Adipose tissue-derived stromal/stem cells transplantation + cholecalciferol in recent-onset type 1 diabetes patients: twelve months follow up.

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Abstract

**OBJECTIVE:** To evaluate safety and therapeutic effect along 12 months of allogenic adipose tissue-derived stromal/stem cells (ASCs) transplantation+cholecalciferol(VITD) in patients with recent-onset type 1 diabetes (T1D).

**METHODS:** Prospective, phase II, open trial, pilot study in which patients with recent onset T1D received ASCs(1Kgx10^6 cells) and VITD 2000UI/day for 12 months(group 1) and were compared to controls with standard insulin therapy (group 2). Adverse events, C-peptide area under the curve(CP AUC), insulin dose, HbA1c and frequency of CD4^+ FoxP3^+ T-cells were evaluated at baseline(T0), after 3(T3), 6(T6) and 12 months(T12).

**RESULTS:** 11 patients completed the 12 months follow up (7:group 1;4:group 2). Group 1 had lower insulin requirement at T3(0.24+/-.018vs0.53+/-.023UI/kg,p=0.04), T6(0.24+/-.015vs0.66+/-.33 UI/kg,p=0.04) and T12(0.39+/-.15vs0.74+/-.29 UI/Kg,p=0.04), HbA1c was lower at T6(6.7+/-.079vs8.75+/-.95%,p=0.01), without significant differences at T12(7.3+/-.11 in group 1 vs 8.90+/-.33 in group 2,p=0.16). CPAUC was not significantly different at T0(p=0.07), higher in group 1 at T3(p=0.04) and T6(p=0.006), but similar at T12(p=0.23).Six patients (85.7%) in group 1 were in partial clinical remission(CR) at T6 vs none in group 2,p=0.01.4 remained in remission until 12 months. Patients with partial CR exhibited higher FOX P3 expression in CD4^+ lymphocytes at T6 and T12(p=0.004 and p=0.02, respectively). VITD levels were higher in patients that underwent partial CR at T6. One patient has a recurrence of a benign teratoma that was surgically removed, not associated to the intervention was observed in a patient from group 1.

**CONCLUSIONS:** ASCs+VITD without immunosuppression was safe and associated lower insulin requirements, a better glycemic control and a transient better pancreatic function in recent onset T1D, but the potential benefits were not sustained.

**Trial registration:** ClinicalTrials.gov NCT03920397

Background

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by progressive destruction of pancreatic β-cells, which leads to a decrease in insulin production and hyperglycemia. Life-long insulin treatment is essential for the survival of these patients and to avoid renal, ophthalmologic and neurologic complications, but is associated with an increased risk of hypoglycemia and reduced quality of life (1). Residual pancreatic function has been associated with better glycemic control, lower frequency of hypoglycemia and chronic complications (2, 3). Pancreatic insulin production may be indirectly evaluated by measurement of serum stimulated C peptide (CP), which is considered a clinically relevant end point in type 1 diabetes prevention trials (4).

Immunosuppressants, antigen-based therapies, pancreatic islet cell and stem cell transplantation have been tried to cure T1D, with variable and transient results in most studies (5). Autologous non-myeloablative hematopoietic stem cell transplantation associated with immunosuppression has shown promising outcomes. However potential adverse events are significant, such as oligospermia and infections, which limits the use in clinical practice (6, 7). Mesenchymal stromal/stem cells (MSC) transplantation have been used in clinical trials for a number of autoimmune diseases (8–13). These cells seems promising due to their intrinsic regenerative capacity and immunomodulatory properties and do not require immunosuppression, even for allogenic cell sources since it does not express co-stimulatory molecules to T cells (14, 15). MSC may achieve arrest of autoimmune β-cells destruction and generate functional β-cells in vitro and in animal models (16, 17). A recent metanalysis evaluated the potential benefit of preserving CP secretion after hematopoietic stem cells, mesenchymal stem cells or umbilical cord blood transplantation (18). A better pancreatic function was obtained with all except umbilical cord blood infusion (18). Neither of the studies that were included used Adipose tissue-derived stromal/stem cells (ASC) as source of MSC without immunosuppression.

Vitamin D (VIT D) has immunomodulatory properties. In vitro and in vivo studies have shown that, inhibits lymphocyte proliferation, cellular autoimmune pathways and stimulates T regulatory response (19, 20). However, results with vitamin D supplementation for patients with T1D are conflicting, with both positive and neutral effects (21–24). A metanalysis with 7 randomized interventional trials with VIT D supplementation observed a positive effect on insulin dose, in basal and stimulated CP (25).

Since the autoimmune process in T1D is complex, an ideal approach to cure the disease will probably include a combination of agents with different mechanism of action to act in multiple points of the immune process. The therapy also has to be safe, have the lowest possible toxicity and long-lasting benefits. The aim of this pilot study was to evaluate the safety and efficacy of a combination of Adipose tissue-derived stromal/stem cells transplantation and VITD, in recent-onset type 1 diabetes patient along 12 months.

Material And Methods

Patient selection and study design

This was a pilot prospective, single center, open trial in which patients with recent onset T1D received one dose of intravenous allogenic ASC and oral VIT D 2000UI/day for 12 months. The sample was selected by convenience. Participants signed an informed consent. The study was approved by the Institutional Review Board (17488313.1.0000.5257, Hospital Universitário Clementino Fraga Filho and registered at ClinicalTrial.gov (NCT03920397). Inclusion criteria were: T1D diagnosis according to American Diabetes Association (ADA) criteria for less than 4 months; age between 16 and 35 years and a positive glutamic acid decarboxylase antibody (GADA). Malignancy, infections, pregnancy, breastfeeding, renal dysfunction, use of immunosuppressors or glucocorticoids and diabetic ketoacidosis at T1D onset were exclusion criteria.

Patients were divided in two groups: Group 1 received ASC infusion + VIT D 2000UI/day (ASC + VIT D) supplementation for 12 months, and group 2 received standard treatment for type 1 diabetes (controls). Standard treatment included NPH insulin or basal insulin analog (glargine) and rapid insulin analog (aspart
or lispro or glulisine), according to ADA recommendation (26). Both groups had insulin adjustments performed by the same care team according to glucose monitoring in each visit. All patients received the same diabetes education, nutritional recommendations and help with management from health care providers. All volunteers received individual face-to-face consultation sessions, which included individualized diet prescriptions based on ADA current recommendations (dietary energy content of 45–55% carbohydrates, 15–20% protein, and 25–35% total fat, ≤7% saturated fatty acids (SFA), 5–15% monounsaturated fatty acids (MUFA); ≤10% polyunsaturated fatty acids (PUFA), and 30–50% total fiber intake and advice on food selection and carbohydrate counting method (27). Exercise recommendation was similar in both groups, according to ADA (28).

**Lipoaspirate human samples and ASC culture**

Adipose tissue samples were obtained through liposuction from three healthy females. Donor's serology testing was negative for syphilis, Chagas disease, Hepatitis B and C, HIV and HTLV. All three donors had Cytomegalovirus IgG positive with negative polymerase chain reaction (PCR) in blood samples and ASCs.

ASCs were isolated, cultured and characterized as previously described (25). Samples were processed at the Core Cell Technology facility of Pontifícia Universidade Católica do Paraná. Briefly, 100 ml of adipose tissue was washed in sterile phosphate-buffered saline (PBS) (Gibco Invitrogen). A one-step digestion by 1 mg/ml collagenase type I (Invitrogen) was performed for 30 minutes at 37°C during permanent shaking, followed by a filtration step through a 100 µm mesh filter (BD Falcon, BD Biosciences Discovery Labware). The cell suspension was centrifuged at 800g for 10 minutes, and erythrocytes were removed through a lysis buffer with pH 7.3. The remaining cells were washed at 400g for 10 minutes and then cultured at a density of 1x10^5 cells/cm² in T75 culture flasks and DEMEM-F12 (Gibco Invitrogen) supplemented with 10% of fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The culture medium was replaced three days after seeding, and then twice a week. ASCs were subcultured after reaching 80% confluence, with 0.5% trypsin/EDTA (Invitrogen) solution. Cells were related at a density of 4x10^3 cells/cm² for expansion (25).

Quality control of cell suspension sterility was evaluated by tests to detect bacteria and fungi (Bact / Alert 3D, Biornerieux), endotoxins (Endosafe™ PTS, Charles River) and Mycoplasma (KIT MycoAlert™ PLUS Mycoplasma Detection, Lonza). Cell viability was performed by flow cytometry using the vital dye 7-AAD (7-Aminoactinomycin D – BD#559925) to determine the percentage of viable cells and Annexin V protein (BD#S1-65875X) to determine the percentage of cells in apoptosis. Cytogenetic analysis was performed using the GTG-banding method.

Cells were phenotypically characterized by flow cytometry before the clinical application, using the following monoclonal antibodies: FITC-labeled CD14 (BD#555397), CD45 (BD#555482), CD19 (BD#555412), CD44 (BD#555478); PE-labeled CD73 (BD#550257), CD90 (BD#555956), CD166 (BD#559263), PerCP-labeled HLA-DR (BD#551375); APC-labeled CD34 (BD#555824), CD105 (BD#562408), CD29 (BD#559883) all purchased from BD (Pharmingen). At least 100,000 events were acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences), and data were analyzed using FlowJo 10 (TreeStar) software and presented in Table 1 (31).

![Table 1](image.png)

<table>
<thead>
<tr>
<th>Marker</th>
<th>CD 105</th>
<th>CD 73</th>
<th>CD 90</th>
<th>CD 29</th>
<th>CD 166</th>
<th>CD 44</th>
<th>CD 36</th>
<th>CD 14</th>
<th>CD 34</th>
<th>CD 45</th>
<th>CD 19</th>
<th>HLA-DR</th>
<th>CD 31</th>
<th>CD 106</th>
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<tbody>
<tr>
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<td>51.16%</td>
<td>49.66%</td>
<td>47.45%</td>
<td>46.09%</td>
<td>12.80%</td>
<td>5.92%</td>
<td>0.71%</td>
<td>0.71%</td>
<td>0.50%</td>
<td>0.57%</td>
<td>2.39%</td>
<td>3.77%</td>
<td></td>
</tr>
<tr>
<td><strong>DP</strong></td>
<td>0.055</td>
<td>0.415</td>
<td>0.434</td>
<td>0.442</td>
<td>0.467</td>
<td>0.395</td>
<td>0.131</td>
<td>0.038</td>
<td>0.005</td>
<td>0.007</td>
<td>0.003</td>
<td>0.005</td>
<td>0.002</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Legend: FITC-labeled CD14 (BD#555397), CD45 (BD#555482), CD19 (BD#555412), CD44 (BD#555478); PE-labeled CD73 (BD#550257), CD90 (BD#555956), CD166 (BD#559263), PerCP-labeled HLA-DR (BD#551375); APC-labeled CD34 (BD#555824), CD105 (BD#562408), CD29 (BD#559883) all purchased from BD (Pharmingen). At least 100,000 events were acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences), and data were analyzed using FlowJo 10 (TreeStar) software11A.

**ASC infusion**

On the day of infusion, the ASC monolayer were dissociated as described above, and 1x10^6 cells/kg of the recipient patient were resuspended in 5 ml of saline solution with 50% albumin and 5% ACD (Anticoagulant Citrate Dextrose Solution). The cell suspension was sent to the hospital in a cooler with recycled ice.

Patients that received ASCs were admitted to hospital on the day of the infusion and discharged 24 hours after infusion. A single dose of ASCs was infused in a peripheral upper arm vein for 15–20 minutes. Patients started taking oral cholecalciferol 2000 UI one day after the infusion of ASCs.

**Clinical and pancreatic function evaluation**

In the first visit (T0), patients were interviewed and underwent a physical exam. Weight, height, body mass index (BMI), blood pressure, heart frequency, and insulin dose/kg of body weight were evaluated at T0 and after 3 (T3), 6 (T6) and 12 (T12) months. Insulin dose adjustments were performed at each visit as necessary by the same the same care team according to glucose monitoring, and the number of severe hypoglycemia episodes were recorded, defined by glucose lower than 70 mg/dl and altered mental and/or physical functioning that requires assistance from another person for recovery (29). Patients received nutritional guidance according to ADA recommendations in each visit (27). Blood samples were drawn at T0, T3, T6 and T12 for the following measurements: HbA1c (High Performance Liquid Chromatography by boronate affinity), blood count and biochemistry analysis, 25 (OH) vitamin D (automated CMD 800 iX1), GADA and tyrosine phosphatase (IA2) (ELISA assay, Euroimmun brand and Molecular Devices Spectra max reader) and CP (Microparticle Chemiluminescent Immunoassay, Architect Abbott) before and after 30, 60, 90 and 120 minutes after liquid mixed meal (Glucerna®). The area under the curve (AUC) for CP was calculated. Adverse events were recorded during hospitalization and at each follow-up visit. Partial clinical remission (CR) was considered when insulin daily doses were lower than 0.5ui/kg and HbA1c < 7.5%, as described elsewhere (30).
Flow cytometry

Mononuclear cells were isolated from peripheral blood samples by density centrifugation on Ficoll (Ficoll-Paque, GE Healthcare) and stained as described previously (31). The following fluorochrome-conjugated anti-human monoclonal antibodies were used: CD45RA-PE (Clone HI100), CD3 PE-CF594 (Clone UCHT1), CD4-PerCP CY5.5 (Clone RPA-T4) and CD8-APC7 (Clone SK1), with anti-human FoxP3 – Alexa 647 (Clone 259 D / C7) (BD Biosciences, Franklin Lakes, NJ, USA). Cells were washed with PBS and followed the intracellular FoxP3 staining protocol, according to manufacturer’s recommendations (BD Biosciences). Data were analyzed using FACSDiva 6.0 software. A forward scatter gate on lymphocytes (FSC) was defined versus side scatter (SSC) dot plot, followed by gating on CD45RA+CD3+ lymphocytes, followed by gating on CD4+ or CD8+ cells. Finally, a gate was set to determine the percentage of FoxP3+ cells among CD45+CD3+CD4+ cells or CD45+CD3+CD8+ cells, as previously described (31). T-cells were evaluated on blood samples before (T0), three (T3), six (T6) and twelve (T12) months after ASCs infusion.

Statistical analysis

The primary outcomes were changes in insulin daily dose, HbA1c, CP AUC and 12 months adverse events. The sample size was established by convenience sampling. Descriptive statistics was used to summarize patients’ characteristics. Data are expressed as mean ± standard deviation. Chi square and Mann Whitney tests were used to compare categorical and continuous variables between groups, respectively. Wilcoxon test was used to compare results at baseline and after follow-up in each group. Spearman test was used to investigate correlation between continuous variables. Statistical tests are based on a 2-sided significance level of 0.05. SPSS software, version 21.0 was used for statistical analyses.

Results

Sixteen patients were interviewed, and three were excluded (one used glucocorticoid, pulmonary tuberculosis and another had renal dysfunction). One patient from each group requested to not continue in the study after three months (Flow chart - Figure 1). In this pilot study, seven patients were included in group 1 (ASC + VIT D) and four in the standard treatment (controls- group 2), with 25.71± 5.79 and 21.75±3.3 years old (p = 0.23), respectively. Patients’ individual characteristics are described in Table 2. Biochemical and metabolic characteristics before and after intervention are reported in Table 3. Age, gender, glycemic control and insulin dose were similar between groups before intervention (p = 0.23, 1.0,0.97, and 0.23, respectively). No patients received vitamin D supplementation before the study. Baseline vitamin D levels were similar in both groups (p = 0.164), and higher in group 1 after intervention (Table 3). One patient in each group used NPH insulin, all others used glargine as basal insulin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age years (Gender)</th>
<th>Ethnicity</th>
<th>BMI</th>
<th>T1D</th>
<th>ASCs</th>
<th>Ins T0</th>
<th>HbA1c T0</th>
<th>CP AUC T0</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>26 (M)</td>
<td>W</td>
<td>26.06</td>
<td>4</td>
<td>78x10^6</td>
<td>0.84</td>
<td>9.90</td>
<td>104.85</td>
</tr>
<tr>
<td>G1</td>
<td>35 (M)</td>
<td>NW</td>
<td>25.91</td>
<td>4</td>
<td>74x10^6</td>
<td>0.17</td>
<td>8.0</td>
<td>148.95</td>
</tr>
<tr>
<td>G1</td>
<td>28 (M)</td>
<td>W</td>
<td>23.38</td>
<td>2</td>
<td>65x10^6</td>
<td>0.21</td>
<td>6.30</td>
<td>340.50</td>
</tr>
<tr>
<td>G1</td>
<td>23 (F)</td>
<td>NW</td>
<td>20.76</td>
<td>1.7</td>
<td>60x10^6</td>
<td>0.15</td>
<td>7.90</td>
<td>178.95</td>
</tr>
<tr>
<td>G1</td>
<td>16 (F)</td>
<td>W</td>
<td>20.96</td>
<td>3.5</td>
<td>55x10^6</td>
<td>0.47</td>
<td>6.90</td>
<td>318.75</td>
</tr>
<tr>
<td>G1</td>
<td>28 (F)</td>
<td>W</td>
<td>23.71</td>
<td>2</td>
<td>69x10^6</td>
<td>0.25</td>
<td>7.60</td>
<td>233.70</td>
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<tr>
<td>G1</td>
<td>24 (M)</td>
<td>W</td>
<td>26.06</td>
<td>4</td>
<td>66x10^6</td>
<td>0.30</td>
<td>7.40</td>
<td>153.00</td>
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<tr>
<td>G2</td>
<td>20 (F)</td>
<td>NW</td>
<td>23.71</td>
<td>3</td>
<td>—</td>
<td>0.92</td>
<td>6.80</td>
<td>90.30</td>
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<tr>
<td>G2</td>
<td>18 (M)</td>
<td>NW</td>
<td>18.20</td>
<td>3</td>
<td>—</td>
<td>0.60</td>
<td>6.90</td>
<td>102.75</td>
</tr>
<tr>
<td>G2</td>
<td>25 (F)</td>
<td>W</td>
<td>20.60</td>
<td>3</td>
<td>—</td>
<td>0.20</td>
<td>7.70</td>
<td>160.2</td>
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<tr>
<td>G2</td>
<td>24 (M)</td>
<td>W</td>
<td>19.30</td>
<td>3</td>
<td>—</td>
<td>0.50</td>
<td>10.10</td>
<td>113.25</td>
</tr>
</tbody>
</table>

Table 2: Characteristics of the study group

Note: Body mass index (BMI) expressed as kilogram of body weight/centimeter^2 height was measured at the time of the transplant. ASCs number of cells was calculated according to patient weightx10^6. Abbreviations: G1, patient that received ASCs + VITD or Group 1; G2, control or Group 2; M, Male, F, Female W, white; NW, non-white; BMI, Body Mass Index; T1D, Type 1 Diabetes duration in months; ASC, Adipose tissue-derived stromal/stem cells; T0, basal period; Ins, insulin dose (IU/Kg); HbA1C, glycated hemoglobin (%); CPAUC, the area under the curve of C-Peptide (ng/ml).
ASC were approved by cytogenetic quality control for therapeutic use, the mean number of cells infused was 6.71x10^6, with 95.10% cell viability. Tests for microorganism's growth control were negative. ASCs were immunophenotypically characterized as follows: CD105:94.18%; CD73:96.46%; D90:99.86%; CD29:99.15%; CD166:94.04%; CD44:89.13%; CD14:1.94%; CD34:0.59%; CD45:0.87%; CD19:0.71%; HLA-DR:0.64% (data not shown). No clonal chromosomal rearrangements were detected.

Acute adverse events were mild and transient during the infusion, and were previously published elsewhere (31). Adverse events up to three months have been previously published: four patients developed local superficial thrombophlebitis within the first week without systemic manifestations and one patient developed central retinal vein occlusion at T3, with complete resolution (31). At T9 one patient developed a recurrence of a benign ovarian teratoma, which was surgically removed. She underwent surgery 2 years before the study entry, with no recurrence until that time. No major adverse events were reported in the study volunteers along the 12-month of follow-up.

### Insulin dose and glycemic control

Mean HbA1c levels were similar between groups at T0 (p = 0.97), T3 (p = 0.07), but lower at T6 in group 1 (p = 0.01), Table 2 and Fig. 2A. After 12 months there was no difference in glycemic control between group 1 and 2, p = 0.16 (Fig. 2A). Group 1 had an improvement in glycemic control in 12 months (p = 0.017) but in group 2, HbA1c remained stable (p = 0.17). There was no severe hypoglycemia in either of the groups.

Insulin daily doses per kg of body weight were similar between groups at T0 (group 1 = 0.34 +/- 0.24 U/kg and group 2 = 0.55 +/- 0.29 U/kg, p = 0.23). Group 1 had a lower insulin dose at T3, T6 and T12 when compared to controls (group 2), p = 0.04; p = 0.04 and p = 0.04, respectively (Fig. 2B). Insulin dose remained stable 12 months after intervention (p = 0.69) in group 1 and increased in group 2 (p = 0.05).

### Pancreatic β-cell function

CP AUC levels before and after intervention is described in Table 2. CP AUC levels was not significantly different before intervention, but it was higher in group 1 than in group 2 at T3 (group 1 = 199.67 +/- 102.59 ng/ml.min; group 2 89.14 +/- 32.71 ng/ml.min, p = 0.04) and T6 (group 1 220.58 +/- 97.06 ng/ml.min and group 2 82.86 +/- 54.62 ng/ml.min, p = 0.23) (Table 3 and Fig. 2C).

Percentual CP AUC alteration from T0 to T3, T6 or T12 did not differ between groups (group 1 = 20.73 +/- 16.09% vs group 2 = 24.06 +/- 23.53%, p = 1.0; group 1 = 12.11 +/- 47.08% vs group 2 = 44.95 +/- 31.78%, p = 0.07; group 1 -26.88 +/- 32.83% vs group 2 -24.92 +/- 54.74%, p = 1.0, respectively). There was no correlation between age and pancreatic function (p = 0.48).

CP AUC was stable until 6 months (T0 vs T6 p = 0.49) in group 1, with a decline from T6 to T12 (T0 vs T12 p = 0.043; T6 vs T12, p = 0.043). In Group 2, CP AUC remained stable (T0 vs T6, p = 0.068; T0 vs T12, p = 0.046).

There was no correlation between VIT D levels and the CP AUC in any of the groups at T0 (group 1 p = 0.64; group 2 p = 0.20), T6 (group 1 p = 0.64; group 2 p = 0.60) or T12 (group 1 = 0.88; group 2 = 0.60). In the sample as a whole (groups 1 and 2), VIT D levels at T6 had a direct correlation with CP AUC (r = 0.67, p = 0.02), but no correlation was found at T0 (p = 0.29), T3 (0.14) and T12 (p = 0.51)

### GADA, IA2 and FOXP3 expressing lymphocytes

GADA and IA2 titers were similar between groups during the study: T0 (GADA p = 0.78; IA2 p = 0.16), T3 (GADA p = 0.41; IA2 = 0.31), T6 (GADA p = 0.45; IA2 p = 0.23) and T12 (GADA p = 0.315; IA2 p = 1.0).
Type 1 diabetes partial clinical remission

Six months after intervention (ASC + VIT D), 6 patients (85.7%) were in partial clinical remission (CR) vs none in the control group, p = 0.015. The CR persisted until 12 months of follow-up in 4 patients (57.17%). Age, HbA1c, CP AUC, VIT D levels, before intervention did not differ between those that remained in partial CR when compared to others. However, patients that had partial CR exhibited higher FOX P3 expression in CD4+ lymphocytes at T6 and T12 (p = 0.004 and p = 0.02, respectively). VIT D levels were higher in the patients that underwent partial CR at T6 (43.75 +/- 15.61 vs 26.44 +/- 7.71 ng/ml p = 0.03), without difference at T12 (p = 1.0).

Discussion

We evaluated the safety and efficacy of a single allogeneic intravenous ASC infusion and daily oral VIT D in recent onset patients with T1D during 12 months. The procedure was safe and we observed a potential benefit in glycemic control, insulin dose and a temporary stability of pancreatic function until 6 months. This pilot study was the first to evaluate long term results of a single ASC infusion + VIT D in patients with recent onset T1D. Allogenic source of cells was chosen due to the possibility of impairment of mesenchymal stromal/stem cells immune properties in individuals with T1D (32, 33).

Acute safety of ASC infusion and 3 months adverse events have been published previously (31). Nine months after the intervention, one patient had a recurrence of a benign ovarian teratoma with a complete surgical removal. ASC has a differentiation potential capacity to mesodermal and other embryonic lineages, including adipocytes, osteocytes, chondrocytes, hepatocytes, neurons, muscle cells and epithelial cells, depending on the surrounding microenvironment (34, 35), however previous in vitro and in vivo studies showed the safety of intravenous infusion of MSC, including ASC (30, 36, 37). No tumor differentiation occurred in those studies (32–35). Mature benign teratoma is a germ line tumor and is the most frequent non-epithelial ovarian tumor with a chance of recurrence of 3–15% (38). This may be due to incomplete previous resection or missed small mature ovarian cysts at the time of removal rather than a recurrence (38).

Patients that received the ASC infusion + VIT D had a better glycemic control after 12 months. The treatment was also associated with a better β cell function during the first 6 months after infusion. Beneficial effects from MSC infusion in recent diagnosed T1D patients have been published previously. Carlsson et al and Hu et al both observed a better CP, lower HbA1c and lower insulin dose/kg 12 months after autologous bone marrow MSC infusion and 21 months after Wharton Jelly umbilical cord mesenchymal stroma cell (WJ-UC MSC) infusion, respectively, in patients with recent onset T1D (39, 40). Even though HbA1c and insulin dose/kg were lower in the intervention group for 12 months, better pancreatic function was observed only until 6 months, which may indicate a temporary effect of the intervention. Therefore, additional ASC infusions might be necessary to maintain that benefit, as seen with other autoimmune disease, such as multiple sclerosis (41). Moreover, other immunotherapies with ciclosporin, rituximab and humanized Fc-mutated anti-CD3 monoclonal antibody hOKT3 also had temporary effects (42–46). In previous studies with MSC, beneficial effects were sustained until 12–24 months, but none of those used ASC.

Different mesenchymal stem cell sources could have distinct immunomodulatory and regenerative properties, contributing to different results in clinical trial (47, 48). In vitro, ASC present a more pronounced cytokine secretion than MSC from other sources, however this was not confirmed in vivo (47–49). The number of cells infused also might have contributed to the transient effect. In a recent metaanalysis, the infusion of > 1 x 10^7 cells were associated to better outcomes (17), and we used 67.71x10^6. It is also possible that the slight difference is due to the lack of immunosuppression (50). Couri and cols evaluated de pancreatic function after autologous nonmyeloablative hematopoietic stem cell transplantation in 23 patients, after a median follow up of 30 months, 12 patients remained insulin-free with an increase of the CP AUC (50). Other authors confirmed the potential benefit of this therapy in inducing partial CR in patients with T1D, however these better results might have been secondary to the immunosuppressants that were used rather than the stem cell therapy itself (50, 51).

A higher pancreatic function is associated with less glycemic variability and severe hypoglycemia (2, 3). This difference could not be observed in our study since none of the patients had severe hypoglycemic episodes.

A significant number of patients were in partial CR in the intervention group, 85.7% and 57.17% in 6 and 12 months respectively. In other observational studies, this frequency varied from 0 to 20% in 6 months to 0 to 10% in 12 months (30). Patients that underwent partial CR presented a lower frequency of chronic microvascular complications after 7 years of follow up (52).

Some potential temporary immunomodulatory effect was observed after ASC infusion + VIT D in FOX P3 expression in CD8+ lymphocytes 1 month after intervention, as published previously by our group (31). However, this was not sustained after 3, 6 and 12 months. Immunoregulatory CD8+ T cells have been reported before (53) and were also associated with clinical response in a T1D trial with humanized Fc-mutated anti-CD3 monoclonal antibody hOKT3 (54). CD4+CD25+FOX P3 T cells are also critical for controlling autoimmunity and tolerance, this defective Treg cells has been associated to many autoimmune diseases, including T1D (55). Patients that were in partial CR after 6 and 12 months had a higher FOX P3 expression in CD4+ lymphocytes, suggesting that the immune activation might have had a role in the ASC + VIT D therapy in patients with a better response. This immune activation has been published previously by Haller et cols after umbilical cord MSC infusion in children with T1D, but was not associated with a better pancreatic function (56). In addition, after immunotherapy with teplizumab and otezolizumab, T1D patients had a transient modification of CD4+ lymphocyte expression profile (57, 58). Since our intervention group was treated with VIT D + ASC, we cannot confirm that this immunologic effect was solely by the ASC cells, by VIT D supplementation or both. We have previously reported a comparison between our patients and individuals that used only VIT D supplementation for 6 months and took part in a previous study. The CP AUC could not be compared since this group had only basal e peak CP, but ASC + VIT D had an increase in basal CP and a better HbA1c after 6 months when compared to VIT D supplementation group and controls (59). VIT D can act in lymphocyte proliferation, cellular autoimmune pathways...
and stimulated T regulatory response (19, 20). In this study, VIT D levels had a direct correlation with CP at T6, and, in those with CR after 6 months, vitamin D levels were higher. Previous clinical trials with vitamin D supplementation were inconsistent and a metaanalysis has shown a small effect of VIT D on glycemic control in patients with recent-onset T1D (21–25).

Our study has some limitations. Firstly, as in most pilot studies, there was a limited size sample, which could explain the lack of statistical significance observed in some comparisons. Even though CP AUC was statistically similar between groups, at T0 2 patients in group 1 had a significant higher CP, which occurred by chance. Individual characteristics could have influenced this better pancreatic function. Moreover, there was not a group of patients that received intervention solely with VIT D, without ASC. Therefore, it is not possible to determine the exact beneficial effect of each intervention. An additional limitation is the location/route of administration of the ASC. In vivo studies have shown that after peripheral venous infusion, MSC tends to migrate to inflamed tissues (60, 61, 62), however some authors reported that only a small proportion of MSC reach the main target, while most migrate to the lungs and later distributed throughout the body (61, 62). Direct infusion in the peripancreatic region would be more effective and with longer lasting results (63). Finally, a longer follow up is still necessary to understand the sustained benefits in glycemic control and CP secretion. On the other hand, this pilot study has shown safety from a single ASC infusion with VIT D supplementation after 12 months, with a transient benefit in pancreatic function and glycemic control. A larger sample and a longer follow up is still necessary to understand the real benefit of this combined therapy. Other infusions could be necessary to maintain CP secretion after 6 months, since better results were observed until that time point.

**Conclusion**

After a twelve-months follow-up, patients with recent onset T1D that underwent intervention with ASC infusion + VIT D without immunosuppression, presented a better glycemic control and lower insulin requirement than patients that received standard treatment, with a transient better pancreatic function. It is possible that more than one ASC infusion and/or peripancreatic infusion could lead to longer lasting results. However, this pilot study is an important 12 months assessment of ASCs and vitamin D supplementation as a potential combined therapy for T1D with no long-term complications associated with the procedure.

**Declarations**

**Ethics approval and consent**

The study was approved by the Hospital Universitário Clementino Fraga Filho Institutional Review Board (17488313.1.0000.5257) and registered at ClinicalTrial.gov (NCT03920397)

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

Authors declare that they have no competing interests in the article that could be perceived as prejudicing the impartiality of the research reported.

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**Author contributions**


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Figures

Assessed for eligibility n=16

Excluded n=3
Not meeting the inclusion criteria

Randomized n=13

Allocated to intervention n=8
Group 1
Withdrawn consent = 1
Analysed= 7

Allocated to control n=5
Group 2
Withdrawn consent = 1
Analysed = 4

Figure 1
Flow Diagram
Figure 2

2A: Glycemic control during 12 months follow up after ASC + VIT D (group 1) and controls (group 2)

Legend: T0= before intervention. T3, T6 and T12 , 3, 6 and 12 months after intervention, respectively. Group 1 = ASC + VIT D, Group 2 = controls. *p < 0.05

2B: Insulin dose during 12 months follow up after ASC + VIT D (group 1) and controls (group 2)

Legend: T0= before intervention. T3, T6 and T12 , 3, 6 and 12 months after intervention, respectively. Group 1 = ASC + VIT D, Group 2 = controls. *p < 0.05

2C: C-Peptide during 12 months follow up after ASC + VIT D (group 1) and controls (group 2)

Legend: C-Peptide area under the curve after a mixed meal test (CP AUC) during follow-up. T0= before intervention. T3, T6 and T12 , 3, 6 and 12 months after intervention, respectively. Group 1 = ASC + VIT D, Group 2 = controls. *p < 0.05; **p < 0.01.