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Insulin-secreting Adipose Derived Mesenchymal Stem Cells With Bone Marrow Derived Hematopoietic Stem Cells From Autologous and Allogenic Sources For Type-1 Diabetes Mellitus

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ABSTRACT

Back ground: Stem cell therapy (SCT) is now the up-coming therapeutic modality for treatment of type-1 diabetes mellitus (T1DM). Material and Methods: Our study was prospective open-labeled 2-armed trial, for 10-T1DM patients in each arm of allogenic and autologous adipose-derived insulin-secreting mesenchymal stem cells (IS-AD-MS-C)+bone-marrow derived hematopoietic stem cells (BM-HSC) infusion. Group-1 received autologous SCT with 9-males, 1-female with mean age, 20.2-years, disease duration (DD) 8.1-years and group-2 received allogenic SCT with 6-males, 4-females with mean age, 19.7-years, and DD 7.9-years. Glycosylated hemoglobin (HbA1c) was 10.99%; Serum(S.) C-peptide, 0.22ng/ml and insulin requirement, 63.9IU/day in group-1 and HbA1c was 11.93%, S.C-peptide 0.028ng/ml and insulin requirement 57.55IU/day in group-2. SC were infused into portal+thymic circulation and subcutaneous tissue under non-myeloablative conditioning. Patients were monitored for blood-sugar, S.C-peptide, GAD antibodies and Hb1Ac at 3-monthly intervals. Results: Group-1 received mean SC 103.14ml with 2.65x10² ISC/ μ L, CD34+ 0.81% and CD45-/90+/73+, 81.55%. Group-2 received mean SC 95.33ml with 2.07x10² ISC/ μ L, CD34+ 0.32% and CD45-/90+/73+ 54.04%. No untoward effect was observed with sustained improvement in HbA1c and S.C-peptide in both groups with decrease in GAD-antibodies and reduction in mean insulin requirement. Conclusion: SCT is safe and viable treatment option for T1DM. Autologous IS-AD-MS-C+BM-HSC co-infusion offers better long-term control of hyperglycemia as compared to allogenic SCT.

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Introduction:

Type-1 Diabetes Mellitus (T1DM) is the second most common chronic disease of childhood believed to be autoimmune in nature, characterized by irreversible destruction of insulin-secreting pancreatic β islet cells, requiring life-long exogenous insulin therapy. Symptoms appear when insulin making β cell mass gets reduced by approximately 90% leading to severe insulin deficiency and hyperglycemia.¹ The incidence of diabetes mellitus (type-2 DM) has been increasing in an epidemic-like fashion in the last two decades globally. India is expected to become the world capital of DM by year 2030.^{2, 3, 4} Sporadic cases of hematopoietic stem-cell transplantation (HSCT) have been reported with limited success.⁵ Stem cell therapy (SCT) offers great promise for cure of many autoimmune diseases including T1DM. Insulin secreting cells (ISC) generated from SC represent an attractive alternative.⁶

Mesenchymal stem cells (MSC) have remarkable paracrine effects which can be divided into trophic ('nurturing'), immunomodulatory, anti-scarring and chemo attractant.⁷ We have successfully generated Insulin secreting adipose tissue derived MSC (IS-AD-MS-C) in vitro.⁸ We now present our experience of insulin replacement therapy (IRT) in T1DM patients by co-infusion of in vitro generated IS-AD-MS-C and bone marrow (BM) derived HSC. In this study we compared the results of autologous SCT with allogenic SCT. We assessed the safety & efficacy of SCT.

Material and Methodology

This study was approved by Institutional Review Board. Inclusion criteria were T1DM of >12 months duration with presence of glutamic acid decarboxylase (GAD) antibodies and age group of 8 to 45 years with low serum (s.) C-peptide levels. Exclusion criteria were positive serology for hepatitis C/ hepatitis B/ HIV infection, other systemic infections/ disorders, malignancy and pregnancy. Diabetic ketoacidosis (DKA) was not a contra-indication to SCT.

Key end-points of study were morbidity, mortality, untoward side effects from SCT, and changes in exogenous insulin requirements. Secondary end-points were GAD antibodies, S.C-peptide levels with mixed-meal tolerance test and glycosylated haemoglobin (HbA1c). Monitoring was done at 3-monthly intervals

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Group-1 included autologous SCT in which patients' own abdominal fat and BM were used. Group-2 with allogenic SCT included healthy non-diabetic volunteer donors from family of recipients with compatible blood group, who were willing to donate fat and BM without any objection after written informed consent.

Patient Data

This was a prospective open-labeled 2-armed clinical trial. Group-1 had 9 males and 1 female with mean age of 20.2 ± 6.9 years, mean disease duration 8.1 ± 3.4 years, mean fasting blood sugar (FBS) 269 ± 93.04 mg/dl, mean postprandial BS (PPBS) 372 ± 68.3 mg/dl, mean HbA1c $10.99 \pm 2.1\%$, mean S.C-peptide 0.22 ± 0.2 ng/ml and mean insulin requirement 63.9 ± 20.95 IU/day.

Group-2 had 6 males and 4 females with mean age of 19.7 ± 9.96 years, mean disease duration 9.9 ± 7.1 years, mean FBS 309.5 ± 67 mg/dl, mean PPBS 334.7 ± 72.1 mg/dl, mean HbA1c $11.93 \pm 1.9\%$, mean S.C-peptide 0.028 ± 0.01 ng/ml and mean insulin requirement 57.55 ± 21.82 IU/day.

Study Design (Figure 1)

Isolation of MSC from h-AD

Ten gram adipose tissue was resected from anterior abdominal wall under local anesthesia after making a small incision on left lateral side below umbilicus. Sutures were taken after hemostasis was secured. This adipose tissue was collected in self-designed proliferation medium comprising of Minimum Essential Medium with alpha modification (a-MEM) (Sigma, St. Louis, MO, USA), 20% human albumin (Reliance Life Sciences, Navi Mumbai, India), 5ng/ml Basic-Human Fibroblast Growth Factor (B-hFGF) (Sigma, USA), 1% Sodium pyruvate (Hi-media, India) and standard antibiotics which included 1% penicillin, streptomycin-cefotaxime solution (Hi-media, Mumbai, India) and anti-fungal fluconazol (1 mg/10ml) (Life sciences, Haryana, India). The adipose tissue was minced with knife into fine pieces with addition of collagenase type-1 (10 mg/ 10 ml) in 75cm² tissue culture dishes. After mincing the tissue, the culture dish was placed in an incubator at 37C with a shaker arranged at 35RPM (selfdesigned) for 1 hour for removal of extra-cellular matrix from the adipose tissue. Subsequently the contents were transferred to 15ml centrifuge tubes and centrifuged at 780RPM for 8min. Subsequently the supernatant and pellets were separately cultured in proliferation medium on 100 and 25cm² cell+ Plates (Sarstedt, Newton, NC, USA), respectively, at 37C with 5% CO₂ for 10 days. Medium was changed every other day without doing any passaging. On 10th day of the culture in proliferation medium, the tissue culture dishes containing cells were washed with Phosphate Buffered Saline (1N, PBS) and MSCs were harvested by means of trypsinization (0.25% Trypsin EDTA solution (Hi Media, India). Harvested MSCs were checked for viability using trypan blue, sterility (Bactec, Franklin Lakes, NJ, USA) and cellcounts in a modified Neubauer chamber. MSC characteristics were confirmed by flow cytometric analysis with CD45 (PerCP) negativity and CD90 (FITC)/ CD73 (PE) (Beckton Dickinson, Franklin Lakes, NJ, USA) positivity. Negative isotype controls were carried out by flow cytometric analysis to confirm the analysis. A small aliquot was also stained by hematoxylin and eosin stain for morphologic analysis.

Differentiation of h-AD-MSc into insulin secreting (IS) MSCs: Harvested MSCs were further subjected to differentiation to ISCs using differentiation medium comprising of Dulbecco's Modified Eagle's Medium (DMEM) (4,500 mg glucose/l) (Sigma, USA), DMEM: F-12, (1:1) (Sigma, USA), growth factors and serum supplements like nicotinamide (10 mM) (Hi-media, India), activin A (2 nM) (Sigma, USA), exendin (10 nM) (Sigma, USA), pentagastrin (10 nM) (Sigma, USA), Hepatocyte Growth Factor (HGF) (100 pM) (Sigma, USA), B-27 (2%) (Sigma, USA), N-2 (2%) (Sigma, USA) and antibiotics. No xenogenic material was used. The cells were kept in differentiation medium for 4 days in and then were subjected to isolation on FicolI Hypaque (density: 1.077 ± 0.007 g/ml; Osmolality: 290 ± 15 ml Osmol) (Invitrogen, Karlsruhe, Germany) by density gradient centrifugation for possible selective isolation of IS-MSc at the 1,000 RPM for 8 min. After centrifugation prepared cell pellet was diluted with Phosphate Buffered Saline (PBS) (1 N, Hi-media). Cellswere tested for sterility, viability and cell counts and subsequently subjected to immunofluorescence (IF) study for paired box genes-6 (Pax-6): key regulator for normal islet cell development, insulin promoter factor-1(Ipf-1) (in humans) or also known as pancreatic and duodenal homobox 1(Pdx-1): regulator of β -cell specific gene expression, function and for self-renewal of β progenitor cells (in animal), islet-1 transcriptional factor (Isl-1): the gene up-regulating expression of insulin. C-peptide and insulin secretion from cells were measured by a chemiluminescence assay (Lumax, Lake Forest, CA, USA). To check glucose responsiveness of the cells, they were first incubated in absence of glucose and then after addition of glucose. Insulin and C-peptide levels secreted by the cells were measured on both occasions by chemiluminescence assay. IF study for Pax-6, Isl-1 and Pdx-1 IF study for Pax-6, Ipf-1 (Pdx-1) and Isl-1 (genes responsible for insulin secretion) was carried out after cells were isolated.1

On day-10, 100 ml BM was aspirated from posterior superior iliac crest under local anesthesia (LA) and sedation after stimulation with granulocyte colony stimulating factor, 300 μ g subcutaneously 12 hourly for 2 days, on day-8 and 9, and subjected to culture for generation of HSC.

The prepared inoculum was then mixed with HSC. Co-infusion of IS-AD-MSc and BM-HSC was carried out on day-14, into superior mesenteric artery to portal route and brachiocephalic artery to thymic circulation via femoral artery catheterization under LA, and into abdominal subcutaneous tissue. Conditioning was done with Bortezomib, 1.3 mg/m² body surface area along with methylprednisone, 125 mg, on days 1, 4, 8 and 11 followed by rabbit anti-thymocyte globulin, 1.5 mg/kg BW on day 12 in both the groups.

Stem cell data

Generated ISCs expressed transcription factors ISL-1, PAX-6 and IPF-1 (Figure 1). In group-1, mean SC quantum infused was 103.14 ± 28.29 ml with ISC $2.65 \pm 0.8 \times 10^2$ / μ L, CD34+ $0.81 \pm 0.61\%$; CD34+ 0.81% and CD45-/90+/73+, $81.55 \pm 24.88\%$. In group-2, mean SC quantum infused was 95.33 ± 14.23 ml with mean ISC $2.07 \pm 0.67 \times 10^2$ / μ L, CD34+ $0.32 \pm 0.33\%$; CD45-/90+/73+, $61.6 \pm 24.99\%$.

Patient Monitoring

Patients were monitored 4 hourly for blood sugar levels for first 2 days after infusion. FBS and PPBS levels after lunch and dinner were monitored for the next 5 days and patients were

discharged at the end of 1 week. Subsequently patients were advised to monitor FBS and PPBS weekly for the first month, fortnightly for the next 2 months and monthly thereafter till the end of 1 year. Subsequently they were advised to check for FBS and PPBS every 3 months or as and when needed.

S-C-peptide and Hb1Ac were measured by chemiluminescence assay before infusion and 3 monthly after infusion. [Hb1Ac reference range: normal: 4.2-6.2%, good control: 5.5-6.8%, fair control: 6.8-7.6%, poor control: >8%] (Erba diagnostics, Germany)]. GAD antibodies were measured before infusion and 3 monthly post-infusion by ELISA technique (normal range: <10 IU/ ml) (Euroimmun -Medizinische Labordiagnostika AG, UK). Body weight and DKA episodes were also regularly monitored. Insulin administration was made on sliding scale with an objective of maintaining FBS \leq 150 mg/dl (reference range: 70-110 mg/dl) and PPBS \leq 200 mg/dl (reference range: 80-140 mg/dl).

Statistical Analysis (Table 1)

Statistical analysis was performed using SPSS version 12. Data are expressed as mean \pm SD (min-max) for continuous variables. Continuous variables were compared using Wilcoxon signed rank test. $p < 0.05$ was considered to be statistically significant. Insulin requirement, Hb1Ac, serum C-peptide levels, FBS and PPBS were monitored.

Results

No untoward effect, morbidity (pulmonary embolism, sepsis) or mortality due to SCT or conditioning was recorded in any patient. Variable and sustained improvement in mean FBS, PPBS, HbA1C and serum C-peptide was noted over a mean follow-up of 33.10 ± 18.38 months in group-1 and 54.24 ± 15.74 months in group-2. Mean GAD antibody decreased from 327.8 ± 652.0 to 107.2 ± 228.0 IU/ ml in group-1 and 722.5 ± 686.6 to 133.1 ± 216.9 IU/ml in group-2. Mean insulin requirement decreased from 63.9 ± 20.9 to 38.6 ± 8.5 IU/day in group-1 and 57.56 ± 21.82 to 40.5 ± 15.99 IU/day in group-2. No functional correlation was observed between exogenous insulin requirement with C. peptide levels and GAD antibody levels. Group-1 showed better response to achieve insulinopenic stage than in group-2 (Figure 2). There was an impressive absence of DKA episodes in all of them with improved subjective energy levels.

Table 1

	Group, n=10	Pre SCT	1 month	3 month	6 month	9 month	12 month	15 month	18 month	21 month	24 month
Insulin Requirement	1	63.9 \pm 2.09	60.1 \pm 0.36	55 \pm 19.8	49.7 \pm 9.41	44.3 \pm 5.47	38.44 \pm 4.56	37.66 \pm 2.15	38.87 \pm 0.49	38 \pm 11.43	39.66 \pm 9.37
	2	57.5 \pm 1.8	31.44 \pm 9.09	31.0 \pm 0.22	29.55 \pm 11.52	32.11 \pm 9.64	32.22 \pm 0.88	31.66 \pm 9.13	33.66 \pm 7.24	35.75 \pm 8.71	38.5 \pm 13.34
	P-value	0.526	0.001	0.004	0.014	0.058	0.319	0.253	0.247	0.672	0.858
HbA1c	1	10.9 \pm 2.15	10.6 \pm 0.99	10.2 \pm 0.01	9.72 \pm 0.66	9.35 \pm 0.63	9.03 \pm 0.65	8.56 \pm 0.65	8.51 \pm 0.4	8.4 \pm 0.87	7.75 \pm 1.05
	2	11.93 \pm 1.9	9.11 \pm 0.76	9.62 \pm 0.54	9.15 \pm 0.61	8.55 \pm 0.56	8.62 \pm 0.38	8.38 \pm 0.48	8.17 \pm 0.1	8.25 \pm 0.01	8.01 \pm 0.04
	P-value	0.328	0.230	0.244	0.464	0.294	0.575	0.813	0.590	0.765	0.651
C-peptide	1	0.22 \pm 0.21	0.36 \pm 0.29	0.43 \pm 0.32	0.53 \pm 0.32	0.61 \pm 0.29	0.63 \pm 0.23	0.71 \pm 0.22	0.79 \pm 0.24	0.84 \pm 0.22	0.93 \pm 0.24
	2	0.02 \pm 0.01	0.19 \pm 0.27	0.31 \pm 0.37	0.35 \pm 0.4	0.38 \pm 0.39	0.43 \pm 0.36	0.43 \pm 0.30	0.4 \pm 0.31	0.49 \pm 0.29	0.46 \pm 0.29
	P-value	0.020	0.20	0.483	0.292	0.172	0.196	0.039	0.013	0.022	0.008
Fasting Blood Sugar	1	269.6 \pm 93.04	260 \pm 8.39	248.9 \pm 81.8	237.8 \pm 80.56	225.2 \pm 81.31	210.22 \pm 80.65	198.4 \pm 6.94	208.6 \pm 6.821	217.14 \pm 68.72	218.0 \pm 7.245
	2	309.5 \pm 67.01	216.8 \pm 59.26	213.11 \pm 56.8	191.11 \pm 44.36	188.3 \pm 49.2	173.33 \pm 48.53	170.11 \pm 43.6	175.77 \pm 47.17	188 \pm 41.53	202.5 \pm 5.134
	P-value	0.285	0.218	0.289	0.142	0.255	0.256	0.315	0.261	0.331	0.646
Post-prandial Blood Sugar	1	372 \pm 68.3	362.2 \pm 64.66	346.7 \pm 65.64	333.6 \pm 69.51	316.0 \pm 59.88	301.33 \pm 60.0	289.77 \pm 62.08	291.12 \pm 44.8	267.14 \pm 61.02	270.0 \pm 5.338
	2	334.77 \pm 72.13	279.44 \pm 74.09	257.55 \pm 72.46	228.11 \pm 50.8	214.88 \pm 39.67	204.77 \pm 36.98	206.00 \pm 35.32	205.4 \pm 2.638	203.5 \pm 2.803	204.37 \pm 27.5
	P-value	0.264	0.018	0.011	0.001	0.0005	0.001	0.003	0.0002	0.034	0.010

Statistical analysis for mean insulin requirement, Hb1Ac, serum C-peptide levels, FBS and PPBS over 24-months follow-up of total 20 patients; 10 from autologous and 10 from allogenic stem cell therapy.

Figure 1. Paradigm of stem cell therapy (SCT) and showing generated ISCs expressed transcription factors ISL-1, PAX-6 and IPF-1

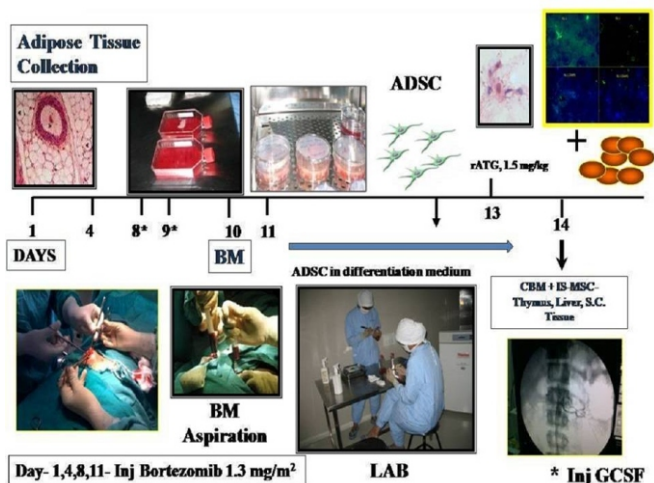


Figure 2. Comparison of autologous and allogenic stem cell Therapy (SCT) response to glycosylated hemoglobin, serum C-peptide, daily insulin requirement and fasting - post prandial blood sugar

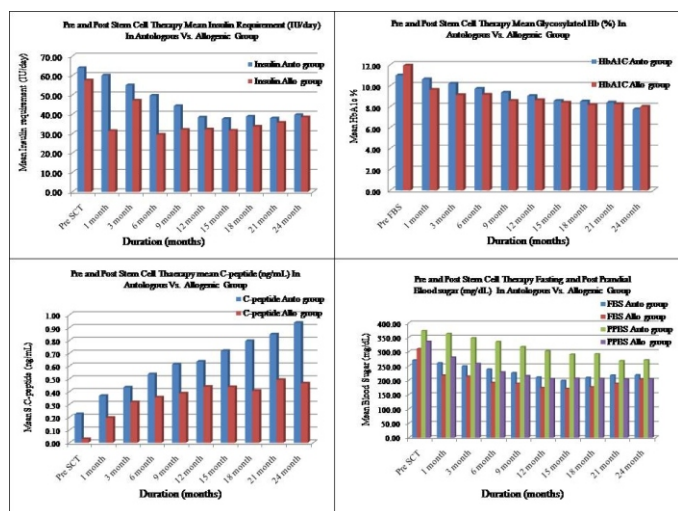


Figure 1. Paradigm of stem cell therapy (SCT) and showing generated ISCs expressed transcription factors ISL-1, PAX-6 and IPF-1

Discussion

Potential therapy for T1DM needs to address insulin-replacement and immune dys-regulation arising in these patients. Islet cell transplantation is a well-known therapeutic option yet not feasible due to the shortage of available organs. 9,10 Optional cell therapy includes HSC and MSC, especially since MSC have the plasticity to adopt to pancreatic endocrine phenotype and migrate to the sites of tissue injury. They are also potent immunomodulators.11,12,13,14,15

In animal models of T1DM, MSC have shown beneficial effects in glycemic control, either isolated16 or combined with HSC17.

We have generated MSC in vitro from human adipose tissue which qualify the definition standardized by the Mesenchymal and Tissue Stem Cell Committee of the International Society for

Cellular Therapy. 1 We further differentiated them to ISCs under defined culture conditions phenotypically identical to pancreatic β -cells. These cells expressed transcription factors ipf-1, pax-6, and isl-1. All three are central controlling genes capable of reprogramming non-pancreatic cells to surrogate β -cell functions. Again our technique is a shortcut to reprogramming non-pancreatic cells as compared to vector-based gene transfer techniques.1 Thymic infusion was carried out in our patients to achieve central tolerance18 and portal circulation was done to take the advantage of tolerogenicity of liver.19 Subcutaneous tissue being an immunologically privileged site, we decided to inject part of the cells in abdominal subcutaneous tissue, so that it will serve as a “back-up reservoir” for insulin supply.20

In our previous experience of using allogenic SCT we did have sustained partial response of decreased insulin requirement along with sustained elevated levels of S.C peptide.21 However we could not establish complete insulin-free status in our patients. One of the belief was that since the source of SC was allogenic, there was a possibility of these cells being rejected, since the major component of SC was HSC. We have never used any immunosuppression post-SCT. Hence we decided to carry out the present study to compare the effect of allogenic vs. autologous SCT.

In the present study, we have established that even if a patient has T1DM, IS-AD-MSC can be generated using the adipose tissue reservoir of SC. Secondly these cells have sustained effect of decreased exogenous insulin status in addition to sustained level of C-peptide. However we have still not been able to establish insulin-free status in this group of patients also.

Conclusion

This is the first report of successfully treating T1DM with co-infusion of autologous vs. allogenic IS-AD-MSC and BM-HSC with relatively simple and easy technique, offers a safe and viable approach. Autologous SC infusion shows better response in patients than allogenic SC infusion.

Abbreviations:

- BM: bone marrow
- BM-HSC: Bone marrow derived hematopoietic stem cells
- CBM: cultured bone marrow
- DKA: diabetic ketoacidosis
- FBS: fasting blood sugar levels
- GAD: glutamic acid decarboxylase
- Hb1Ac: glycosylated hemoglobin
- IPF-1: Insulin promoter factor 1
- IS-AD-MSC: Insulin secreting adipose tissue derived mesenchymal stem cells
- ISC: insulin-secreting cell
- ISL-1: Islet-1
- MSC: mesenchymal stem cells
- PPBS: postprandial blood sugar level
- PAX-6: Paired box gene 6
- SCs: Stem cells
- T1DM: type 1 diabetes mellitus

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We would like to state that none of the authors have any conflict of interest regarding this manuscript.

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