Stem cell therapy for diabetes mellitus

Júlio C. Voltarelli[1], Carlos E.B. Couri[1], Maria C. Oliveira[1], Daniela A. Moraes[1], Ana B.P.L. Stracieri[1], Fabiano Pieroni[1], George M.N. Barros[1], Kelen C.R. Malmegrim[1], Belinda P. Simões[1], Angela M.O. Leal[2] and Milton C. Foss[1]

[1]*Department of Clinical Medicine, Ribeirão Preto School of Medicine, University of São Paulo, São Paulo, Brazil and* [2]*Department of Medicine, Federal University of São Carlos, São Carlos, Brazil*

In this review, we present (1) a brief discussion of hematopoietic stem cell transplantation (HSCT) for severe and refractory autoimmune diseases (AIDs) from its beginning in 1996 through recently initiated prospective randomized clinical trials; (2) an update (up to July 2009) of clinical and laboratory outcomes of 23 patients with newly diagnosed type 1 diabetes mellitus (T1DM), who underwent autologous HSCT at the Bone Marrow Transplantation Unit of the Ribeirão Preto Medical School, University of São Paulo, Brazil; (3) a discussion of possible mechanisms of action of HSCT in AIDs, including preliminary laboratory data obtained from our patients; and (4) a discussion of future perspectives of stem cell therapy for T1DM and type 2 DM, including the use of stem cell sources other than adult bone marrow and the combination of cell therapy with regenerative compounds.

Kidney International Supplements (2011) **1,** 94–98; doi:10.1038/kisup.2011.22

KEYWORDS: diabetes mellitus; hematopoietic stem cell; stem cell therapy

TO CITE THIS ARTICLE:
Voltarelli JC, Couri CEB, Oliveira MC *et al*. Stem cell therapy for diabetes mellitus. *Kidney inter., Suppl.* 2011; **1**: 94–98.

Correspondence: *Júlio C. Voltarelli, Blood Center (Hemocenter), Campus USP (University of São Paulo), CEP (ZIP) 14051-140 Ribeirão Preto, São Paulo, Brazil. E-mail: jcvoltar@fmrp.usp.br*

On the basis of the successful therapy of animals with autoimmune diseases (AIDs), using high-dose immunosuppression plus hematopoietic stem cells (HSC) (autologous or allogeneic), and the observed remission of concurrent AID in patients treated for hematological disorders (reviewed in refs. 1–5), the first patients with isolated AID were *a priori* treated with HSC transplantation (HSCT). Since then, more than 1000 patients with severe and refractory AID have been treated in open-phase I/II trials,[5] mostly with autologous HSCT (AHSCT). Between one- and two-thirds of patients experienced sustained remission of their disease, mostly neurological disorders (multiple sclerosis and others), rheumatic disorders (systemic lupus erythematosus, adult and juvenile rheumatoid arthritis, systemic sclerosis, vasculitis, and others), and hematological cytopenias (autoimmune hemolytic anemia, immune-mediated thrombocytopenic purpura, Evans syndrome, and others). Relapses and mortality rates varied with type and status of the disease, and with the intensity of immunosuppression used before transplant (myeloablative versus non-myeloablative conditioning regimens). Currently, prospective randomized clinical trials are being planned or already underway to test safety and efficacy of AHSCT for severe and refractory AID, compared with the best available pharmacological treatment (Table 1). Such a trial for adult rheumatoid arthritis (ASTIRA) was halted in Europe because of the lack of patients who were not already involved in other trials with biological agents.

A number of studies that were conducted after HSCT for AID suggest that the regenerated immune system is more immune tolerant with a regulatory phenotype, characterized by increased numbers of naïve and regulatory T cells, and more diverse T-cell receptor repertoire diversity.[6,7]

HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR NEWLY DIAGNOSED TYPE 1 DIABETES MELLITUS

After more than 10 years of clinical use of HSCT for severe and refractory AID, animal studies suggested that this approach could be beneficial for human type 1 diabetes mellitus (T1DM). In addition, further support for this approach was derived from clinical studies in which the effectiveness of immunosuppression for early-onset T1DM and the beneficial effects of HSCT in diabetic patients with

Table 1 | Randomized clinical trials (ongoing or planned) testing safety and efficacy of autologous hematopoietic stem cell transplantation for autoimmune diseases compared with the best available pharmacological treatment

Disease	Name	Region of the world	Control arm
Multiple sclerosis	ASTIMS	Europe	Mitoxantrone
Multiple sclerosis	MIST	USA, Canada, Brazil	Best available drugs
Systemic sclerosis	ASTIS	Europe	IV cyclophosphamide
Systemic sclerosis	SCOT	USA	IV cyclophosphamide
Crohn's disease	KISS	USA	Best available drugs
Crohn's disease	ASTIC	Europe	Best available drugs
Systemic lupus	ASTIL	Europe	Rituximab

Adapted from refs. 5 and 6.

hematological disorders were observed. Specifically, beneficial effects of HSCT in various AIDs,[2–5] and in experimental models of T1DM,[1,8,9] together with low/moderate doses of immunosuppressive drugs in human T1DM,[10–14] were documented, whereas HSCT in individuals with long-standing T1DM[15] was ineffective, and transfer of T1DM from donor to recipient in allogeneic HSCT[16] was noted.

On the basis of the evidence discussed above, HSCT for early-onset T1DM was proposed in review articles in 2001 (ref. 17) and 2002 (ref. 18), and a clinical protocol, designed by investigators at Northwestern University in Chicago, USA (Richard Burt) and the University of São Paulo in Ribeirão Preto, Brazil (Júlio Voltarelli and others) was approved by local and national Institutional Review Boards, permitting the initiation of this study in 2003 in Brazil.

Since 2003, our research group in Brazil has been conducting an original study of non-myeloablative AHSCT in patients with newly diagnosed T1DM. The objective of the treatment is to stop autoimmune destruction of β-cells with high-dose immunosuppressive drugs (cyclophosphamide and rabbit antithymocyte globulin), and to 'reset' the deleterious immunological system with one that is reconstituted by an AHSCT.[19] The rationale for this therapy is to both preserve residual β-cell mass and to facilitate endogenous mechanisms of β-cell regeneration. Hematopoietic stem cells probably do not have the capacity to differentiate into large numbers of β cells; therefore, these cells are used solely to regenerate a new immune system that lacks autoreactive memory cells against pancreatic antigens (see below). The exact mechanisms of action that operate in this treatment are still unclear. However, it has been suggested that AHSCT may shift the balance from a destructive immune response to immune tolerance through clonal exhaustion, regulatory cells, cytokine alterations, and changes in T- or B-cell repertoires.[1,2]

The procedure of AHSCT consists of several steps from patient selection through long-term follow-up. Most patients interested in the study were excluded for not fulfilling enrollment criteria, especially the required short time period (6 weeks) between the first diagnosis of T1DM and therapy, positivity for anti-glutamic acid decarboxylase antibodies, or a lack of a complete understanding of and compliance with the study protocol. Patients enrolled in the study underwent HSC mobilization from the bone marrow, using granulocyte colony-stimulating factor (10 mcg/day) and cyclophosphamide (2 g/m^2). Mobilized HSC were collected by leukapheresis, cryopreserved, thawed, and infused unmanipulated (minimum of 2.0 million/kg) after preconditioning with cyclophosphamide (200 mg/kg) and rabbit antithymocyte globulin (4.5 mg/kg). Apart from the diabetic status, all the treated patients were in good health before transplantation, which explains in part the low frequency and severity of adverse effects (see below). This outcome is also explained by the rapid engraftment of neutrophils (mean of 9 days) and platelets (mean of 10 days).[19,20]

The first patient enrolled and treated in December 2003 showed a discouraging response. His insulin requirements increased progressively up to 12 months following transplantation, when he abandoned follow-up and when his insulin dose was 250% above baseline. His hemoglobin A1c level was 11.1% at 12 months and his C-peptide concentration did not increase. A possible cause for this poor clinical response may have been his very low β-cell reserve, predicted by the previous diagnosis of diabetic ketoacidosis, and further impaired by the apoptotic effect of glucocorticoids used to prevent rabbit antithymocyte globulin reactions. Considering these possibilities, we decided not to use glucocorticoids in the conditioning regimen in the following patients and did not include those with previous diabetic ketoacidosis.

In July 2009, after a mean follow-up of 36.1 months (range 12 to 65 months), all but two of the subsequent 22 patients became independent of exogenous insulin, most of them shortly after being placed on high-dose immunosuppression and occasionally even before SC infusion. Nine patients remained insulin-independent following its discontinuation for a mean period of 40 months (range from 23 to 59 months; Figure 1). Eleven out of 20 patients had to resume insulin injections after varying periods of insulin independence, ranging from 6 to 47 months. Ten of the subjects required 30–50% of their baseline insulin doses after, and patient no. 11 was using a higher dose than that needed before transplantation.

Two patients (nos. 2 and 4) became transiently insulin independent for 47 months and 43 months, respectively. After 4 and 2 months of resuming insulin injections at daily doses of 0.25 IU/kg and 0.20 IU/kg, respectively, we opted to treat these individuals with sitagliptin at 100 mg/day orally. Both became completely insulin independent after 2 months (patient no. 2) and after 1 month (patient no. 4), and both remained independent of exogenous insulin for 12 months and 13 months, respectively, (Figure 2). Sitagliptin is a dipeptidyl peptidase-4 inhibitor, a member of a novel class of oral antihyperglycemic agents. This medication enhances meal-stimulated active glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide levels, improves measures of β-cell function, suppresses glucagon levels produced

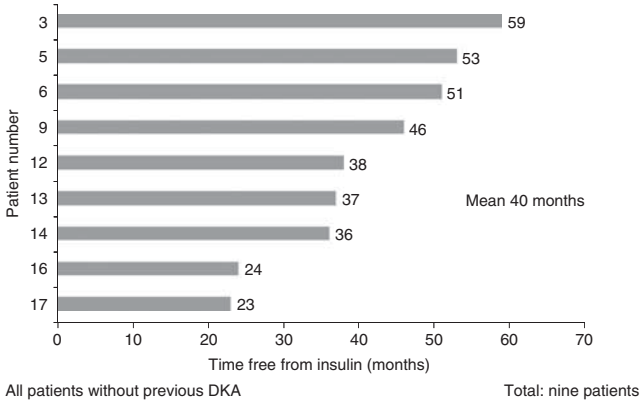

Figure 1 | Sustained insulin independence after autologous hematopoietic stem cell transplantation in nine type 1 diabetic patients. No patient had diabetic ketoacidosis or used corticosteroids during the transplantation procedure.
DKA, diabetic ketoacidosis.

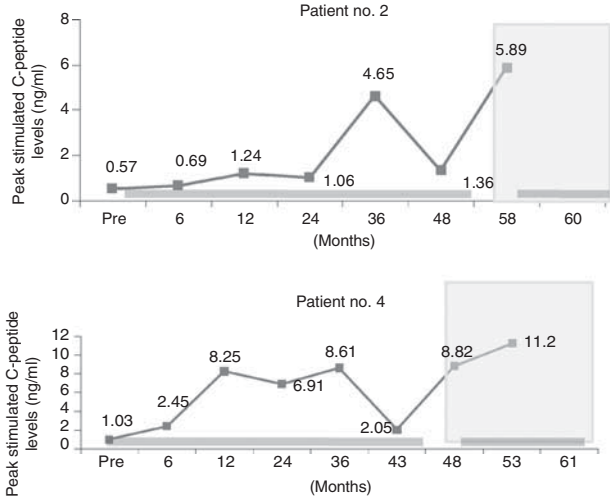

Figure 2 | Time-course peak stimulated C-peptide levels, period free from insulin (blue lines), and period of use of sitagliptin (box) in patients no. 2 and 4.

by α-cells, and increases β-cell mass in rodent models. It also inhibits T-cell aggression against β-cells.[21–23]

The 21st patient presented with previous ketoacidosis, and the 20th patient received steroids (300 mg hydrocortisone) along with stem cell infusion to prevent reactions to rabbit antithymocyte globulin. Neither patient ever became insulin independent.

There was a statistically significant reduction of mean hemoglobin A1c levels after transplantation, and all insulin-independent patients achieved A1c levels of $<7\%$ (upper limit of good glucose control) during follow-up. β-Cell function was analyzed by measuring the area under the curve of serum C-peptide levels during the mixed-meal tolerance test. During the follow-up period, both group of patients, continuously and transiently insulin independent, showed a significant elevation of C-peptide levels[20] of more than two times of the mean area under the curve.

In face of the good metabolic results that were obtained, the adverse effects appeared acceptable. With respect to acute complications, most patients had febrile neutropenia, nausea, vomiting, and alopecia due to the immunosuppressive agents used in the mobilization and conditioning phases of the protocol. Bilateral pneumonia of unidentified etiology, requiring supplementary oxygen, responded completely to broad-spectrum antibiotics in patient nos. 2 and 22. These were the only severe acute complications of the transplantation procedure. During long-term follow-up, patient no. 2 presented with Graves' disease at 3.5 years post-transplantation, and patient no. 3 developed autoimmune hypothyroidism and transient renal dysfunction due to rhabdomyolysis, a complication that was successfully treated with levothyroxine. Patient no. 10 presented with mild transient hypergonadotropic hypogonadism at 12 months after transplantation. These late-onset endocrine abnormalities presented by these three patients may have been caused by the transplant procedure itself or may have been part of an autoimmune polyendocrine syndrome that is frequently associated with T1DM. Of the 16 enrolled men, 13 had sperm examinations done before treatment: 11/13 had abnormal sperm morphology and abnormal motility scores, and 2/13 had low sperm-cell counts (one patient presented with bilateral cryptorchidism). During the follow-up, eight patients presented with oligospermia. Two male patients had children after transplantation (one of them did not have spermograms before and after transplantation). There was no mortality.

We are currently performing exhaustive studies of immunoreconstitution (phenotypic and functional) in the transplanted patients in order to unravel the mechanisms by which AHSCT produces clinical benefits in type 1 diabetic patients. Compared with pretransplant profiles, preliminary results show that after transplantation there is an increase in the number of regulatory $CD4^+CD25^+Foxp3^+$ T cells and Th2 cytokine-producing cells. In addition, we detected after AHSCT, profound qualitative and quantitative changes in T-cell receptor repertoire, as well as alterations in the expression of pro- and anti-apoptotic genes (Malmegrim, personal communication). Anti-glutamic acid decarboxylase autoantibodies decreased in most patients but did not correlate with clinical response. Further preliminary data from our ongoing immune reconstitution studies resemble those observed in other AIDs after AHSCT.[6,7] These results support the suggested hypothesis that a new and more tolerant immune system is generated after this treatment, explaining the reduction of autoimmune destruction of insulin-producing cells and clinical improvement (mechanism no. 2, Table 2). Other possible mechanisms by which HSCT may operate in AID are the specific actions of high-dose immunosuppression (mechanism no. 1, Table 2) and/or tissue repair mediated by transdifferentiation and engraftment of administered bone marrow stem cells (mechanism no. 3, Table 2). However, in the presence of anti-glutamic acid decarboxylase autoantibodies and without specific immunological reactivity tests to β-cell antigens, we cannot be sure that our treatment

Table 2 | Possible mechanisms of action of autologous hematopoietic stem cell transplantation for type 1 diabetes mellitus

1. Ablation of autoreactive immune system by high-dose immunosuppression
2. Regeneration of a naïve immune system from autologous hematopoietic stem cells after lymphoablation by high-dose immunosuppression
3. Regeneration of pancreatic β-cells from autologous bone marrow stem cells mobilized to the peripheral blood

blocks autoimmune attack to endocrine pancreas more efficiently and for longer time than other immunosuppressive interventions, particularly anti-T-cell therapies.

Our clinical trial was approved by the National Institutional Review Board and it was registered at http://clinicaltrials.gov under number NCT00315133.

CONCLUSIONS AND PROSPECTIVE

Type 1 diabetes mellitus

Our preliminary study of AHSCT in a subset of non-ketoacidotic newly diagnosed T1DM patients yielded a number of unexpected positive results: 20/21 patients were able to stop insulin use after initiation of high-dose immunosuppression, and nine patients maintained this status after a mean follow-up of 40 months (maximum of 59 months). Ten patients relapsed after stopping insulin use, and one patient who received steroids during conditioning never became insulin independent. In this group, two patients regained insulin independence after treatment with sitagliptin, which was associated with an increase in C-peptide levels. Longer follow-up and controlled studies are needed to evaluate the full potential of this novel therapy of early T1DM.

The underlying mechanism of action of the various components of the AHSCT (cyclophosphamide, ATG, and stem cells) cannot be studied by direct methods in humans; however, our current immune reconstitution studies and similar studies in other AIDs suggest that the immune system is reset toward a tolerant phenotype, as evidenced by increased regulatory T-cell numbers and by the regeneration of a different and more diverse T-cell receptor repertoire. We hypothesize that the combination of high-dose immunosuppression and HSC infusions function synergistically to downregulate autoreactive T cells, to renew the immune system, and to improve the immune regulatory networks.

Although our approach provides the proof of principle that high-dose immunosuppression coupled with autologous HSC boosting can revert early-onset T1DM in humans, it will hardly solve the overall problem of the disease. First, only a small subset of patients with early-onset T1DM were successfully treated with AHSCT, whereas millions of patients with long-standing T1DM will likely need another type of stem cell to regenerate pancreatic β-cells and other damaged tissues. Second, AHSCT is an expensive, cumbersome, and complex procedure performed in specialized bone marrow transplantation facilities, and has the potential of causing life-threatening short- and long-term complications. In the future, more simple approaches such as pharmacological, biological, or cellular immunoregulatory interventions may accomplish the same therapeutic goal in millions of type 1 diabetic patients. Besides HSC, other types of stem cells, such as embryonic, mesenchymal, or those from umbilical cord blood, may be promising candidates to fulfill this role (reviewed in ref. 11). At this point, AHSCT and islet/pancreas transplantation remain currently the only treatment options to durably reverse the disease in humans. However, the utility of AHSCT will have to be tested in other groups of patients (with previous ketoacidosis, with longer duration of the disease, and in young children). The potential of incretin enhancers, such as sitagliptin, to restore insulin independence, as observed in two of our patients who relapsed after HSCT, illustrates the possible role of combination therapies (stem cells plus immunosuppression plus regenerative compounds) in the future treatment of T1DM.[24]

Type 2 diabetes mellitus

Type 2 diabetes mellitus (DM2) is associated with insulin resistance and secretory dysfunction of the β cells. There are now experimental, epidemiological, and clinical evidences, suggesting the involvement of immune system and inflammatory mediators in both pathogenic mechanisms of DM2.[25–29] Although the initiating events of β-cells lesion are different in DM1 and DM2, the final pathway of pancreatic β-cell death have similarities in DM1 and DM2, suggesting the potential of immunological and regenerative interventions in DM2 as well.

Cellular therapy with stem cells for glycemic control has been tested in experimental models for some years,[30–32] and various sources of stem cells to replace pancreatic β-cells are now under investigation. However, there are only few studies of such approaches reported in humans. Estrada *et al.*[33] reported improvements in glycemic control and C-peptide levels and reduction in insulin requirements in patients with DM2 submitted to combined therapy of intrapancreatic autologous stem cell infusion and hyperbaric oxygen therapy. Bhansali *et al.*[34] reported the same benefits in patients with DM2 after intra-arterial infusion of autologous bone marrow-derived stem cells.

Although there are many obstacles to be overcome before regenerative therapy becomes a real option to treat DM2, in the future it may become an important strategy to attain metabolic control and in prevention of chronic complications.

DISCLOSURE

BPS has received consulting fees and lecture fees from Novartis and BMS. The remaining authors declared no competing interest.

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