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Combination treatment of autologous bone marrow stem cell transplantation and hyperbaric oxygen therapy for type 2 diabetes mellitus: A randomized controlled trial

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Abstract

The objective of this study was to compare standard treatment versus the combination of intrapancreatic autologous stem cell (ASC) infusion and hyperbaric oxygen treatment (HBOT) before and after ASC in the metabolic control of patients with type 2 diabetes mellitus (T2DM). This study was a prospective, randomized controlled trial. The combined intervention consisted of 10 sessions of HBOT before the intrapancreatic infusion of ASC and 10 sessions afterwards. ASCs were infused into the main arterial supply of the pancreas to maximize the presence of the stem cells where the therapeutic effect is most desired. A total of 23 patients were included (control group = 10, intervention group = 13). Age, gender, diabetes duration, number of medications taken, body weight and height, and insulin requirements were recorded at baseline and every three months. Also, body mass index, fasting plasma glucose, C-peptide, and HbA1c, C-peptide/glucose ratio (CPGR) were measured every three months for one year. HbA1c was significantly lower in the intervention group compared with control throughout follow-up. Overall, 77% of patients in the intervention group and 30% of patients in the control group demonstrated a decrease of HbA1c at 180 days (compared with baseline) of at least 1 unit. Glucose levels were significantly lower in the intervention group at all timepoints during follow-up. C-peptide levels were significantly higher in the intervention group during follow-up and at one year: 1.9 $\pm$ 1.0 ng/mL versus 0.7 $\pm$ 0.4 ng/mL in intervention versus control groups, respectively, $p = 0.0021$. CPGR was higher in the intervention group at all controls during follow-up. The requirement for insulin was significantly lower in the intervention group at 90, 180, 270, and 365 days. Combined therapy of intrapancreatic ASC infusion and HBOT showed increased metabolic control and reduced insulin requirements in patients with T2DM compared with standard treatment.

Keywords

diabetes, stem cell therapy, autologous transplantation, clinical trial

Introduction

Type 2 diabetes mellitus (T2DM) is a common and underdiagnosed condition that poses challenges for treatment[1]. The introduction of new oral drugs has expanded the range of options to treat this disease, but their safety is yet to be established[2]. The current cellular-based therapeutic method to treat diabetes focuses on the transplantation of the pancreas or islet cells to reconstitute the insulin-secreting functional β-cells[3]. However, the shortage of organs poses an obstacle to this treatment.

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Autologous stem cell (ASC) therapies are promising options with only a few undesirable side effects[4]. Indeed, adult stem cell therapies have been studied for a variety of conditions[5–8]. Infused cells can differentiate into the desired tissues or may simply provide the damaged tissues with trophic signals that promote their self-regeneration. In the case of T2DM, both embryonic and adult stem cells have been induced to form insulin-producing cells and/or islet cell-like clusters in vitro[9–11].

Islet precursor cells exist in the pancreas; there is an ongoing debate as to whether they can be induced to form β-cells under the right conditions[12]. Hence, therapeutic options are based on inducing differentiation of existing stem cells in vivo or providing stem cells to replace those that are not differentiating adequately[13–16]. In fact, multipotential adult stem cells are capable of producing a whole spectrum of cell types, regardless of whether these tissues are derived from the same germ layer, highlighting the opportunity to manipulate stem cells for therapeutic use[13,14]. Bone marrow (BM)-derived stem cells have been capable of inducing endogenous pancreatic tissue regeneration in a mouse model of streptozotocin-induced diabetes[17]. Similarly, multipotent stromal cells from human BM infused into mice treated with streptozotocin have resulted in higher levels of mouse insulin and in an increase in mouse islets and β-cells, although it was unclear whether this effect was due to cell protection or new cell formation[15].

Hyperbaric oxygen therapy (HBOT) has been used to treat ischemic wounds and decompression sickness; more recently, it has been shown to increase BM nitric oxide (NO) synthase, which causes stem cell mobilization/endothelial progenitor cell (EPC) release[18,19]. It is believed that these cells are attracted to sites of inflammation, such as the pancreas in the case of T2DM, as a result of local factors including the presence of cytokines or chemokines. At the site of the lesion, mobilized stem cells/EPCs may cause angiogenesis and the release of factors for the local differentiation of progenitor cells[20]. The infusion of intra-arterial pancreatic stem cells may increase the local levels of stem cells/EPCs, enhancing this effect[21]. In fact, this technique has been described in animal models and humans[4,22–24]. Moreover, diabetes has also been shown to impair progenitor cell mobilization, which adds to the importance of local stem cell infusion[25]. Cell differentiation into β-cells may lead to increased C-peptide production and improved metabolic control; this would probably be associated with decreased insulin requirement as well as reduced metformin dosage. A pilot study performed by our group showed that the combination of ASC and HBOT was associated with a consistent improvement of metabolic variables when comparing baseline with 12 months' follow-up in patients with T2DM[24]. The purpose of this study was to confirm these findings in a randomized controlled trial.

Materials and Methods

Design and Setting

This study was a prospective, randomized controlled trial comparing the benefit of combined HBOT+stem cells (SC)+standard medical treatment (SMT) versus SMT alone for T2DM. A total of 23 patients with T2DM (duration 5–15 years at baseline) were randomized. Patients were enrolled in December 2009, and the randomization was performed in March 2010, with the follow-up occurring through March 2011. This study was performed at a third-level hospital in the city of Formosa, Argentina.

Ethical Aspects

Ethical approval was obtained from the Independent Ethics Committee of Hospital Alta Complejidad Pte JD Perón, Formosa, Argentina. All procedures in this study were conducted in accordance with the Independent Ethics Committee of Hospital Alta Complejidad Pte JD Perón, Formosa, Argentina and the approved protocol as well as with the provisions of Law 25.326 /Habeas Data and the provisions issued by Argentine authorities. Written informed consent was obtained from patients or their representatives participating in this study for their anonymized information to be published in this article.

Inclusion and Exclusion Criteria

Patients who met all the following criteria were eligible for participation in the study: age 45 to 65 years, provided written informed consent, were mentally stable and able to comply with the procedures of the study, clinical history compatible with T2DM, onset of T2DM disease at $\geq$40 years of age, T2DM duration $\geq$5 and $\leq$15 years at the time of enrollment, basal C-peptide 0.5–2.0 ng/mL, HbA1c $\geq$7.5% and $\leq$11% before SMT, treated with SMT for $\geq$four months prior to randomization with stable insulin and metformin doses over three months prior to randomization, HbA1c at $\geq$7.5 and $\leq$9.5% at randomization, and a total insulin daily dose (TDD) $\leq$100 units(U)/day at the time of randomization. Patients were not eligible if their body mass index (BMI) was >35 kg/m^2, their insulin requirement was >100 U/day, HbA1c was >9.5% at the time of randomization, or status of type 1(GAD-65) glutamate decarboxylase was seropositive.

Assessment and Follow-Up

Patients were assessed over a period of one to four months before randomization. All patients were recommended a 1500-calorie diet and an exercise routine (walking or similar for 45 min, three times a week, for the duration of the study). Patients were assessed every three months for one year after randomization. Baseline (day 1) corresponds to time of randomization. Age, gender, diabetes duration, number of

medications taken, body weight and height, and insulin requirements were recorded at baseline and every three months. BMI was calculated as weight/height^2 (kg/m^2). Similarly, fasting plasma glucose (enzymatic colorimetric method glucose oxidase/ peroxidase), C-peptide (chemiluminescence assay), and HbA1c (immunoturbidimetry method) were measured, and the C-peptide/glucose ratio (CPGR) was calculated by the formula C-peptide $\times$ 100/ glucose, to evaluate glycemic profile at the different time points.

Randomization

At 17 weeks, patients with HbA1c level 7.5%–9.5% were eligible for randomization to SMT alone (control group) or HBOT+SC+SMT (intervention group). Simple randomization was applied using a random number table.

Standard Treatment

In all patients, dyslipidemia was managed following guidelines of the American Diabetes Association, the Executive Summary of the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults, and the ESC/EAS Guidelines for the management of dyslipidemias[26–28]. Every effort was made to have all patients on a statin medication as per standard care to achieve low-density lipoprotein levels $\leq$100 mg/dL. In addition, aspirin therapy at 81 mg/day was prescribed to all patients unless contraindicated. Aspirin (Bayaspirina 81 mg, Bayer, Argentina) was discontinued one week before BM aspiration and the autologous intrapancreatic stem cell infusion to reduce the risk of bleeding when the femoral artery was punctured.

SMT for T2DM was managed by an endocrinologist following the standards of care according to the guidelines of the American Diabetes Association and included patient education, medical nutrition therapy, and lifestyle interventions to decrease weight and increase physical activity[26]. Patients were required to complete 45 minutes of walking or similar exercise three times/week from enrollment until 3–16 months after the harvest. At enrollment, diabetes therapy was assessed, and all prohibited medications were discontinued. If the patient was not on metformin, it was initiated and increased as tolerated to a minimum dose of 1000 mg/day, preferably 1000 mg twice per day. Metformin was discontinued and restarted 48 hours after administration of iodine contrast (Iopamiron 300, Schering, Mexico) (e.g., intrapancreatic ASC infusion). Insulin was adjusted as necessary to achieve target fasting and pre-prandial glucose values of 70–130 mg/dL, two-hour postprandial glucose values of less than 180 mg/dL, and HbA1c values of 7.0% without hypoglycemia. Adjustments to metformin and insulin glargine were made every three months throughout the study. In SMT, patients received insulin (Glargina, Lantus Sanofi Aventis, Germany) and metformin (Glucophage 1000 mg, Merck, Argentina) during the four-month evaluation phase. All patients started vitamin C (Redoxon, Roche, Argentina) at 1000 mg/day and vitamin E (Evion 200, Merck, Argentina) at 200 mg/day at the final visit prior to randomization and continued until the three-month post intervention visit to minimize HBOT potential adverse effects secondary to oxygen free radicals.

Hyperbaric Oxygen Therapy

HBOT was administered over three weeks according to standard operating procedure. Briefly, each session of HBOT was administered in a hyperbaric chamber (Multiseat 302, Hypermed Argentina), at a target pressure of 2.5 atmospheres with a minimum pressure of 2.0 atmospheres at 100% FiO_2. The combined intervention consisted of 10 sessions before the intrapancreatic infusion of ASC and 10 sessions afterwards.

Autologous Stem Cell Harvesting

BM ASCs were harvested under local or general anesthesia using multiple bone marrow aspirations from both iliac crests according to standard operating procedure. Briefly, a minimum of 100 mL and a maximum (target) of 375 mL of BM was mixed with 20,000 units of anticoagulant heparin (Fresenius Kabi, Argentina) as a preservative in a quadruple collection bag (Rivero, Argentina). If the target volume of 375 mL could not be reached, difference was completed with peripheral blood up to 375 mL. As exercise is known to facilitate BM harvest, patients were advised to perform a minimum of 45 minutes of walking three times a week from four months before harvest to three months after the harvest. Units were processed using centrifugation according to standard operating procedures to separate red blood cells, plasma, and fat from the buffy coat layer and to subsequently obtain a buffy coat cell count. Briefly, using centrifugation, gravity flow, and the various bags of the quadruple bag system, red cells were discarded in the second bag, the buffy coat was collected in the third bag, and the plasma and fat were discarded with the first bag. The buffy coat was washed and resuspended in isotonic normal saline in the third bag for the final product. The aspirate was a buffy coat pool containing EPCs and a few mesenchymal stem cells. This was transported for immediate infusion into the pancreatic artery. The standard operation procedure was designed and discussed in conjunction with Diabetes Research Institute, Miami, USA, and was based on a procedure used by Sakai and Reboredo[29,30].

Intra-Arterial Pancreatic Autologous Stem Cell Infusion

ASCs were infused into the main arterial supply of the pancreas to maximize the presence of the stem cells where the therapeutic effect is most desired. Because the dorsal pancreatic artery supplies the largest volume of pancreatic tissue

and is an end organ vessel without supply to other organs, it was selected as the site of the infusion. Briefly, access to this vessel was achieved by a groin puncture into the common femoral artery. The celiac axis was selected using wire and catheter techniques, and sub-selection of the dorsal pancreatic artery was performed. Depending on the caliber of this vessel in each patient, an appropriately sized catheter (JR4, Johnson & Johnson, New Brunswick, NJ, USA) was used.

Outcomes and Statistical Analysis

We assessed the glycemic profile measured by plasma glucose levels and HbA1c, and β-cell function measured by basal C-peptide levels. Clinical and laboratory variables were indexed by three-month periods. Data are expressed as median and interquartile range or mean and standard error. Baseline and post-treatment data were compared using a paired Student's *t* test or Wilcoxon rank-sum (Mann–Whitney) test and chi-squared or Fisher's exact test, as appropriate. Where indicated, measures of clinical efficacy are reported as the percentage change from baseline evaluation. Values of $p < 0.05$ were considered statistically significant. Analysis was performed using Stata v14 (Stata Corp., College Station, TX, USA).

Results

Ten patients in the control group and 13 in the intervention group were included. Age of patients in the intervention group (n = 13, 7 men) was 59 ± 9 years and in the control group (n = 10, 5 men) was 59 ± 6 years, with no significant difference. At baseline, levels of HbA1c, glucose, C-peptide, CPGR, and insulin requirements were comparable between the two groups, although BMI was significantly higher in the intervention group: 27.0 ± 4.0 versus 23.1 ± 2.5, respectively ($p = 0.0134$). This difference was maintained throughout follow-up. Although there was a body weight increase, the difference between groups was not statistically significant.

HbA1c was significantly lower in the intervention group compared with the control group throughout follow-up, with the most significant differences at 180 days: 6.7 ± 1.0% versus 8.2 ± 1.0% in intervention versus control groups, respectively, $p = 0.0025$. At 365 days, HbA1c percentages were 7.3 ± 0.9% versus 8.0 ± 0.7% in intervention and control groups, respectively, $p = 0.0366$. HbA1c at all timepoints was significantly lower than baseline in the intervention group. In the control group, this difference did not reach statistical significance at any timepoint.

Overall, 77% (10/13) of patients in the intervention group and 30% (3/10) of patients in the control group demonstrated a decrease of HbA1c at 180 days (compared with baseline) of at least 1 unit. A significant difference between the groups in terms of the proportion achieving a 1-unit reduction of HbA1c over the first six months ($p = 0.02$) was found. At 270 days, these proportions were 69% (9/13) in the intervention group and 40% (4/10) in the control group, and at 365 days, 77% (10/13) and 40% (4/10), respectively (Figure 1).

Glucose levels were significantly lower in the intervention group at all timepoints during follow-up, with the most significant difference between groups at 90 days: 119.7 ± 26.2 mg/dL versus 178.7 ± 17.9 mg/dL in the intervention and the control group, respectively, $p = 0.0000$ (Figure 1). The change in glucose from baseline was significantly different in the intervention group at all timepoints; however, in the control group, the difference never achieved significance.

C-peptide levels were significantly higher in the intervention group during follow-up. At one year, C-peptide level was 1.9 ± 1.0 ng/mL versus 0.7 ± 0.4 ng/mL in intervention versus control groups, respectively, $p = 0.0021$ (Figure 1). A significant difference compared with baseline levels was observed in the intervention group at 90 days, whereas in the control group a difference was seen at 90,180, 270, and 365 days.

CPGR was comparable in both groups at baseline but differed significantly during follow-up. CPGR was higher in the intervention group at all controls during follow-up, with its highest level at 180 days: 1.5 ± 0.4 versus 0.5 ± 0.3 in intervention versus control group, respectively ($p = 0.0003$). At 365 days, CPGR was 1.7 ± 1.0 versus 0.4 ± 0.3 in intervention versus control group, respectively ($p = 0.0002$). In the intervention group, CPGR was significantly higher than at baseline throughout follow-up (Figure 1).

The requirement for insulin (mean units per 24 hours) was significantly lower in the intervention group at 90, 180, 270, and 365 days. The most significant difference was found at 180 days with 17.8 ± 13.6 U versus 29.4 ± 11.3 U in the intervention group versus the control group, respectively, $p = 0.0254$ (Figure 1). In the intervention group, the insulin level remained significantly lower than the baseline level throughout follow-up, whereas in the control group, this difference never achieved significance.

Discussion

This study confirmed that the combination of HBOT+SC+SMT could safely improve the control of plasma glucose, HbA1c, and C-peptide in T2DM patients when compared with patients who received SMT alone. This study validates the pilot study and shows that BM ASC infusion is a safe option for the treatment of T2DM[24]. Moreover, no complications were observed during the BM harvest, the ASC infusion, or the HBOT sessions.

We had hypothesized that delivering SC intra-arterially and activating them with systemic in vivo HBOT might activate at least one crucial reparative step to reduce the chronic injuries that attack the EPC and the islets in the diabetes phenotype. Our hypothesis was based on the fact that neogenesis of cells in the adult pancreas from non-β progenitor cells has been documented postnatally in

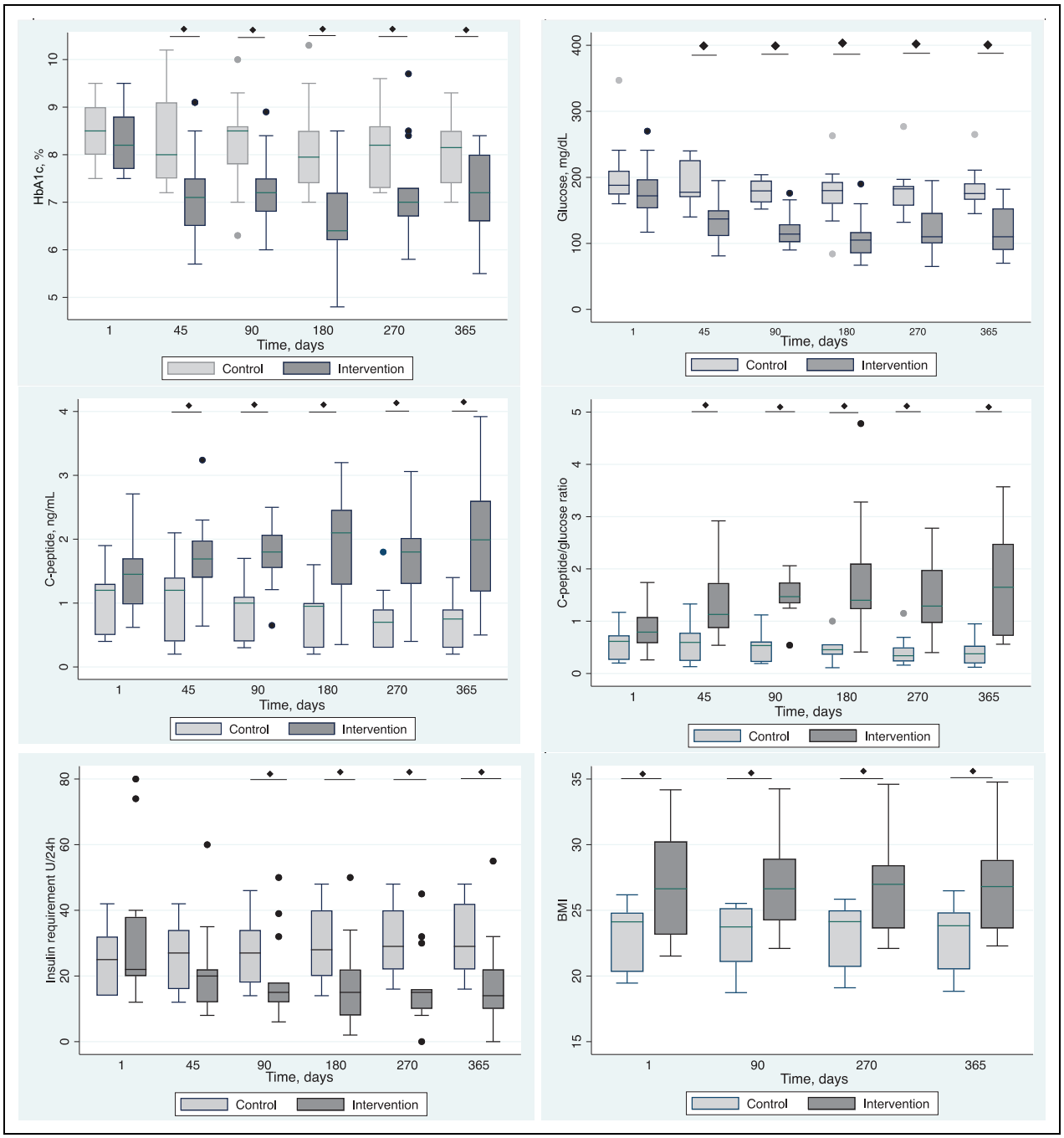

Figure 1. Comparison of groups in parameters variation.

experimental rodent models of pancreatic damage[31–33]. Importantly, it has also been demonstrated that HBOT efficiently mobilizes stem cells from the BM contributing to tissue-repairing processes[18,34]. The authors had proposed that the exposure to hyperbaric oxygen mobilized stem/progenitor cells from the BM by an NO-dependent mechanism[18]. Then, ASC can activate pancreatic embryonic-like cells and/or homing, with later differentiation by means of their own plasticity, or generate neovascularization, improving pancreatic islets and function.

Traditional approaches to T2DM include using external insulin and oral antidiabetic drugs, with the complication of insulin resistance; moreover, the transplantation of islet cells, considered as a promising treatment, faces lack of donors, ethical conflict, and risk of immunogenicity[35]. In this context, stem cell-based therapies are good candidates for treatment with increasing evidence[35,36].

In 2007, Voltarelli et al. reported the use of autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus with promising

results[37]. Bhansali et al. targeted autologous BM-derived stem cell transplantation (SCT) as β-cell replacement therapy in patients with T2DM, showing that after a single administration, SCT was able to reduce the daily requirement of insulin dosage[4,38]. Stem cells were injected into gastroduodenal arteries, and patients showed a significant reduction in insulin requirement, the mean HbA1c showed a reduction of 1%, fasting and glucagon-stimulated C-peptide levels improved significantly, and the homeostatic model assessment for β-cell function increased, while insulin resistance did not change significantly[4,38]. Another randomized study assessed the effect of BM mononuclear cells and HBOT on T2DM, and the results showed that these cells improved islet function and metabolic control; unlike our findings, HBOT did not have a synergistic effect with the infusion[35].

The application of HBOT has been shown to enhance the engraftment and the tissue repair effects of SC therapies in experimental models of heart infarction and nerve regeneration[5,20,39]. Moreover, HBOT may precondition organs after brain death, making them less susceptible to ischemic injury[40], and promote liver regeneration in living donors or in liver recipients[41–43]. In the case of pancreatic islets, endocrine cell clusters are richly vascularized, which most likely reflects the high metabolic rate of the endocrine cells and the need to promptly respond to the metabolic needs. A key role of oxygen in the maturation of endocrine cells in the developing pancreas has been suggested in an animal model, with the emergence of endocrine cells in the embryonic pancreas coinciding with the formation of new blood vessels[10]. Indeed, the availability of adequate oxygen concentrations in vitro enhances the maturation of pancreatic endocrine precursors into insulin- and glucagon-producing cells[44,45], possibly via the modulation of the hypoxia-inducible factor-1 alpha pathway[46]. In addition, mice transplanted with pancreatic islets and treated with HBOT showed increased islet engraftment, probably through the modulation of local inflammation, the improvement of tissue remodeling, and the vascularization at the implantation site[47,48].

Experimental evidence indicates that HBOT may have an impact on immune cell function; indeed, its administration to immunocompetent mice has resulted in a significant decrease in the cellularity of the spleen and thymus, particularly with a reduction of CD4+CD8+ cell numbers compared with CD4+ or CD8+ single positive T cells in the thymus, as well as a more dramatic reduction of β-cells (B220+) compared with Thy-1+ T cells in the spleen[43]. In a murine model prone to autoimmunity (MRL-lpr/lpr mice), HBOT resulted in a marked reduction in cellularity in otherwise enlarged spleens and lymph nodes compared with untreated controls[43]. In a model of graft versus host disease following BM transplantation in lethally irradiated mice, HBOT improved recipient survival and was associated with reduced numbers of CD3+, CD4+, CD8+, CD4+CD11a+, CD4+CD18+, CD8+CD11a+, and CD8+CD18+ T lymphocytes in the spleens of treated animals[49]. Interestingly, it has been reported that HBOT was the only effective intervention in the treatment of a case of mutilating and resistant vasculitis[50]. Pre-treatment of islets or human fetal pancreas with high oxygen in culture has been associated with reduced immunogenicity and indefinite survival upon transplantation into allogeneic or xenogeneic recipients[51,52]. Similar effects have been described in a model of corneal transplantation, in which HBOT resulted in the depletion of the Langerhans cells and the long-term acceptance of allogeneic grafts[53]. Furthermore, combined treatment of experimental animals with cyclosporine and HBOT prevented rejection of allogeneic skin grafts[54]. The rationale of adding SC is based on the fact that HBO may not be sufficient to reduce β-cell apoptosis, and that the duration of the hypoglycemic effect is limited to post administration hours. Moreover, the addition of stem cells possibly actives intrapancreatic embryonic latent SC, generating new β-cells and secreting factors favorable to the pancreatic environment.

In a pilot trial study, we evaluated the impact of HBOT combined with intra-arterial injection of BM mononuclear cells in patients with T2DM and observed improved glycemic control and reduced insulin requirements[24]. The effect of HBOT alone on the metabolic control of patients with T2DM has also been studied; in a series of 28 patients with T2DM with diabetic foot ulcers, exposure to HBOT was associated with significantly improved glycemic control, and increased peripheral insulin sensitivity in humans, equivalent to moderate weight loss[55,56].

Our study has important limitations that should be acknowledged: a relatively small sample of patients, a short follow-up, and a lack of metabolic clamp studies to assess the β-cell function and insulin sensitivity. Although follow-up ends in 2011, missing data prevented the reporting of longer follow-up results. Oxidative stress is reduced by HBO, and this counters the glucotoxicity and the apoptosis of β-cell pancreatic islets. Unfortunately, we did not have enough resources to measure oxidative stress in this study. On the other hand, its main strength is the inclusion of a control group.

In conclusion, autologous bone marrow-derived stem cell therapy in combination with HBOT in patients with T2DM resulted in significantly improved metabolic parameters and reduced insulin requirements. We think the improvement was due to the interaction of both factors, but this approach warrants further investigation in larger studies and the addition of groups to identify the effects of these techniques both separately and in combination.

Ethical Approval

This study was approved by the Independent Ethics Committee of Hospital Alta Complejidad Pte JD Perón, Formosa, Argentina.

Statement of Human and Animal Rights

All the experimental procedures involving human subjects were conducted in accordance with the protocol approved by the Independent Ethics Committee of Hospital Alta Complejidad Pte JD Perón, Formosa, Argentina, with the provisions of Act 25.326 / Habeas Data and the provisions issued by Argentine authorities. There were no animals involved in this study.

Statement of Informed Consent

Written informed consent was obtained from patients or their representatives participating in this study for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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