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Efficacy of Autologous Bone Marrow-Derived Mesenchymal Stem Cells and Mononuclear Cells Transplantation in Type 2 Diabetes Mellitus: A Randomized Placebo-Controlled Comparative Study

Short Running Title- Stem cells transplantation in T2DM

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Abstract

Drugs targeting β-cells have provided new options in the management of T2DM; however, their role in β-cell regeneration remains elusive. The recent emergence of cell-based therapies like autologous bone marrow-derived mesenchymal stem cells (ABM-MSCs) and mononuclear cells (ABM-MNCs) seems to offer a pragmatic approach to augment β-cell function/mass. This study aims to examine the efficacy and safety of ABM-MSCs and ABM-MNCs transplantation in T2DM and explores the alterations in glucose-insulin homeostasis by metabolic studies. Thirty patients of T2DM with duration of disease≥5yrs, receiving triple oral anti-diabetic drugs along with insulin (≥0.4IU per Kg per day) with HbA1c ≤7.5%(≤58.0mmol/mol) were randomized to receive ABM-MSCs or ABM-MNCs through targeted approach and a *sham* procedure (n=10 each). The primary end-point was a reduction in insulin requirement by ≥50% from baseline, while maintaining HbA1c <7.0% (<53.0mmol/mol) during one-year follow-up. Six out of 10 (60%) patients in both the ABM-MSCs and ABM-MNCs groups, but none in the control group achieved the primary end point. At 12 months, there was a significant reduction in insulin requirement in ABM-MSCs ($p<0.05$) and ABM-MNCs group ($p<0.05$) but not in controls ($p=0.447$). There was a significant increase in 2^{nd} phase C-peptide response during hyperglycemic clamp in ABM-MNCs ($p<0.05$) group, whereas a significant improvement in insulin sensitivity index ($p<0.05$) accompanied with an increase in IRS-1 gene expression was observed in the ABM-MSCs group. In conclusion, both ABM-MSCs and ABM-MNCs result in sustained reduction in insulin doses in T2DM. Improvement in insulin sensitivity with MSCs and increase in C-peptide response with MNCs provide newer insights in cell-based therapies.

Keywords- Stem cells, T2DM, β-cells, Insulin sensitivity

Introduction

Despite advances in understanding the pathophysiology of T2DM and consequent development of plethora of new anti-diabetic medications, drugs targeting β-cell function and/or mass are still wanting. Intensive insulin therapy (IIT) [1], thiazolidinedione [2], dipeptidyl peptidase4 inhibitors (DPP4i) [3] and glucagon-like peptide1 (GLP-1) agonists [4] have exhibited glycemic durability, if used in early stages of disease. IIT is usually not accepted by the patients at diagnosis of diabetes, and is associated with weight gain and hypoglycemia, whereas weight gain, increased risk of heart failure and atypical fractures are accompanied with use of thiazolidinedione. DPP4i and GLP1 agonists fail to sustain glycemic durability with increasing duration of disease and are associated with gastrointestinal intolerance, pancreatitis and C-cell hyperplasia [5].

β-cell failure is progressive and inexorable with advancing duration of diabetes. Therefore, targeting β-cells through pancreatic/islet transplantation and novel β-cells regenerative therapies like stem cells seems to be a pragmatic approach[6,7]. Limitations of pancreatic/islet transplantation include restricted availability of cadaveric pancreata, progressive decline in insulin independence, graft rejection and immunosuppression-associated complications [8]. Therefore, interest is growing in stem cells that possess homing-in, differentiation and transdifferentiation properties.

Bone marrow is enriched with mononuclear cells (MNCs), hematopoietic stem cells (HSCs) and a few mesenchymal stem cells (MSCs) [9,10]. Several studies have demonstrated that use of bone marrow derived-MNCs transplantation in patients with T2DM resulted in significant decrease in insulin requirement, though C-peptide response was variable [11,12]. MSCs are multipotent stem cells that have ability to differentiate into variety of cell types, thereby making these cells an attractive therapeutic tool for cell transplantation. Jiang et al. studied the efficacy of placenta-derived MSCs (PD-MSCs) in patients with long-standing T2DM and showed a significant increase in C-peptide by 40% with achievement of ≥50% reduction in insulin requirement. However, the heterogeneity in glycemic control at inclusion, lack of a placebo arm and short follow-up were limitation of this study [13]. Recently, a study demonstrated the dose-response relationship of allogenic bone marrow–derived mesenchymal precursor cells in patients with T2DM. The higher the dose of MSCs, greater was the reduction in HbA1c level at all time points as compared to placebo. However, this study had a follow-up of only 3 months[14].

Although a study in alloxan-induced diabetes in rats compared the efficacy of MNCs and MSCs transplantation and showed that MNCs transplantation was associated with a significant decrease in fasting plasma glucose and greater number of differentiated insulin producing cells as compared to MSCs [15]. No human studies have compared the efficacy and safety of these different autologous bone marrow-derived stem cells in patients with T2DM. Further, while homeostatic model assessment (HOMA) based measures are used to assess the efficacy of therapeutic interventions in patients with diabetes, these tools are recognized to have limitations as longitudinal measures of insulin sensitivity/β-cell function in response to treatment modalities [16] and until now, metabolic studies have not been performed to elucidate the mechanism of reduction in insulin doses with these cell-based therapies.

This prospective, randomized, single-blinded, placebo-controlled study was designed to compare the efficacy and safety of autologous bone marrow-derived mesenchymal stem cell (ABM-MSCs) and mononuclear cells (ABM-MNCs) in patients with T2DM and examines the alterations in glucose-insulin indices by metabolic studies.

Materials and Methods

Study Design

Seventy patients were screened at out-patient department of the Postgraduate Institute of Medical Education and Research, Chandigarh, India. Informed consent was obtained from the study subjects, Stem Cell Ethics Committee of the institute approved the study and trial was registered at Clinicaltrials.gov (ID number, NCT01759823). The inclusion criteria were: patients of either sex with T2DM, aged between 30 and 60years with duration of diabetes ≥5 years, and failure to achieve HbA1c ≤7.5% (≤58.0mmol/mol), while receiving triple oral anti-diabetic drugs in optimal doses along with insulin (≥0.4 IU per Kg per day) for the last 6 months. Prior to randomization, patients were on stable doses of vildagliptin, metformin, pioglitazone and insulin (≥0.4 IU per Kg per day) during run-in-period for at least 3 months with HbA1c ≤7.5%(≤58.0mmol/mol). Patients with T1DM, glutamate decarboxylase-65 seropositive, abnormal liver and renal function tests, active infections, malignancy, or acute coronary syndrome in the past 3 months were excluded. The study was randomized, blinded for intervention, placebo controlled trial evaluating the efficacy and safety of ABM-MSCs and ABM-MNCs in patients with T2DM. Out of 40 patients, 30 were randomized into three groups in the ratio of 1:1:1 by random allocation software; ABM-MSCs group (Group I), ABM-MNCs

group (Group II) and the Control group (Group III)].

Baseline evaluation

All subjects underwent clinical and biochemical assessment regarding glycemic control and for micro-, and macrovascular complications.

Efficacy Studies

A) Hyperglycemic Clamp Study

Subjects were requested to refrain from vigorous exercise, and anti-diabetic medications were omitted 24h prior to the procedure. Patients reported at 0630h after an overnight fast of 10h for hyperglycemic clamp study[17]. Dextrose solution (20%) was rapidly infused intravenously to increase the plasma glucose level to the target level (15.5mmol/L) for 180min. Blood samples for glucose and C-peptide were drawn at -5, 2, 4, 6, 8, 10, 30, 60, 120, 140, 160, and 180 min relative to the beginning of dextrose infusion. 1st phase C-peptide (nmol/L) response was calculated as the area under the curve from 2 to 10 min and 2nd phase C-peptide response as the AUC from 120 to 180 min during the hyperglycemic clamp. An insulin sensitivity index (ISI) was calculated by dividing the steady state (140–180 min) of average glucose infusion rate (μmoles per Kg body weight per min) by the average insulin concentration (pmol/L) [18-20]. C-peptide and plasma insulin was estimated by electrochemiluminescence immunoassay (Elecsys 2010, Roche, Mannheim, Germany) and HbA1c (Bio-Rad D-10 system, Hercules, CA, USA).

B) Glucagon Stimulated C-peptide

The test was performed in fasting state after intravenous (IV) administration of 1mg glucagon, and blood samples were drawn at −15, 0, and 6 min after injection. The homeostatic model assessment (HOMA) of insulin resistance (HOMA-IR), β cell function (HOMA-β) and insulin sensitivity (HOMA-S) were used to assess these indices [21].

C) *Gene expression analysis of Glucose Transporter Type 4 (GLUT-4) and Insulin Receptor Substrate-1 (IRS-1)*

Skeletal muscle biopsy tissue sample was obtained from vastus lateralis under local anesthesia from the study patients. Total cellular RNA was extracted from the cryopreserved skeletal muscle tissue by TRIZOL method (Invitrogen, USA) and cDNA was synthesized using the RevertAid First strand cDNA Synthesis kit (Fermentas Life Sciences, USA) according to manufacturer's instructions. Real-time PCR was performed by 7500 Real Time PCR System SYBR Green I master detection

(Applied Biosystems, USA) method to analyze the gene expression of GLUT-4 and IRS-1 in skeletal muscle tissue. 18S ribosomal RNA was used as an endogenous control. The primer sequence of IRS-1 were (from 5' to 3') CTTCTGTCAGGTGTCCATCC and CTCTGCAGCAATGCCTGTTC; for GLUT-4, CCTGCCAGAAAGAGTCTGAAGC and ATCCTTCAGCTCAGCCAGCA and for 18S rRNA, AACGGCTACCACATCCAAG and CGTCCCAAGATCCAACTAC. The analysis of the relative gene expression data was done by the $2^{-\Delta CT}$ method to produce the data as fold change up-or-down regulation [22].

Preparation of ABM-MSCs

Approximately 100 ml of autologous bone marrow was aspirated from the posterior superior iliac spine under local anesthesia following aseptic precautions from the patients in Group I. MNCs were suspended in α-minimum essential medium (α-MEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% pooled human platelet lysate (PHPL). After attaining 80% confluency, cells were trypsinized and expanded in T-225 flasks. These cells were expanded up to 4-5 passages to obtain targeted cell numbers (1 million cells per Kg body weight) for infusion. Cultured media were aspirated and tested for aerobic and anaerobic (Bactec, BD Bioscience), fungal and mycoplasma infection before the infusion. Cell viability was tested by using trypan blue (Sigma Aldrich). Cells were stained with antibodies conjugated with fluorescent markers for characterization of mesenchymal stem cells like anti-CD 73-PE, CD90-PE, CD105-FITC, CD34-PE and CD45-FITC and analyzed by 4-colour flow cytometer (FACS Calibur, BD Bioscience, USA) [23].

Preparation of ABM-MNCs

Approximately 200-250 ml of autologous bone marrow was aspirated from the posterior superior iliac spine under local anesthesia following aseptic precautions. The mononuclear cells (MNCs) were separated by centrifugation after layering on density-gradient medium (Ficoll-Hypaque, Sigma- Aldrich, St. Louis, MO, USA), and were washed using phosphate-buffered saline (PBS; Himedia Laboratories Private Limited, Mumbai, India) and resuspended in normal saline with a final product volume of 8–10 ml. Aliquots (1 ml) were taken for MNCs count, viability testing by trypan blue (Sigma-Aldrich) dye exclusion test and for phenotyping: cells were stained with antibodies conjugated with fluorescent markers like anti-CD34-PE and CD45-FITC and acquired and analyzed onto 4-colour flow cytometer (FACS Calibur, BD

Bioscience). Five milliliters of final wash supernatant was used for sterility testing (aerobic and anaerobic culture, Bactec, BD Bioscience) [11,24].

Stem Cells Transplantation

The procedure of stem cell transplantation has been published earlier [11,24]. Briefly, a 5F catheter (Sim1, Beacon®, USA) was selectively navigated through transfemoral route into the celiac trunk. Within the Sim1 catheter, another caliber catheter (Progreat microcatheter, Terumo, Japan) was selectively advanced into superior pancreatico-duodenal (SPD) artery and cells were injected accordingly unless anatomical variations in vasculature were noted. MSCs were infused at a dose of one million cells per Kg body weight [13], whereas MNCs at a dose of approximately one billion cells per patients [11,24] as reported in our previous studies.

Control Group (Group III)

Patients in Group III underwent a *sham* procedure, which has been described previously [11]. Approximately 20ml of bone marrow was aspirated and after 5h of marrow aspiration, 10 ml of diluted vitamin B complex was injected through transfemoral route into the femoral artery under sterile precautions in the same ambience. An acellular vehicle was preferred as a control to eliminate the "placebo–like effect", if any, of these cell-based therapies

Follow-up

All patients were followed up every 2 weeks for the first month, monthly for the next 3 months, and at three monthly intervals thereafter. Lifestyle modification advice was reinforced during each visit to all the patients. Self-monitoring of blood glucose (SMBG) was advised at least 5 points per week and at the time of suspected hypoglycemia. Fasting plasma glucose (FPG) levels were targeted between 5.0 and 7.2mmol/L, postmeal glucose levels <9.9mmol/L and HbA1c <7.0% (<53.0 mmol/mol). Insulin doses were tapered whenever the patient reported hypoglycemic episodes or when the FPG was <3.8mmol/L and a postmeal glucose <6.6mmol/L, without any alterations in oral anti-diabetic drugs. Hyperglycemic clamp study and muscle biopsy were repeated after 6 months and glucagon-stimulated C-peptide test was performed at 3, 6 and 12 months. All patients were followed for a period of 12 months.

Outcomes

The primary end point was a reduction in insulin requirement by ≥50%, while maintaining HbA1c <7.0% (<53.0mmol/mol) [11], and the secondary end points included change in weight, HbA1c, metabolic indices including C-peptide and insulin sensitivity as compared to the baseline.

Safety Study

All patients underwent whole body fluorine 18-fluorodeoxyglucose positron emission tomography-computed tomography (^{18}F-FDG PET-CT) examination after completion of the study to look for any untoward event.

Statistical Analysis

All the data are expressed as median and interquartile range. Baseline and post treatment data within the groups were compared using Friedman's test with post hoc Wilcoxon's signed rank test (p value corrected using Bonferroni procedure). Data between the groups were analyzed using the Kruskal Wallis test for continuous variables and Fisher exact tests for categorical variables. Spearman's rank correlation test was used to identify an association between two variables. Generalized linear model was applied for adjustment of baseline variables. The p value <0.05 was considered significant. Statistical analysis was carried out using the SPSS version 22 for window (SPSS Inc., Chicago, USA).

Results

All recruited patients completed the study except one in ABM-MSCs group who was lost to follow-up after 6 months of stem cells transplantation (Figure 1).

ABM-MSCs Group (Group I)

Baseline clinical and biochemical characteristics of the study patients are summarized in the Table 1. All patients had neuropathy, four had nonproliferative diabetic retinopathy (NPDR), two had proliferative diabetic retinopathy (PDR) and three had microproteinuria. All patients had hypertension, and one had coronary artery disease. The median volume of the bone marrow harvested for culturing of MSCs was 110.0 (92.5–137.3) ml, which yielded 83.5 (72.0–91.5) X 10^6 MSCs (Table S1). More than 90% of MSCs expressed CD73+, CD90+ and CD105+, and were negative for CD34 and CD45 (Figure S1).

In 9 out of 10 patients (90%), MSCs were injected into superior pancreatico-

duodenal (SPD) artery, except in one in whom MSCs were injected into the splenic artery due to anatomical malformation. Post-procedure and follow-up study period was uneventful.

A total of six (60%) patients achieved the primary end point (responders) over a mean duration of 5.5(2.8 to 6.0) months, while maintaining HbA1c <7.0% (<53.0 mmol/mol) till the end of the study. There was a 52% reduction in insulin dose in the first 6 months of SCT ($p<0.05$) followed by a 58% reduction in the next 3 months ($p<0.05$) and finally by 54% at 12 months ($p<0.05$) (Table 2). There was a non-significant decrease in HbA1c from 6.9% (52.0mmol/mol) to 6.4% (46.0mmol/mol) at 12 months (Table 2). The glucagon stimulated C-peptide modestly increased from 0.7 to 0.8 nmol/L at 12 months, however, there was no significant change in HOMA-IR, HOMA-β and HOMA-S at the end of the study (Table 2)

Hyperglycemic clamp, a measure to assess β-cell function, showed that C-peptide response at baseline and AUC of 1st phase were insignificant before and after SCT; whereas AUC of 2nd phase C-peptide response was significantly lower after SCT as compared to baseline ($p<0.05$) (Table 2). Further, generalized linear model analysis showed significant improvement in the insulin sensitivity index (ISI) at the end of the study ($p<0.05$) (Table 2).

On sub-group analysis, patients in the responder group (n=6) had a significant weight loss -2.8Kg (-5.1 to -2.4) ($p<0.05$) at the achievement of the primary end point and they continued to maintain the weight deficit -5.4Kg (-6.0 to -4.0) ($p<0.05$) even at one year. However, weight loss neither correlated with reduction in insulin requirement ($r=0.530$, $p=0.280$) or improvement in the ISI ($r=0.771$,$p=0.072$) in these patients.

Skeletal muscle biopsy was performed in 7 patients. GLUT4 and IRS1 mRNA expression in the skeletal muscle was higher but insignificant after SCT as compared to baseline 0.0 (0.0–1.1) to 0.4 (0.0–1.0) ($p=0.866$) and 0.0 (0.0–2.3) to 1.3 (0.4–3.7) ($p=0.128$), respectively.

ABM-MNCs Group (Group II)

Baseline clinical and biochemical characteristics of the study patients are summarized in Table 1. All patients had neuropathy, two had nonproliferative diabetic retinopathy and four had microproteinuria. Seven patients had hypertension, and one had coronary artery disease. The median volume of the bone marrow harvested for transplantation was 223.5(209.3-227.0) ml, which yielded 1.1(1.0–1.4)X10^9 MNCs

(Table S1) and 1.8(1.4-2.2)X10^7 of these MNCs expressed CD34+.

In 9 out of 10 patients (90%), MNCs were injected into SPD artery, except in one in whom stem cells were injected into the splenic artery due to non-visualization of SPD artery.

After SCT, 6 patients (60%) achieved the primary end point (responders) over a mean duration of 3.5(3.0 to 4.8) months, while maintaining HbA1c <7.0% (<53.0 mmol/mol) till the end of the study period. There was a decrease in the total daily insulin requirement by 51% at 6 months ($p<0.05$) and maintained at the same dose at 9 ($p<0.05$) and at 12 months ($p<0.05$) (Table 3). There was an insignificant increase in HbA1c from 6.7% (50.0mmol/mol) to 7.0% (53.0mmol/mol) at 12 months (Table 3). The glucagon stimulated C-peptide significantly increased from 0.7 to 1.1 nmol/L at 12 months ($p<0.05$) (Table 3). In a sub-group analysis, patients in the responder group (n=6) had insignificant weight loss -0.7Kg (-2.7 to 1.3) (p=0.463) at the achievement of the primary end point and it remained insignificant even at one year -2.4Kg (-3.3 to -0.8) (p=0.173) and did not correlate with reduction in insulin requirement (r=0.551, p=0.257). There was no significant alteration in HOMA-IR, HOMA- β and HOMA-S at the end of the study (Table 3).

The β-cell function by hyperglycemic clamp study at baseline and AUC of 1st phase C-peptide response were insignificantly before and after SCT; whereas AUC of 2nd phase C-peptide response was significantly increased after MNCs transplantation as compared to baseline ($p<0.05$) (Table 3). Further, generalized linear model analysis showed a nonsignificant decrease in ISI at the end of the study (p=0.066) (Table 3).

Five patients underwent muscle biopsy. GLUT4 and IRS1 mRNA expression in the skeletal muscle was lower but insignificant after stem cells treatment as compared to baseline 0.5 (0.2–2.1) to 0.2 (0.2–4.5) (p=0.892) and 0.1 (0.1–2.4) to 0.0 (0.0–1.1) (p=0.666).

Control Group (Group III)

Baseline clinical and biochemical characteristics of the study patients are summarized in the Table 1. All patients had neuropathy, five subjects had microproteinuria, two had nonproliferative diabetic retinopathy and one had macroproteinuria. Nine patients had hypertension.

After the "*sham procedure*", insulin requirement decreased by 10% at the end of the study. However, none of the patients could achieve ≥ 50% reduction in insulin requirement, while maintaining HbA1c <7% ("nonresponders"). The decrease in

insulin doses was significant at 6 months ($p<0.05$), however it could not be sustained at 9 and 12 months (Table 4). There was a modest but non-significant weight gain at 9 and 12 months compared to the baseline (Table 5). The HbA1c decreased from 6.5% (47.5mmol/mol) to 6.2% (43.0mmol/mol) without any significant alterations in HOMA-IR, HOMA-β, HOMA-S and stimulated C-peptide at 12 months (Table 4).

The β-cell function as assessed by hyperglycemic clamp study displayed insignificant alterations in basal, AUC 1st phase and 2nd phase C-peptide response and ISI at the end of the study (Table 4).

Seven patients underwent muscle biopsy. GLUT4 mRNA expression in the skeletal muscle was lower but insignificant [0.6 (0.2–1.6) to 0.2 (0.0–2.5) ($p=0.866$)] after *sham* treatment as compared to baseline. Further, IRS1 mRNA expression in skeletal muscle was significantly lower after *sham* treatment as compared to baseline [0.6 (0.3–1.5) to 0.1 (0.0–0.4) ($p<0.05$)].

Comparison among the groups

All the groups were comparable at baseline with respect to clinical and biochemical parameters (Table 1). On comparing between the groups at 6 and 12 months, the changes in clinical parameters were not statistically significant. The Δ change in insulin dose was significantly more in the ABM-MNCs group compared to the controls at 6 months ($p<0.05$) as well as at 12 months ($p<0.05$). However, there was no significant Δ change in insulin dose in ABM-MSCs group as compared to ABM-MNCs and control group at 6 and at 12 months. The Δ change in HbA1c was not significantly different between the groups (Table 5). In hyperglycemic clamp, the increase in Δ change in AUC of 2nd phase C-peptide response in ABM-MNCs group was significantly higher as compared to ABM-MSCs ($p<0.05$), while Δ change in ISI significantly improved in ABM-MSCs as compared to ABM-MNCs ($p<0.05$) and control groups ($p<0.05$) (Table S2). On gene expression analysis at 6 months, Δ change in GLUT4 gene expression showed a rising trend but not statistically significant in ABM-MSCs group as compared to others. However, Δ change in IRS1 gene expression was statistically significant in ABM-MSCs as compared to ABM-MNCs ($p<0.05$) group.

Adverse events

Two patients had nausea and vomiting following glucagon administration, which was used for the assessment of β-cell reserve. One patient had local extravasation of blood following the targeted approached through the transfemoral

route for the administration of stem cells, which subsided after a week time. None of the patients developed major hypoglycemia, however, average incidence of minor hypoglycemic episodes were 19, 15 and 9 in ABM-MSCs, ABM-MNCs and control groups, respectively, throughout the study period (Table–2,3,4). Further, whole body ^{18}F-FDG PET-CT did not reveal any abnormality till the end of the study.

Discussion

This study demonstrates that both ABM-MSCs and ABM-MNCs transplantation resulted in reduction in exogenous insulin requirement by ≥ 50%, while maintaining HbA1c <7.0% (<53.0mmol/mol) in almost two-third of the patients. This was accompanied by significant improvement in C-peptide response in the MNCs group and insulin sensitivity in MSCs group on hyperglycemic clamp study. No adverse events were noted during one-year follow-up. To our knowledge this is the first study to compare the efficacy and safety of ABM-MSCs and ABM-MNCs transplantation in patients with T2DM and explores the alterations in glucose-insulin homeostasis by metabolic studies.

A very few studies have examined the utility of ABM-SCT for the treatment of T2DM in humans. Estrada et al. [12] and Wang et al. [25] showed that combined use of ABM-SCT and hyperbaric oxygen therapy into the dorsal pancreatic artery resulted in reduction in fasting plasma glucose and insulin doses, and an increase in C-peptide levels. However, these studies had certain limitations including lack of placebo arm, heterogeneity in study population at inclusion regarding anti-diabetic medication and the majority of patients were lost to follow-up. A recent study showed that the ABM-MNCs treatment could achieve significant reduction in HbA1c with a decrease in oral hypoglycemic drugs and insulin doses compared to intensive insulin therapy [26]. In our previous study, we showed ≥50% reduction in the insulin requirement in 75% of patients with a significant reduction in HbA1c and increase in stimulated C-peptide level after ABM-MNCs therapy, which could be maintained for at least 15 months in almost two third of the patients[24,27]. Another study from our center and the present study also confirm the previous results with the use of ABM-MNCs [11].

There are animal studies showing beneficial effects of MSCs on glucose-insulin homeostasis. Streptozotocin-induced diabetic rats showed a reduction in blood glucose after allogenic MSCs transplantation and transdifferentiation/fusion of MSCs into insulin-producing cells [28,29]. Other studies have shown this effect to be short-

lived [15]. A recent study demonstrated that MSCs transplantation in early phase of diabetes in a rat model resulted in enhanced insulin secretion, increase in islets number with higher number of insulin positive cells and improved insulin sensitivity, whereas administration in late phase of disease only improved insulin sensitivity by upregulation of GLUT-4 and IRS-1 expression in insulin target tissues [30]. Although there are a few studies that have explored the usefulness of MSCs in patients with T2DM showing a significant decrease in insulin requirement and increase in C-peptide [13,14], however, these were limited by lack of placebo arm and short duration of follow-up. The present study showed reduction in insulin doses in 60% of patients with the use of ABM-MSCs and these results could be sustained up to one year.

Our study was intentionally designed to enroll patients with HbA1c ≤7.5% (≤ 58.0mmol/mol) in order to mitigate the effect of glucotoxicity on β-cell function and insulin sensitivity, thereby providing an exclusive opportunity to examine the effect of stem cells on these indices. Further, hyperglycemia per se has been shown to have detrimental effect on functionality and survival of stem cells [31]. Although there were no differences in terms of reduction in insulin requirement and achievement of primary end-point between MSCs and MNCs groups, our study provides newer insights into their action. We showed that administration of MSCs was associated with improvement in insulin sensitivity index, thereby resulting in reduction in insulin doses. Furthermore, the adaptive decrease in AUC of 2^{nd} phase C-peptide response is a surrogate evidence of improvement in insulin sensitivity. In addition, enhancement of IRS-1 expression in patients treated with MSCs suggests that insulin sensitivity may improve through IRS-1-dependent mechanism. On the contrary, the ABM-MNCs administration was associated with augmented C-peptide response on clamp study and this was further supported by significant increase in glucagon-stimulated C-peptide response. Reduction in insulin doses in the early phases in the control group can be attributed to placebo-like effect, as weight loss of 1.0-3.0 Kg and regular insistence on life-style modification was uniformly observed in all the groups. However, the decrease in insulin requirement could not be sustained during later half of the study and none of the patients could achieve the primary endpoint. These observations, though a small sample size, suggest that "cocktail" therapy by combining MNCs and MSCs together may be useful in targeting both the defects and could translate into better outcome in terms of reduction in insulin doses and glycemic

durability.

Various investigators including us, have used variable doses of stem cells in their clinical studies and it was shown that response was greater with higher doses [11,13,14,24,27,32] going up to 200-400ml [12,26,33]. However, the cell count was not mentioned in these studies. One study used MSCs in dosage varying from 0.3 $X10^6$ to 2 $X10^6$ per Kg body weight and showed better response with higher doses [14]. We have used higher doses for MNCs as compared to MSCs, because bone marrow-derived MNCs consist of heterogeneous mixtures of cells including hematopoietic stem cells (CD34+,1%), MSCs and endothelial progenitor cells, whereas MSCs were expanded in-vitro and it consisted of >90% of purified cellular mass. Both these dose schedules in our study elicited favorable clinical response that were sustained up to one year.

Approximately 5-10% of weight loss has been shown to be associated with improvement in glycemic profile, lipids and effective control of blood pressure [34]. There was a significant weight loss (-7.0%) in patients who achieved the primary end point after ABM-MSCs transplantation and it was accompanied with a reduction in insulin doses. This can be attributed to improvement in insulin sensitivity as demonstrated by increase in insulin sensitivity index. However, weight loss did not correlate with decline in the insulin doses as well as with insulin sensitivity index in these patients. Therefore, reduction in insulin doses may be a direct effect of ABM-MSCs on insulin sensitivity index rather than mediated by weight loss. On the contrary, patients in the ABM-MNCs group also had a weight loss (-2.9%) but it was insignificant; however, there was a significant reduction in insulin requirement in the responders group which can be attributed to beneficial effects of ABM-MNCs on β-cell function as evidenced by significant improvement in C-peptide response on hyperglycemic clamp. There was also a significant reduction in the insulin requirement for a short duration in the control group without any significant weight loss and this may be attributed to placebo-like effect and initial enthusiasm to adapt life-style modification.

The proposed mechanism/s for the improvement in β-cell function/mass following stem cells transplantation have been examined in animal studies, as it is difficult to perform morphometric studies in human subjects. These include i) regeneration of endothelial progenitor cells and promotion of angiogenesis in the damaged islets by the bone marrow-derived hematopoietic stem cell ii) pancreatic and duodenal

homeobox 1 (PDX-1) up regulation, thereby enhancing islet differentiation, iii) direct differentiation of MSCs into pancreatic endocrine cells, iv) improvement in insulin signaling transduction by up regulation of GLUT4 expression and IRS-1 in insulin target tissues, (V) and rarely, trans-differentiation into β-cells [11,30,35]**.** However, these mechanisms are further to be explored for better understanding of stem cells action.

The strengths of our study include presence of a placebo arm, head to head comparison between ABM-MSCs and ABM-MNCs and use of metabolic clamp studies to assess the β-cell function. The limitations include small sample size (given the intensive nature of the study), short duration of follow-up and limited skeletal muscle tissue samples for Western blot to validate our data at protein levels.

In conclusion, both ABM-MSCs and ABM-MNCs therapies in T2DM result in significant decreases in insulin dose requirement accompanied with improvement in insulin sensitivity and β-cells function, respectively. Larger numbers of patients with a longer duration of follow-up are required to determine the place of cell-based therapies as "standard of care" for the management of T2DM.

Acknowledgements

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Competing interests

The authors declare that they have no competing interests.

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Legends-

Figure 1- Schema of the study. Seventy patients were screened, 30 eligible patients were randomized, and were divided into three groups. All patients underwent complete follow-up and analysis excluding one patient lost to follow-up in Group I after 6 months.

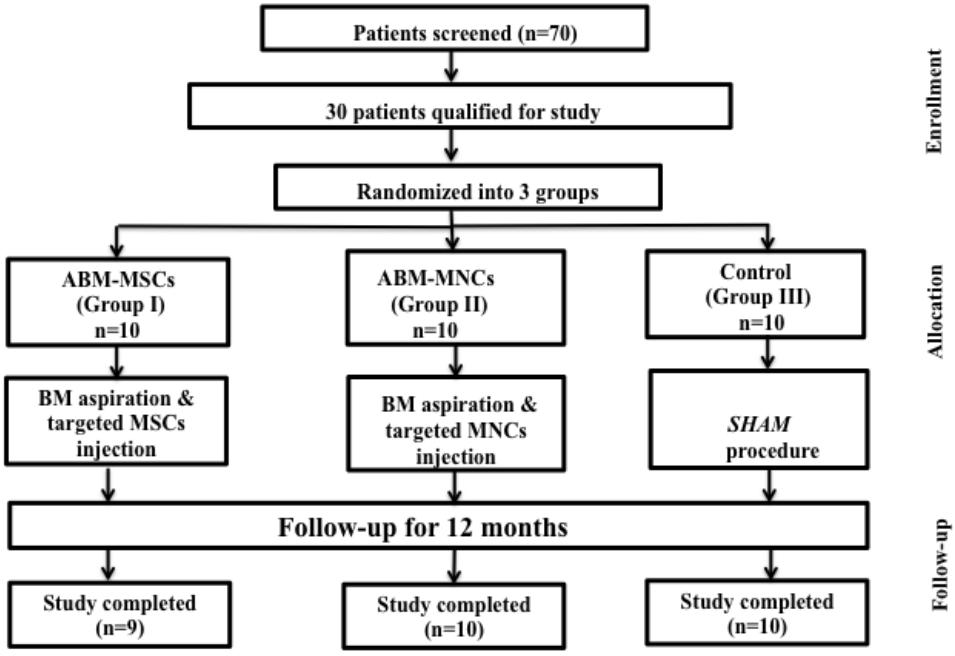

Figure 1- Schema of the study. Seventy patients were screened, 30 eligible patients were randomized, and were divided into three groups. All patients underwent complete follow-up and analysis excluding one patient lost to follow-up in Group I after 6 months.

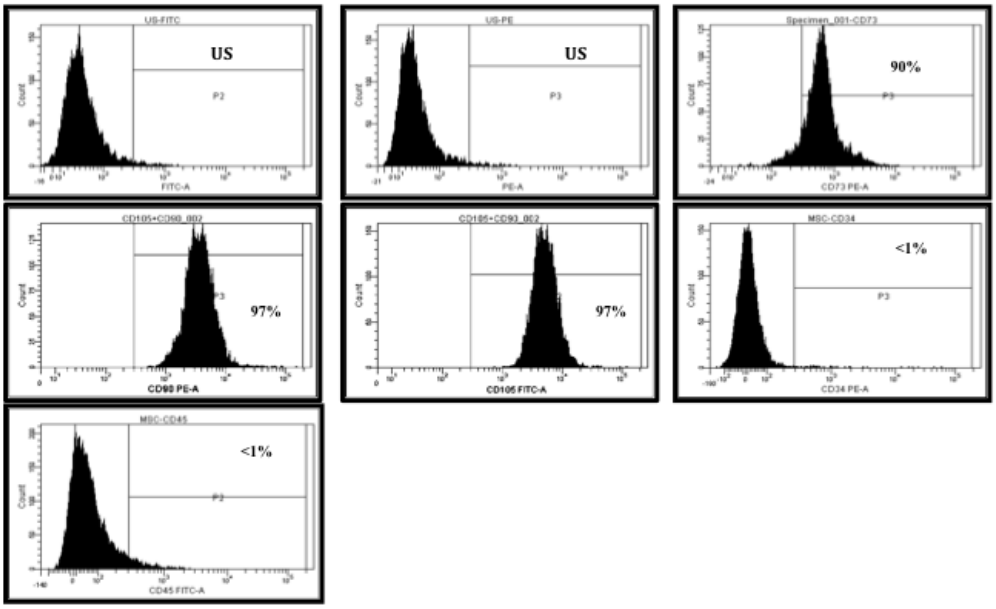

Figure S1 - Immunophenotype of autologous bone marrow-derived mesenchymal stem cells (ABM-MSCs). The ABM-MSCs were isolated from bone marrow, cultured and expanded in GMP Laboratory for 4 passages, and analyzed by FACS. ABM-MSCs were positive for the cell surface markers CD73, CD90 and CD105 and negative for the markers CD45 and CD34.

Table 1 - Baseline clinical and biochemical parameters of study groups

Parameters	ABM-MSCs (n=10)	ABM-MNCs (n=10)	Controls (n=10)	P value
Age (years)	50.5 (36.0-58.0)	44.5(39.5-49.8)	53.5 (43.3-58.8)	0.456
Sex (M: F)	8:2	7:3	6:4	
Duration of Diabetes (Years)	15.0 (8.0-22.8)	13.0 (8.5-15.0)	14.0 (9.0-15.0)	0.586
Duration of insulin therapy (Years)	6.5 (2.5-11.0)	5.0 (3.0-6.5)	2.0 (1.1-5.3)	0.121
Weight (Kg)	81.5 (70.2-91.3)	80.8 (68.1-85.6)	69.3 (64.9-72.9)	0.265
BMI (kg/m^2)	28.1 (26.5-31.6)	28.9 (25.0-30.2)	25.7 (24.5-28.9)	0.533
FPG (mmol/L)	5.8 (5.3-6.2)	6.3(5.5-6.4)	6.0(5.5-6.7)	0.600
Insulin requirement (IU/day)	47.5(34.0-52.3)	68.0(50.5-84.3)	48.5(29.5-76.0)	0.198
HbA1c [%(mmol/mol)]	6.9(6.6-7.0) [52.0(47.8-53.0)]	6.7(6.4 -7.3) [50.0(45.5-57.3)]	6.5(6.2-6.8) [47.5(42.3-52.0)]	0.514
Fasting C-pep (nmol/L)	0.4(0.3-0.4)	0.5(0.4-0.6)	0.5(0.4-0.7)	0.177
Stimulated C-pep (nm/L)	0.7(0.6-0.8)	0.7(0.5-1.1)	0.9(0.7-1.3)	0.226
HOMA-IR (%)	0.8(0.8-0.9)	1.1(0.9-1.3)	1.2(0.9- 1.7)	0.145
HOMA-β (%)	70.0(60.3-80.2)	50.4 (42.8-75.0)	79.7(59.7-97.3)	0.687
HOMA-S (%)	120.7(100.1-132.0)	88.6(74.7-112.1)	84.1(60.0-103.7)	0.145

All values are expressed as median and interquartile range

Table 2- Comparison of clinical and biochemical parameters in ABM-MSCs (Group I) treated group during follow-up

Parameters	Baseline (n=10)	3 months (n=10	6 months (n=10)	9 months (n=9)	12 months (n=9)	P value 3 months	P value 6 months	P value 9 months	P value 12 months
Weight (Kg)	81.5(70.2-91.3)	80.6(65.9-89.6)	79.8(64.6-88.0)	75.4(64.1-88.2)	74.0(64.1-88.8)	1.000	0.139	0.209	0.091
Insulin requirement (IU/day)	47.5(34.0-52.3)	31.5(14.0-36.0)	24.5(19.5-32.5)	22.0(12.0-28.0)	24.0(12.0-33.0)	0.046*	0.022*	0.017*	0.021*
HbA1c [%(mmol/mol)]	6.9(6.6-7.0) [52.0(47.8-53.0)]	6.9(6.4-7.3) [51.5(44.0-57.0)]	6.8(6.1-7.2) [51.0(40.8-56.3)]	6.6(5.9-7.5) [49.0(40.5-58.0)]	6.4(6.0-7.1) [46.0(40.5-54.0)]	0.522	0.441	0.441	0.284
BMI (kg/m^2)	28.1(26.5-31.6)	28.0(25.0-30.4)	27.5(24.5-30.0)	27.0(24.2-30.8)	26.5(23.8-30.5)	1.000	0.139	0.209	0.091
FPG (mmol/L)	5.8 (5.3-6.2)	5.8(5.6-5.6)	6.1(5.6-6.4)	6.4(5.5-6.7)	6.7(6.4-6.7)	0.161	0.314	0.514	0.128
Fasting C-pep (nmol/L)	0.4(0.3-0.4)	0.5(0.4-0.6)	0.5(0.5-0.7)	0.4(0.3-0.5)	0.4(0.4-0.5)	0.110	0.188	0.767	0.515
Stimulated C-pep (nmol/L)	0.7(0.6-0.8)	0.6(0.5-1.0)	0.9(0.6-1.1)	-	0.8(0.8-1.1)	0.508	0.074	-	0.110
HOMA-IR (%)	0.8(0.8-0.9)	1.1(0.8-1.2)	1.2(1.0-1.3)	0.9(0.7-1.3)	0.9 (0.8-1.3)	0.139	0.188	0.515	0.374
HOMA-β (%)	70.0(60.3-80.2)	75.4(64.1-82.6)	65.6(63.5-73.3)	66.8(58.2-78.6)	61.8(49.3-77.4)	0.508	0.646	0.953	0.441
HOMA-S (%)	120.7(100.1-132.0)	89.8(78.0-118.8)	80.2(76.6-91.3)	117.0(78.8-137.1)	105.8(76.8-125.1)	0.139	0.093	0.767	0.441
Hypoglycemic episodes / patient /month	2.0(0.3-2.8)	8.0(4.3-13.8)	1.5(0.0-4.5)	0.0(0.0-0.0)	0.0(0.0-0.1)	0.091	1.000	1.000	1.000
Hyperglycemic Clamp Data									
Fasting C-pep (nmol/L)	0.6(0.5-0.7)		0.6(0.5-0.6)				1.000		

AUC of 1st – Phase C-peptide (nmol/L)	**4.7(3.9-5.3)**		**4.4(3.6-5.2)**				**0.241**		
AUC of 2nd – Phase C-peptide (nmol/L)	**67.7(49.3-90.3)**		**56.2(37.4-61.8)**				**0.013***		
ISI μmoles. kg^{-1}. min^{-1} / pmol L^{-1}	**0.1(0.1-0.5)**		**0.4(0.2-0.6)**				**<0.001***		

All values are expressed as median and interquartile range.

- Significant difference from baseline.

Table 3- Comparison of clinical and biochemical parameters in ABM-MNCs (Group II) treated group during follow-up

Parameters	Baseline (n=10)	3 months (n=10)	6 months (n=10)	9 months (n=10)	12 months (n=10)	P value 3 months	P value 6 months	P value 9 months	P value 12 months
Weight (Kg)	80.8(68.1-85.6)	79.5(68.1-85.6)	78.3(69.4-84.5)	77.3(67.1-84.3)	75.2(67.4-85.2)	0.260	0.153	0.028*	0.123
Insulin requirement (IU/day)	68.0(50.5-84.3)	38.0(24.0-47.3)	34.0(22.5-41.5)*	39.0(22.5-41.5)	38.0(24.3-41.5)	0.089	0.0018*	0.001*	0.007*
HbA1c [%(mmol/mol)]	6.7 (6.4 -7.3) [50.0(45.5-57.3)]	6.5(6.3-6.7) [48.0(44.8-50.3)]	6.8 (6.6-6.9) [51.0(48.8-53.5)]	7.0(6.7-7.5) [53.0(49.0-56.3)]	7.0(6.7-7.5) [53.0(49.0-56.3)]	0.759	0.574	0.475	0.444
BMI (kg/m^2)	28.9(25.0-30.2)	27.9(25.3-30.3)	28.0(25.5-29.4)	27.9(24.7-29.5)	27.9(23.9-30.4)	0.260	0.169	0.028*	0.139
FPG (mmol/L))	6.3(5.5-6.4)	6.7(6.0-6.7)	6.7(6.3-6.7)	6.2(5.5-6.6)	6.5(6.2-6.7)	0.260	0.260	0.906	0.050*
Fasting C-pep (nm/L)	0.5(0.4-0.6)	0.4(0.3-0.6)	0.4(0.3-0.6)	0.5(0.3-0.8)	0.7(0.4-1.1)	1.000	0.109	1.000	0.339
Stimulated C-pep (nmol/L)	0.7(0.5-1.1)	0.7(0.5-0.8)	0.7(0.5-1.0)	-	1.1(0.7-1.7)	1.000	1.000	-	0.001*
HOMA-IR (%)	1.1(0.9-1.3)	1.0(0.7-1.3)	0.9(0.7-1.5)	1.1(0.6-1.8)	1.5(0.9-2.5)	0.799	0.721	0.799	0.088
HOMA-β (%)	50.4(42.8-75.0)	54.3(46.5-78.5)	50.4(42.8-75.0)	74.8(56.0-91.1)	86.2(53.7-101.3)	0.386	0.445	0.575	0.646
HOMA-S (%)	88.6(74.7-112.1)	99.1(76.9-200.8)	108.3(67.3-144.6)	90.2(55.8-281.5)	70.6(39.8-115.9)	0.799	0.721	0.455	0.093
Hypoglycemic episodes / patient /month	1.8 (0.0-3.8)	10.0(5.8-14.5)	1.5(0.0-3.8)	1.0(0.0-2.0)	0.0(0.0-0.8)	0.024*	1.000	1.000	1.000

Hyperglycemic Clamp Data									
Fasting C-pep (nmol/L)	**0.6(0.5-0.8)**		**0.6(0.5-0.9)**				**0.320**		
AUC of 1st – Phase C-peptide (nmol/L)	**4.1(2.8-5.4)**		**4.6(2.9-6.4)**				**0.385**		
AUC of 2nd – Phase C-peptide (nmol/L)	**52.6(32.2-74.6)**		**74.0(39.2-86.3)**				**0.017***		
ISI µmoles. kg^{-1}. min^{-1} / pmol L^{-1}	**0.2(0.1-0.6)**		**0.2(0.1-0.3)**				**0.066**		

All values are expressed as median and interquartile range.

*Significant difference from baseline.

Table 4- Comparison of clinical and biochemical parameters in control group (Group III) during follow-up

Parameters	Baseline (n=10)	3 months (n=10)	6 months (n=10)	9months (n=10)	12 months (n=10)	P value 3 months	P value 6 months	P value 9 months	P value 12 months
Weight (Kg)	69.3(64.9-72.9)	67.8(64.4-72.8)	69.3(63.1-75.6)	72.3(65.0-74.5)	71.0(66.5-74.1)	0.240	0.959	0.508	0.342
Insulin Requirement (IU/day)	48.5(29.5-76.0)	45.0(26.3-59.0)	45.0(25.3-57.0)	45.0(28.0-64.5)	45.0(26.3-67.5)	0.133	0.030*	0.226	0.447
HbA1c [%(mmol/mol)]	6.5(6.2-6.8) [47.5(42.3-52.0)]	6.3(5.9-6.3) [43.5(40.0-45.3)]	6.2(6.0-6.2) [44.0(41.5-45.3)]	6.3(6.0-6.8) [44.5(41.0-56.0)]	6.1(6.0-6.8) [43.0(41.3-47.3)]	0.202	0.262	0.798	0.284
BMI (kg/m^2)	25.7(24.5-28.9)	25.2(23.9-30.2)	25.7(24.0-28.8)	25.7(24.6-29.8)	25.6(24.7-29.3)	0.285	0.959	0.646	0.374
FPG (mmol/L))	6.0(5.5-6.7)	6.0(5.7-6.5)	5.6(5.2-6.6)	6.0(5.6-6.5)	6.3(5.5-6.7)	0.906	0.397	0.610	0.678
Fasting C-pep (nmol/L)	0.5(0.4-0.7)	0.8(0.6-1.0)	0.6(0.5-0.8)	0.7(0.5-0.8)	0.7(0.4-0.9)	0.188	0.066	0.333	0.386
Stimulated C-pep (nmol/L)	0.9(0.7-1.3)	1.1(0.8-1.2)	1.0(0.8-1.4)	-	0.9(0.8-1.2)	0.646	0.241	-	0.838
HOMA-IR (%)	1.2(0.9- 1.7)	1.9(1.4-2.2)	1.3(1.0-1.9)	1.5(1.2-1.8)	1.7(0.9-2.1)	0.074	0.093	0.333	0.241
HOMA-β (%)	79.7(59.7-97.3)	93.8(81.9-116.6)	87.5(74.6-100.9)	79.1(69.5-108.9)	88.9(54.2-120.9)	0.241	0.086	0.445	0.333
HOMA-S (%)	84.1(60.0-103.7)	53.4(46.6-70.6)	75.2(51.1-92.6)	66.8(55.5-84.6)	60.0(48.1-112.7)	0.093	0.173	0.508	0.386
Hypoglycemic episodes / patient/month	1.1(0.0-1.8)	2.5(1.0-5.5)	0.0(0.0-1.8)	0.0(0.0-0.8)	0.0(0.0-2.3)	0.068	0.671	0.391	0.666

Hyperglycemic Clamp Data									
Fasting C-pep (nmol/L)	**0.6(0.5-0.8)**		**0.7(0.6-1.1)**				**0.121**		
AUC of 1st –Phase C-peptide (nmol/L)	**4.6(3.4-6.8)**		**5(4.2-7.9)**				**0.720**		
AUC of 2nd – Phase C-peptide (nmol/L)	**64.6(54.7-82.1)**		**63.8(55.3-95.8)**				**0.878**		
ISI µmoles. kg^{-1}. min^{-1} / pmol L^{-1}	**0.2(0.1-0.4)**		**0.2(0.1-0.3)**				**0.455**		

All values are expressed as median and interquartile range.

*Significant difference from baseline.

Table 5- Comparison of parameters among the groups during the study period at 12 months

Parameters	ABM-MSCs (n=9)	ABM-MNCs (n=10)	Controls (n=10)	P value ABM-MSCs v/s Controls	P value ABM-MNCs v/s Controls	P value ABM-MSCs v/s ABM-MNCs
Primary End Point Achieved, n (%)	6 (60%)	6 (60%)	-	0.011*	0.011*	1.000
Off-insulin n (%)	-	1(10%)	-	-	-	-
Δ Insulin requirement (IU/day)	-20.0 (-28.0 to -16.0)	-34.0(-42.8 to -26.5)	-5.5(-16.0 to -2.5)	0.910	0.006*	0.144
ΔHbA1c [%(mmol/mol)]	-0.2(-0.7 to 0.2) [-3.0(-11.5 to 2.5)]	0.5(-0.6 to 0.9) [5.5(-8.0 to 8.8)]	-0.4(-0.8 to 0.3) [-5.0(-10.3 to 3.5)]	0.315	0.353	0.133
ΔFasting C-pep (nmol/L)	0.0 (-0.1 to 0.1)	0.1(0.0 to 0.3)	0.1(-0.2 to 0.2)	0.968	0.481	0.182
ΔStimulated C-pep (nmol/L)	0.0(-0.1 to 0.1)	0.2(0.1 to 0.3)	0.0(-0.3 to 0.2)	0.400	0.105	0.356

+ Increase from baseline. −Decrease from baseline. All values are expressed as median and interquartile range.

Table S1- Details of stem cell therapy procedure

Parameters	ABM-MSCs (n=10)	ABM-MNCs (n=10)
Volume of bone marrow aspirated (mL)	110.0(92.5-137.3)	223.5(209.3-227.0)
Aspiration		
Unilateral	9	8
Bilateral	1	2
Total Cell Count	83.5 (72.0-91.5) X 10^6	1.1 (1.0-1.4) X 10^9
Procedure Time (min)	45.0(35.5-50.0)	40.0(31.3-44.8)
Artery injected		
Superior pancreaticoduodenal	9	9
Splenic	1	1

All values are expressed as median and interquartile range

Table S2- Comparison of parameters between the groups during hyperglycemic clamp study

Parameters	ABM-MSCs (n=10)	ABM-MNCs (n=10)	Controls (n=10)	P value ABM-MSCs v/s Controls	P value ABM-MNCs v/s Controls	P value ABM-MSCs v/s ABM-MNCs
ΔFasting C-pep (nmol/L)	0.0 (0.0 to 0.1)	0.1(-0.1 to0.2)	0.1 (0.0 to 0.3)	0.351	0.853	0.280
ΔAUC of 1st – Phase C-peptide (nmol/L)	-0.1 (-0.8 to 0.4)	0.1(-0.5 to 2.1)	0.0(-0.7to 1.3)	0.393	0.684	0.280
Δ AUC of 2nd – Phase C-peptide (nmol/L)	-14.8 (-27.3 to -3.5)	6.9 (2.8 to 23.1)	0.6(-8.7to 10.9)	0.023*	0.105	0.0001*
Δ ISI µmoles. kg^{-1}. min^{-1} / pmol L^{-1}	0.1 (0.0to 0.3)	-0.1 (-0.2to 0.0)	0.0 (-0.1 to 0.0)	0.009*	0.592	0.009*

+ Increase from baseline. −Decrease from baseline. All values are expressed as median and interquartile range.

*Significant difference among the groups

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