who received placebo and exercised less than the recommendations (No, Placebo)

416 (P=0.0088). Moreover, patients in the MSC group who had adequate exercise levels (Yes, MSC

417 group) had significantly higher C-peptide levels than the placebo group who exercised less (No,

418 Placebo group). The No, MSC group had higher C-peptide levels compared to the No, Placebo

419 group (P=0.0016, 0.0019, respectively, Figure 8d).

420 IL-10 was significantly higher in patients who received MSC than the placebo group, regardless
421 of the level of exercise (Yes, MSC vs. Yes, Placebo: P=0.0156; Yes, MSC vs. No, Placebo:
422 P=0.0228; No, MSC vs. Yes, Placebo: P=0.0070; No, MSC vs. No, Placebo: P=0.0097; Figure
423 8j). The No, MSC group had significantly higher TGF- β 1 levels compared to the Yes, Placebo
424 group (P=0.0438, Figure 8k). All other changes were not significant (Figure 8a, b, e-i).

a **b** **c** **d** **e** **f** **g****h**

Figure 8. Comparison of metabolic indices and cytokine levels in the mesenchymal stem cell (MSC) and placebo groups based on patient exercise. a. Fasting blood sugar (FBS), **b.** Two-hour postprandial (2hpp), **c.** Glycated hemoglobin (HbA1c), **d.** C-peptide, **e.** Exogenous insulin (EI), **f.** Lability index (LI), **g.** Interleukin 6 (IL-6), **h.** Tumor necrosis factor-alpha (TNF-α), **i.** Interleukin 4 (IL-4), **j.** Interleukin 10 (IL-10), **k.** Transforming growth factor-beta 1 (TGF-β1). Data are presented as mean ± standard deviation with an exercise cut-off of 3.5 h/week. Yes, means more exercise than the cut-off. No, means less exercise than the cut-off. Chart legend for the entire picture is shown in part a. *P<0.05, **P<0.01

Discussion

To the best of our knowledge, this is the first randomized triple-blinded, placebo-controlled clinical trial that assesses the safety and efficacy of transplanting autologous bone marrow-derived MSCs as treatment of newly diagnosed T1D patients.

Our results showed that transplantation of bone marrow-derived MSCs in diabetic children is safe and well-tolerated over the study follow-up period. We followed the safety indices for 24 months and found that this procedure's long-term safety was acceptable (data are not shown). There were no major complications and transplantation-associated adverse events reported, supported by previous studies harboring MSC transplantations for various diseases (50-60).

Interestingly, we observed that the MSC transplantation could significantly reduce the number of hypoglycemic events in recipients as a major life-threatening complication of T1D in children. This effect was reported in a preclinical study of MSC transplantation for post-hepatectomy liver failure (61). Additionally, for the first time, we showed that early transplantation of MSCs significantly reduced the rate of grade II hypoglycemic events in T1D patients compared to late transplantation of MSCs.

The efficacy of MSC transplantation in our study was favorable. We observed a significant reduction of HbA1c at month 12 along with its considerable reduction in month 9. There were higher, but not significant, serum C-peptide levels, a significant increase in serum levels of IL-4 and IL-10, and a slight increase in TGF- β 1 after 12 months as anti-inflammatory cytokines. Besides, there was a significant reduction in serum TNF- α at month 6 and a considerable reduction at month 12, along with a moderate decrease in IL-6 levels at 12 months, as pro-inflammatory cytokines. Previous studies showed a decreased level of IL-4 in newly diagnosed T1D patients (62). We showed that MSCs could increase serum IL-4 levels. The anti-inflammatory properties of IL-4 might prevent disease progression. We did not observe any significant differences in FBS levels between the groups, which was confirmed by previous

studies likely attributed to fluctuations in FBS levels, which are influenced by many variables (11).

Although IL-10 has an anti-inflammatory effect that prevents inflammation as the main pathogenesis of autoimmune disorders, its role in modulating disease progression in T1D is debatable (63). The results of several studies suggest a correlation between elevated IL-10 and disease attenuation (63-65). The proposed mechanism of action for IL-10 mediated T1D regulation includes increasing the numbers of T reg cells and serum levels of T-helper 2-type cytokines (IL-4 and IL-10) while reducing serum levels of T-helper 1-type cytokines (IL-2 and IFN- γ) (66, 67).

Potential immunosuppressive effects of TGF- β are accepted in autoimmune diseases used against most immune cells through the development and peripheral differentiation of Tregs and induced expression of FOXP3 in the context of T1D (63, 68).

IL-6 is a pro-inflammatory cytokine that plays a role in autoimmune disease progression. The results of preclinical studies report a correlation between IL-6 production and β -cell destruction (63, 69, 70). Chen et al. conducted a systematic review and concluded that a link existed between serum IL-6 levels and T1D (71). An ongoing clinical trial aims to preserve β cells through an IL-6 blockade by tocilizumab administration (NCT02293837).

TNF- α is a pro-inflammatory cytokine produced by dendritic cells and macrophages; its effects on MHC-I upregulation results in accelerating β -cell death and initiation of T1D (72, 73). Mastrandrea et al., in a randomized pilot study, have reported preservation of β -cell function in newly diagnosed T1D patients who received TNF blockers (74).

Of note is an increase in anti-inflammatory cytokines (TGF- β , IL-4, and IL-10) and a decrease in pro-inflammatory cytokines (TNF- α and IL-6) with the promotion of T reg cells, which has been reported in other studies as some of the immunomodulatory functions of MSCs. These findings align with our results (13, 36, 39, 42, 45, 48, 75-77).

476 We also observed an improved QOL in the patients in terms of significantly reduced DQOL-
477 worry and improved SF36-Role-Physical evaluation at month 6 as well as SF36-General Health
478 at month 12.

479 In 2013, Hu et al. conducted a randomized clinical trial to evaluate the effects of Wharton's
480 jelly-derived MSC injection in 29 new-onset T1D patients, with 15 patients in the treatment
481 group and 14 patients in the control group. They observed higher C-peptide levels and improved
482 glycemic control after two years of follow-up (11). Carlsson et al. reported that systemic
483 injection of autologous bone marrow-derived MSCs in 9 patients compared to a control group
484 could preserve or even increase C-peptide levels in response to a mixed-meal tolerance test
485 (MMTT) during the first year (54). Our data support the results of these studies.

486 The results of some studies showed that hyperglycemia is caused by reduced β -cell mass and
487 β -cell dysfunction. Its extent varies between patients and is based on patient age and level of
488 insulinitis. More than 80% loss of beta-cell mass at the onset of T1D is a common finding in
489 studies related to T1D pathogenesis, which suggested a preserved β -cell mass with up to 20%
490 residual insulin-secreting cells. Some researchers have stated that β -cell mass might be
491 underestimated due to insulin degranulation, which occurs in exhausted β cells (78, 79).

492 From this point of view, we evaluated the efficiency of early versus late transplantation of
493 MSCs following the occurrence of the first clinical symptoms of T1D, in addition to comparing
494 MSC and placebo groups.

495 We found that early transplantation of MSCs in newly diagnosed patients had benefits
496 compared to late transplantation. These benefits included a significant decrease in HbA1c at
497 months 3, 6, and 12; significantly higher C-peptide levels at months 3, 6, and 9; significantly
498 lower level of TNF- α at 12 months and slightly lower level of IL-6 at month 6 as pro-
499 inflammatory cytokines. There were significant increases in the anti-inflammatory cytokines
500 IL-4, IL-10, and TGF- β 1 at 12 months. We also observed significantly reduced QOL in terms

of DQOL and SF36-Social functioning in the Early Tx group, likely due to the time (one year) passed from the onset of diabetes in the Late Tx group. The patients were more adapted to their disease situation and more stable than newly diagnosed patients.

Another factor that affected blood glucose management in T1D patients is the activity level. Regular exercise should be encouraged in people with insulin-dependent diabetes to decrease cardiovascular disease risk and improve QOL. In addition, mild exercise increases insulin action without influencing BMI. Although physical exercise should be undertaken in T1D patients with safety recommendations for blood glucose monitoring before and after exercise to prevent hypoglycemia or hyperglycemia, this challenge could be mostly overcome with a regular, anticipated exercise program (80, 81).

We found that more than 3.5 h/week of exercise in addition to MSC transplantation could significantly decrease HbA1c levels and increase C-peptide and IL-10 levels when compared to patients who did not have enough exercise and received the placebo. Furthermore, the effects of the MSC transplantation are superior to exercise alone since patients who did not have enough exercise but received MSC transplantation had significantly higher levels of TGF- β 1 and IL-10 compared to the placebo group patients who had enough exercise.

This study had several limitations. First, a limited number of patients met the defined eligibility criteria that caused a longer than expected recruitment process. T1D is a multifactorial disease probably influenced by patients' lifestyle, socioeconomic status, stress level, exercise, diet, and other known and unknown factors that we did not consider. Thus, some studies are needed to assess the effects of these factors on disease progression. International Society for Pediatric and Adolescent Diabetes (ISPAD) noted the importance of partial remission in the early stages of type-1 diabetes following insulin treatment initiation, known as the honeymoon phase. This period reflects the partial recovery of β -cell with increased insulin secretion, in which the metabolic indices, notably HbA1c and C-peptide, could improve towards near normoglycemia

condition (82, 83). In this study, since all participants were enrolled in their early stage of diagnosis, their condition could be affected by the honeymoon phase, which could have obscured the MSC's effects. Finally, regarding the possible reduced therapeutic potential of diabetics' MSCs, further evaluation should be considered for allogeneic MSCs transplantation. In this study, we found that transplantation of MSCs in T1D patients is safe and tolerable during the follow-up period. MSC transplantation can improve some of the patients' metabolic indices and modulate their immune responses. Early transplantation of MSCs can greatly improve metabolic indices and alleviate immune responses by increasing anti-inflammatory cytokines and decreasing pro-inflammatory cytokines compared to late transplantation of MSCs. We observed a noticeable pro-inflammatory to anti-inflammatory cytokine shift in addition to an increased T reg cell population, which provided additional evidence for the benefits of early transplantation of MSCs. However, a detailed study with different cell doses and repeated injections is required to achieve superior clinical results. Although it was hard to control lifestyle variables in patients, we recommended that all study participants exercise by considering safety concerns, resulting in enhanced glycemic control in a limited number of patients who exercised more than 3.5 h/week. Therefore, controlling patients' lifestyles in addition to MSC transplantation could improve patients' responses to therapy, which should be evaluated in a greater number of participants. A phase III study with a higher number of patients who receive multiple doses of MSC injections and longer follow-ups are needed to elucidate the therapeutic effects of MSCs in T1D and shed light on its molecular mechanisms and pathways behind this process. We conclude that autologous bone marrow-derived MSC transplantation is a safe therapeutic choice for newly diagnosed labile T1D patients who have high blood glucose fluctuations and experience several episodes of hypoglycemia.

551 **Declarations**

552 **Ethics approval and consent to participate**

553 The Research Ethics Committee at Royan Institute approved this study (approval code:
554 IR.ACECR.ROYAN.REC.1394.50), and the study was conducted under the Declaration of
555 Helsinki. All participants or their parents or legal guardians (when required for pediatric
556 participants) received comprehensive oral and written information about the nature and possible
557 consequences of the study. They signed a written informed consent form before participating
558 in this clinical trial.

559 **Consent for publication**

560 Not applicable.

561 **Availability of data**

562 The data that supports the findings of this study are available in the supplementary material of
563 this article.

564 **Competing interest**

565 none

566 **Funding**

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570 **Author contributions**

571 MI: Conceptualization, Methodology, Investigation, Resources, Data Curation, Visualization,
572 Writing - Original Draft; ASH: Conceptualization, Methodology, Investigation, Resources,
573 Data Curation, Visualization, Writing - Original Draft; MM: Conceptualization, Methodology,
574 Investigation, Resources, Data Curation, Visualization, Writing - Original Draft; SM: Formal
575 analysis, Data Curation; AR: Supervision, Resources, Project administration; FK:
576 Investigation; AAHA: Investigation; FA: Investigation; NJ: Investigation; MAMM:

Investigation; AS: Investigation, Resources; FA: Investigation, Resources; SEH: Investigation; FMN: Investigation; RSY: Investigation; RN: Investigation; EHS: Conceptualization, Resources, Supervision, Methodology, Validation, Funding acquisition, Project administration, Writing - Review & Editing; HB: Conceptualization, Supervision, Methodology, Funding acquisition, Writing - Review & Editing. All authors read and approved the final manuscript.

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Registration of clinical trial

The trial was registered at the Iranian Registry of Clinical Trials (IRCT) with the identifier IRCT ID: [IRCT2016070428786N1](#)(84) and at the U.S. National Institutes of Health (ClinicalTrials.gov) with the related identifier [NCT04078308](#) (85).

Supplementary materials

Table S1. Complete List of assessed Adverse Events

Table S2. Comparison of metabolic indices in a 12-month follow-up of mesenchymal stem cells (MSCs) versus placebo

Table S3. Comparison of immunologic indices in 12 months follow-up of MSCs versus placebo

Table S4. Comparing the quality of life (QOL) questionnaires scores in 12 months follow-up of MSCs versus Placebo

Table S5. Comparison of quality of life (QOL) questionnaire scores in 12 months of follow-up between the early (Early Tx) and late (Late Tx) transplantation groups

Table S6. Comparison of metabolic indices in 12 months of follow-up between early versus late transplantation of MSCs

602 **Table S7.** Comparison of immunologic indices in 12 months of follow-up for the early (Early
603 Tx) and late (Late Tx) transplantation groups
604 **Table S8.** List of follow up events

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