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Original Article

Bone marrow transplantation reverses new-onset immunoinflammatory diabetes in a mouse model

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Abstract: Bone marrow transplantation might be an effective method to cure type 1 diabetes mellitus. This study aimed to investigate whether bone marrow transplantation could reverse hyperglycemia in diabetic mice and whether high-dose total body irradiation followed by high-dose bone marrow mononuclear cell infusion could improve the efficiency of bone marrow transplantation in treating diabetic mice. Diabetic mice after multiple low doses of streptozotocin injection were irradiated followed by infusion with approximately 1×10^7 bone marrow mononuclear cells intravenously. Before and after bone marrow transplantation, fasting blood glucose, intraperitoneal glucose tolerance test, serum insulin, pancreatic histology, and the examination of insulin and glucagon in islets were processed. All recipients returned to near euglycemic within 1 week after undergoing bone marrow transplantation. No mice became hyperglycemia again during investigation period. The change of serum insulin, glucose tolerance test, pancreatic histology and the expression of insulin and glucagon in recipient islets after bone marrow transplantation all revealed islets regeneration and significant amelioration when compared respectively with those of diabetic mice without bone marrow transplantation. Bone marrow transplantation contributed to reduce blood glucose, prevent further blood glucose hike in diabetic recipients, and promote islets regeneration. High-dose total body irradiation in combination with high-dose bone marrow monoclear cell infusion could improve the efficiency of bone marrow transplantation in treating streptozotocin-induced diabetes.

Keywords: Type 1 diabetes, bone marrow transplantation, streptozotocin, total body irradiation

Introduction

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease that results in selective destruction of insulin-producing beta cells in pancreatic islets. Repeated injection of multiple low doses of streptozotocin (mld-SZ) is a mature method in the study of T1D [1, 2]. There are some evidences shown that T-cell mediated immunological process might play an important role in the development of SZ-induced diabetes. First, lymphocytic infiltration of the islets appeared earlier than overt hyperglycemia after treating with mld-SZ [3]; second, nude athymic mice did not develop diabetes when treated with mld-SZ [4]; and third, transfer of splenic cells from SZ-induced diabetic mice to normal mice could induce diabetes in the latter [5]; and forth, young non-obese diabetes (NOD) mice lacking T cell receptor cells developed diabetes at low frequency after injection of mld-SZ [6]. Hence, SZ-induced diabetes is caused by a T-cell mediated immunological process, other than direct toxicity of SZ.

Bone marrow transplantation has been performed in patients with autoimmune diseases, including T1D for many years. It was based on immune ablation aimed to destroy self-reacting immune cells, and regeneration of a new functional immune system in which immune tolerance could normally effect. Using SZ-induced diabetic mouse model used to be controversial about whether bone marrow transplantation can cure T1D [7, 8]. Some studies reported that there was no improvement in hyperglycemia after bone marrow transplantation in SZ-induced diabetic mice [9, 10]. In previous studies, hyper-

glycemia got reversal when syngeneic bone marrow transplantation was administrated to SZ-diabetic mice [11-13]. In clinical study, after autologous hematopoietic stem cell transplantation, eighty-seven percent of patients achieved different periods of extogenic insulin independence, and the maximum amelioration period lasted for 58 months [14]. De Oliveira et al. [15] also demonstrated clinical remission of type 1 diabetic patients after autologous hematopoietic stem cell transplantation accompanied with upregulation of fas and fasL proapoptotic genes expression. In another retrospective clinical study, 13 new-onset type 1 diabetic patients were administrated autologous hematopoietic stem cell transplantation which showed to regulate the immune system, and they also found that 11 of 13 patients required significantly reduced dose of insulin for adequate glycemic control [16].

Our work was aimed at investigating whether bone marrow transplantation can reverse new-onset mld-SZ-induced diabetes in mouse and the curative effect of bone marrow transplantation, and further exploring the hypothesis that high-dose total body irradiation (TBI) in combination with high-dose bone marrow mononuclear cells (BMCs) infusion might improve the efficiency of bone marrow transplantation therapy for treating mld-SZ-induced diabetes.

Materials and methods

Induction of experimental diabetes

Male C57BL/6J mice, 6 weeks old (Model Animal Research Center of Nanjing University, Nanjing, China), were injected SZ (Sigma, USA) at 50 mg/kg body weight/day intraperitoneally for five consecutive days. SZ was solubilized in 0.1 ml chilled citrate buffer (0.1 M trisodium citrate, 0.1 M citric acid, pH 4.5) and injected within 5 minutes after solution. Fasting blood glucose was measured twice-weekly with a glucometer (Accu-Chek Blood Glucose Meter, Roche Diagnostic, Germany). Mice were considered as overtly diabetic if fasting blood glucose was confirmed to be higher than 13.9 mM (250 mg/dl) for 2 consecutive days. All mice were bred in sterile pathogen free barrier and fed sterily.

Bone marrow transplantation

Donor homologous non-diabetic male mice were euthanized by CO_2 narcosis, and both femurs and tibias were collected into cold phosphate buffer saline. Bone marrow cells were flushed into ice-cold RPMI 1640 medium. BMCs suspension was gathered after removing erythrocytes by lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2-EDTA, pH 7.4). BMCs were washed in RPMI 1640 medium and collected by centrifugation. Viability of BMCs after isolation was checked by Trypan blue (Sigma) dye exclusion test. Recipient mice (n=6) at 10 days after diabetes onset were irradiated (800 cGy from a ^{60}Co source, 1 Gy/min) all over the body and then injected approximately 1×10^7 BMCs through tail vein within 6 h after irradiation. Mice were still bred in sterile pathogen free barrier and fed sterily posttransplantation. Mice without treated with transplantation were raised in the same circumstances as those received transplantation, and diabetic mice were raised to 40 days after diabetes onset to ensure they were at similar age as diabetic mice at 30 days posttransplantation when they received tests or evaluations.

Fasting blood glucose monitoring and glucose tolerance test

Fasting blood glucose was measured every 3 days in mice treated with bone marrow transplantation (BMT mice, n=6), normal mice (NC mice, n=6), and untreated SZ-diabetic mice. At 30 days posttransplantation, BMT mice, mice at 40 days after diabetes onset (DM40 mice, n=6) and NC mice were injected 2 g/kg body weight glucose intraperitoneally after 8 hours of fasting. After injection, blood glucose was measured at different time points: 0 min, 30 min, 60 min, and 120 min, respectively.

Serum insulin test

Peripheral whole blood were collected from retro-orbital vein of the following four groups of mice: BMT mice, DM40 mice, NC mice at 30 days posttransplantation and mice at 10 days after diabetes onset (DM10 mice, n=6). Serum was obtained after centrifugation of peripheral whole blood specimens. Serum insulin was quantified by a rat/mouse insulin-specific enzyme-linked immunosorbent assay kit (Adlitteram Diagnostic Laboratories, USA).

Pancreatic histopathology and estimation of insulin, glucagon and pancreatic duodenum homeobox-1 in islets

Pancreas were removed from the aforementioned four groups of mice, and then fixed in

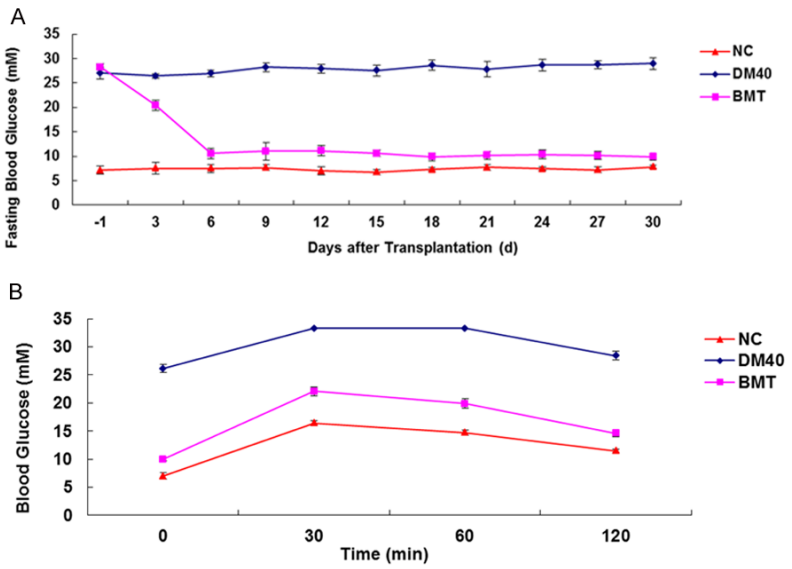

Figure 1. Fasting blood glucose and glucose tolerance test of normal control mice (NC mice), SZ-diabetic mice (DM10 mice) and mice administrated with bone marrow transplantation (BMT mice). A. Fasting blood glucose of NC mice (triangle), DM40 mice (diamond), and BMT mice (square) was measured every 3 days; B. Glucose tolerance test of NC mice, T1D mice, and BMT mice at 30 days posttransplatation.

10% buffered formalin. Paraffin-embedded sections from each pancreas were routinely stained by hematoxylin and eosin dye stain (HE).

Adjacent sections were used for immunohistochemical studies. Deparaffinized sections were boiled in 750 W microwaves to retrieve antigen when immerging in 0.01 M sodium citrate buffer (pH 6.0). After blockage of endogenous peroxidase with 3% H_2O_2 in methanol, the slides were incubated overnight at 4°C with appropriate dilution of primary antibodies. Primary antibodies were as follows: guinea pig anti-mouse insulin antibody (1:200; Sigma, USA), rabbit anti-mouse glucagon antibody (1:200; Sigma, USA). After incubation with primary antibodies, the slides were incubated for 30 mins at 37°C with proper dilution of secondary antibodies. Secondary antibodies were peroxidase conjugated goat anti guinea pig IgG (H+L) antibody and peroxidase conjugated goat anti rabbit IgG (H+L) antibody (Jackson ImmunoResearch, USA) correspongdingly. Diaminobenzidine was used as color substrate. Image-Pro Plus 6.0 software was applied to the evaluation of mean integrated optical density (IOD) of insulin and glucagon expression in islets.

Statistical analyses

One-way ANOVA was used for comparisons between groups. *P* values less than 0.05 were considered significant. All statistical analysis was performed with SPSS 13.0 software. Error bars represent mean±standard error of the mean (SEM).

Results

SZ-induced diabetes in mice

After five-day SZ-injection, fasting blood glucose was increased up to severe hyperglycemia from euglycemia (**Figure 1A**) and the number of islets in pancreas rapidly decreased. In our previous study, about 40% of mice developed diabetes at 14-18 days after 40 mg/kg body weight SZ injection. In our present study, SZ was injected at 50 mg/kg body weight, and about 80% of mice developed diabetes at 14-18 days after injection. Pancreatic sections stained with HE stain showed that hyperglycemia could be related to destruction of islets under diffused infiltration of inflammatory cells in islets (**Figure 2A** and **2B**).

Effects of BMT on immunoinflammatory diabetic mice and induction of diabetes amelioration

In order to evaluate the effects of high-dose TBI and subsequent high-dose BMCs infusion, we applied new-onset SZ-induced diabetic mice to bone marrow transplantation. After transplantation, BMT mice obtained a significant clinical amelioration of fasting blood glucose when compared with T1D mice ($P < 0.001$). This amelioration of blood glucose kept being close to euglycemia which remained steady at 30 days posttransplantation without further hike (**Figure 1A**).

In intraperitoneal glucose tolerance test, BMT mice at 30 days posttransplantation, which had a glucose curve similar to normal mice,

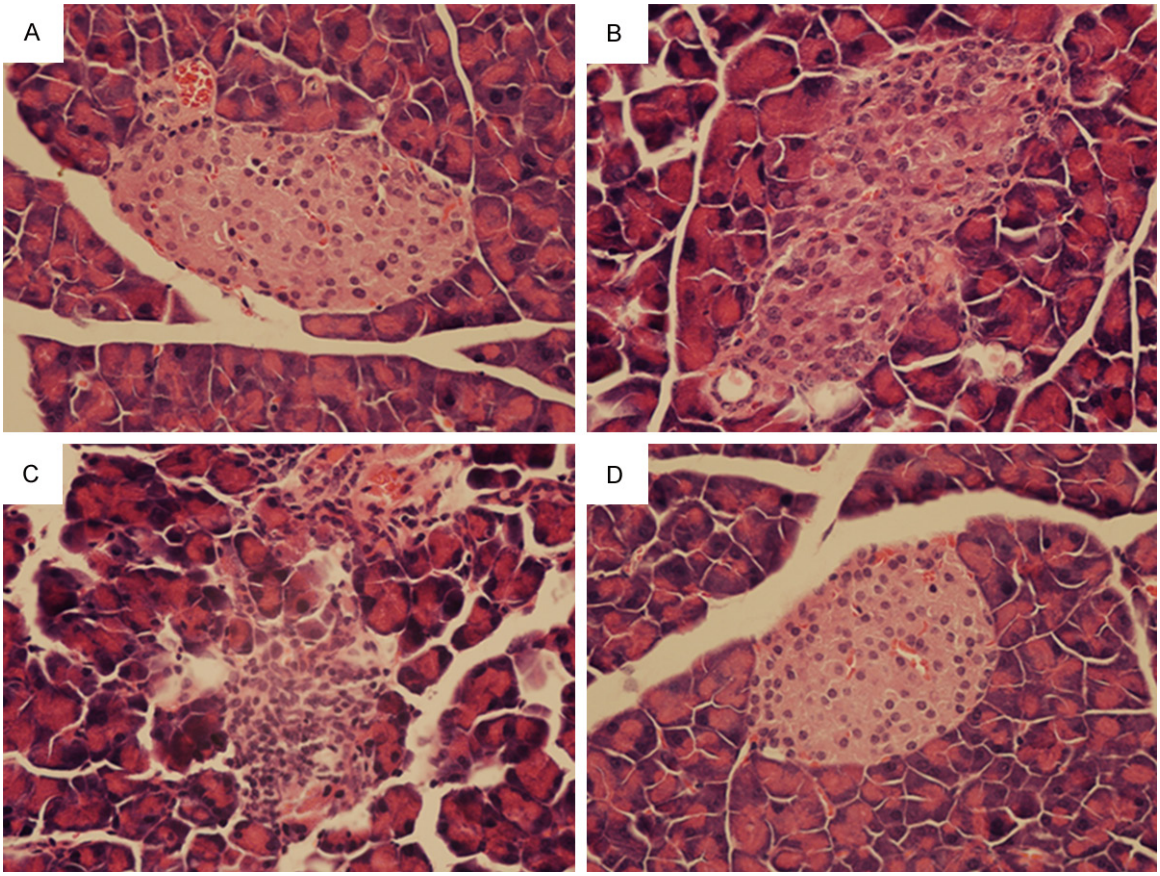

Figure 2. HE staining for islets of every group of mice (EnVision, ×400). At 10 days after diabetes onset (DM10) (B), insulitis, characterized by peri- and intra-islet infiltration of inflammatory cells was frequently observed in most islets, and the margins of islets became obscure; at 40 days after diabetes onset (DM40) (C), islets were severely destructed by the infiltration of inflammatory cells, and most islets lost clear margins, and the total number of islets obviously reduced. Islets of BMT mice at 30 days posttransplantation (D) showed no obvious infiltration of inflammatory cells compared with that of NC mice (A).

showed improved tolerance to glucose stimulation whereas T1D mice remained glucose intolerant throughout the whole 120 mins test (**Figure 1B**).

Serum insulin dropped in DM10 mice ($P < 0.05$), and remained decreased in DM40 mice compared with NC mice ($P < 0.01$). Serum insulin restoration in BMT mice was correlated with normalization of fasting blood glucose (**Figure 3**).

Consistent with these findings, pancreatic histopathological analyses of BMT mice and T1D mice revealed significant differences. As shown and illustrated in **Figure 2**, peri- and intra-islet infiltration of inflammatory cells was frequently observed in most islets of DM10 mice; the margins of most islets became obscure, and the total number of islets obviously reduced. Up to the end of disease monitoring (30 days after BMT), the destructive process progressed, resulting in paucity of architecturally distorted insulin-positive beta cells (**Figure 4C** and **Table 1**), whereas the remanent cells were mostly glucagon-producing cells (**Figure 5C** and **Table 1**). By contrast, at 30 days posttransplantation, the majority of islets of BMT mice appeared normal, showing typical diffuse insulin staining with no signs of inflammation (**Figures 4D**, **5D** and **Table 1**). The glucagon expression in islets had no significant differences among DM10, DM40 and BMT mice (**Figure 5** and **Table 1**).

Discussion

T1D is diagnosed when beta-cell destruction is almost complete and patients need strategies including insulin replacement therapy to control blood glucose. However, Blood glucose is not

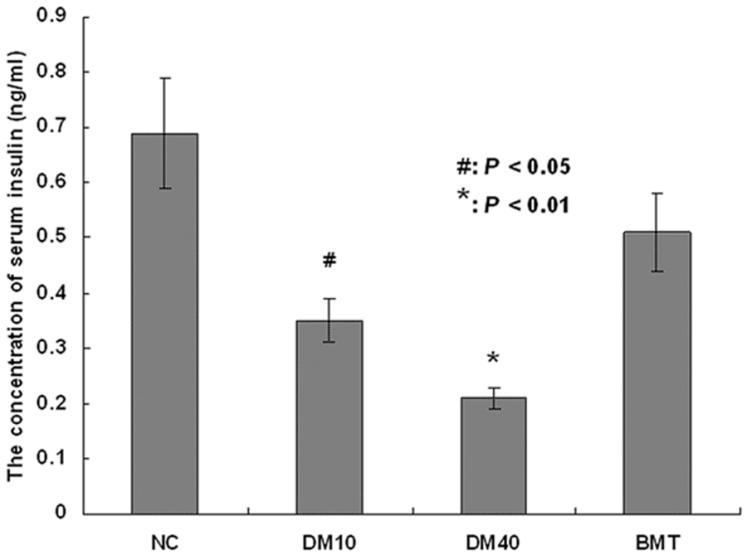

Figure 3. Serum insulin concentration of NC mice, DM10 mice, DM40 mice and BMT mice. Elisa essay was used to detect serum insulin concentration. *$P < 0.01$, and #: $P < 0.05$ vs. NC mice.

always properly regulated with traditional therapies, and chronic hyperglycemia leads to severe microvascular, macrovascular, and neurological complications. These devastating complications can be prevented by normalization of blood glucose. Therefore, successful reversal of T1D will require both beta-cell regeneration from islet cell precursors and prevention of autoimmunity against residual and newly formed beta-cells, if any. Although the causes of T1D are not fully understood, several environmental factors such as viral infection may induce beta-cell destructive immune response in genetically susceptible hosts [17, 18]. In humans, T1D is a multifactorial disease in which genetic factors such as multiple susceptibility genes and environmental factors all contribute to inducing T1D. It was suggested that T1D in humans might be caused more importantly by environmental exposure [19]. After inducing immune ablation and regeneration of brand new immune system with hematopoietic stem cell transplantation, most of T1D patients achieved insulin independence but to different extents [14, 16].

It is pointed out that dose of TBI and BMCs play important roles in bone marrow transplantation [20]. It was shown that syngeneic bone marrow transplantation ameliorated hyperglycemia to near euglycemia, prevented pancreatic destruction, and restored beta cells during a subsequent 30-day follow-up in our study. Bone marrow transplantation led to disease attenuation, and prevented further blood glucose hike, which strongly suggested that bone marrow transplantation might be an alternative option for treating T1D in humans.

Bone marrow transplantation involves TBI and subsequent BMCs infusion, which are both elementary and important. High-dose TBI is expected to give the best therapeutic results, presumably because it kills off most T lymphocytes. The most likely candidate target cells in overt autoimmune diseases are activated T lymphocytes and memory T lymphocytes [21], and both of them are sensitive to irradiation [22, 23]. A lethal dose of TBI is effective in both arthritis model and experimental autoimmune encephalomyelitis model, but partial body irradiation has only short-term effects, if any [24, 25]. In addition, high-dose rate irradiation is very important. Low dose rate irradiation shows more damaging effects, such as lipid peroxidation, antioxidant enzyme activity, and DNA damage [26]. We noticed that elevation of blood glucose decreased during the first week after high-dose irradiation followed by BMCs infusion for diabetic mice, which suggested that insulitis might be terminated. Using high-dose TBI, autologous bone marrow probably not required T-cell depletion when the number of autoreactive T lymphocytes in the grafts is less than that required for a relapse. We applied TBI preconditioning regimen to ensure similar conditioning dose among individuals, and side effects were not seen in our research. But we did not examine objective indicators to evaluate the side effects of TBI because of restricted experimental supply. Some clinical trials for bone marrow transplantation did not suggest TBI conditioning which may cause irreversible lesion to lung in humans. Therefore present ideas are prone to drug conditioning regimen such as cyclophosphamide and ATG as Couri et al. did [14].

In our present study, BMCs were not sorted. Bone marrow stem cells include two distinct

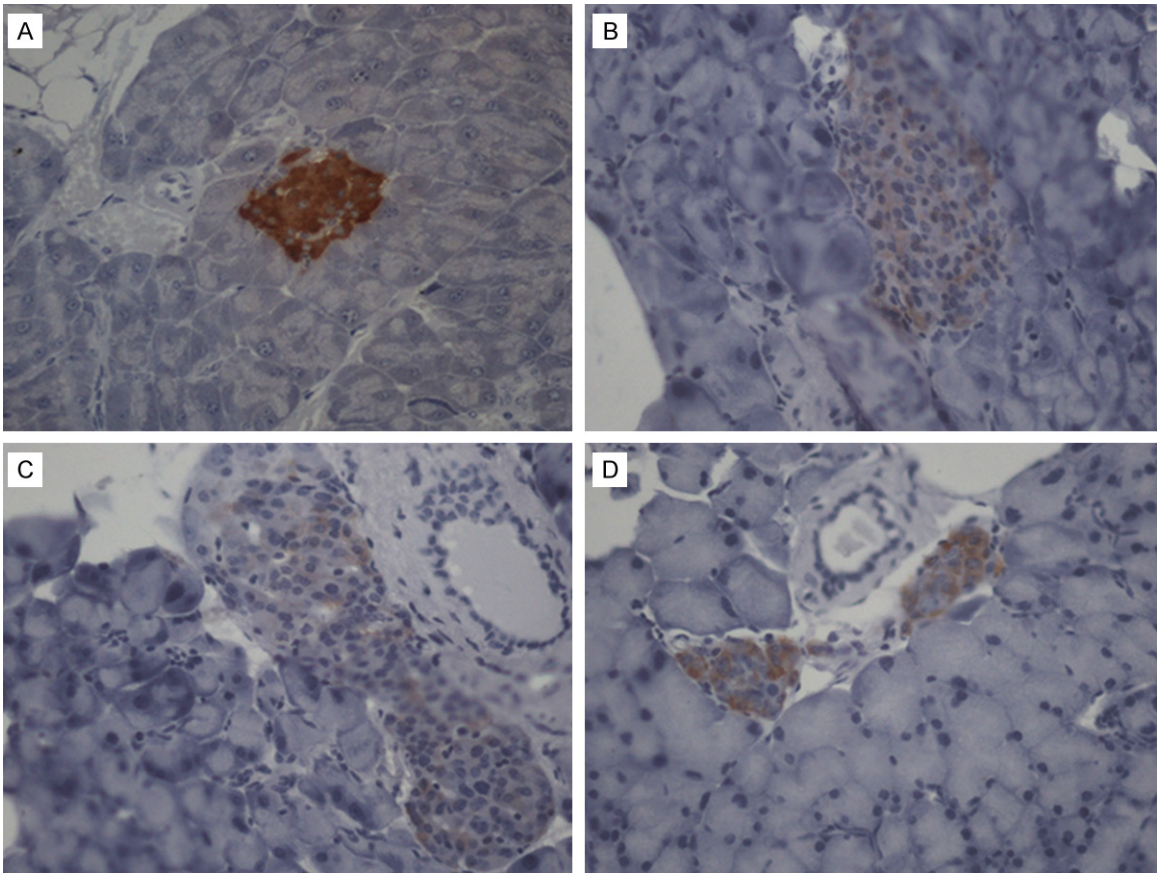

Figure 4. Insulin expression in islets (EnVision, ×400). Pancreas were removed from NC mice (A), BMT mice (D) at 30 days posttransplantation, DM10 mice (B) and DM40 mice (C), and immunohistochemistry essay was used to evaluate insulin expression in islets.

Table 1. Insulin and glucagon expression in islets (*mean IOD,* $\bar{x}$±s)

Group	Insulin	Glucagon
NC	0.3966±0.0991	0.1810±0.0568
DM10	0.1646±0.0282*	0.1928±0.0597
DM40	0.1459±0.0412*	0.1943±0.0585
BMT	0.3197±0.0999	0.2120±0.0353

Abbreviation: NC=normal control mice; DM10=mice at 10 days after diabetes onset; DM40=mice at 40 days after diabetes onset; BMT=mice at 30 days posttransplantation. *: $P < 0.05$.

multipotent cell populations: hematopoietic stem cells (HSCs) which differentiate into all hematopoietic lineages, and mesenchymal stem cells (MSCs) which give rise to different hematopoietic microenvironmental cells, including adipocytes, osteoblasts and, more controversially, endothelial cells [27, 28]. Homing of primitive HSCs to bone marrow is a nonselective and inefficient process. Engraftment and reconstitution of normal hematopoiesis posttransplantation rely on the ability of the HSCs to lodge within specialized bone marrow niches where proliferation and self-renewal process of stem cells commence [29]. When BMCs are intravenously injected, most are trapped in the lung [30] and liver [31]. As a result, only very few cells can migrate into bone marrow. Rapid hematopoiesis and immune reconstitution rely on the number (quantity) and function (quality) of immunocompetent hematopoietic cells. More BMCs infused were associated with more rapid regeneration of T cells [32]. MSCs represent a rare population cells that consist of only 0.001 to 0.01% of total nucleated cells in bone marrow. MSCs produce growth factors and cytokines that promote hematopoietic cells expansion and differentiation, and have an ability to modify the inflammatory response [33]. The declined blood glucose of diabetic mice irradiated at 5Gy and infused with approximate-

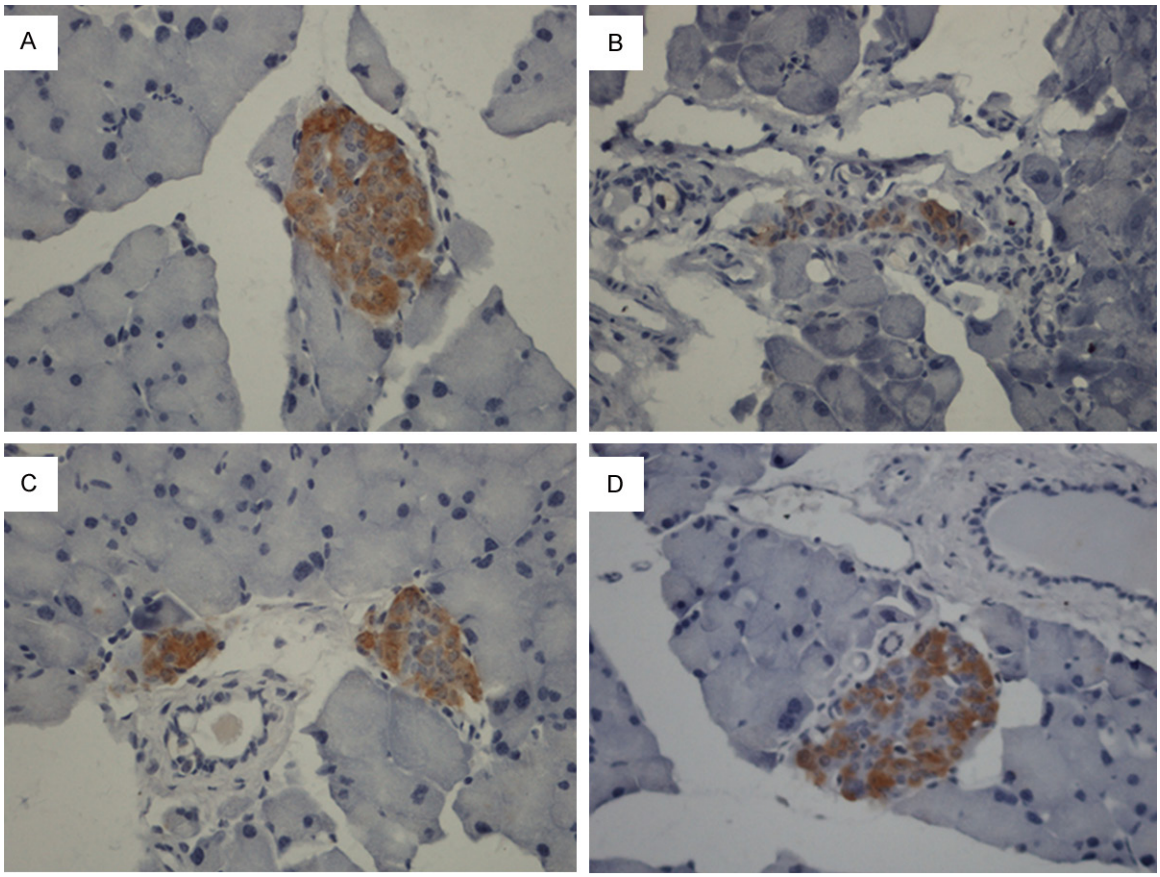

Figure 5. Glucagon expression in islets (EnVision, ×400). Pancreas were removed from NC mice (A), BMT mice (D) at 30 days posttransplantation, DM10 mice (B) and DM40 mice (C), and immunohistochemistry essay was used to evaluate glucagon expression in islets.

ly 6.5×10^6 BMCs was stable [34]. However, blood glucose of diabetic mice irradiated at similar dose followed by low-dose BMCs infusion increased rapidly after a transient delay [10]. The hematopoiesis recovery time after moderate dose TBI (5Gy) followed by bone marrow transplantation was about 18 days (data not shown). Otherwise, it was approximately 30 days to achieve hematopoiesis recovery after high-dose TBI (8Gy). When using high-does TBI, you may choose more BMCs for infusion.

In Urban et al.'s study [10], high-dose TBI combined with low-dose MSCs resulted in total loss of diabetic mice within a few days after treatment. We considered whether TBI or BMCs infusion alone could ameliorate diabetes, so we gave TBI without BMCs infusion to SZ-diabetic mice. Eventually, fasting blood glucose was lower than that of diabetic mice with no treatment, but all died within 20 days after irradiation because of high-dose lethal irradiation, which might due to delayed hematopoiesis. We also administrated BMCs infusion without TBI to diabetic mice, but got no transient or durable improvement of fasting blood glucose during investigation period (**Figure 1A**). Hasegawa et al. [35] also found that BMCs infusion without TBI or TBI without BMCs infusion could not reverse hyperglycemia. We figured out that TBI, as well as BMCs infusion were both essential for attenuation of diabetes.

Unger and Orci [36] proposed a bihormonal-abnormality hypothesis, which highlighted that both deficient insulin and excessive glucagon secretion contributed to hyperglycemia. Alpha cell neoformation has taken place as has been reported in NOD mice pancreas [37], and an experimental model of T1D was known to display hyperglucagonemia similar to that found in human T1D [38]. Li et al. [39] demonstrated that an expansion of alpha cells accompanied by the development of diabetes in C57BL/Ks mice subjected to mld-SZ treatment. Lack of insulin may lead to a decreased suppression of

glucagon secretion by alpha cells and subsequent hyperglucagonemia [40, 41]. In our study, we showed different result of pancreatic glucagon expression which remained stable level when compared with that of normal controls, SZ-diabetic mice and BMT mice. In SZ-diabetic mice, when insulin expression in islets reduced, together with stable glucagon expression, alpha cells constituted the main cells besides inflammatory cells in islets. It was suggested that alpha cells might not be devastated by autoreactive inflammatory cells or SZ toxicity, and that gulcagon expression did not increase just because of alleviated suppressive effect of insulin.

In conclusion, our results of bone marrow transplantation made it particularly interesting candidates for curing T1D in animal studies. However, to date, the role of bone marrow transplantation in T1D remains completely unclarified. We are optimistic that future efforts in this area including more clinical trials and animal researches will shed light on the transplant-related morbidity and mortality, and reveal intimate mechanisms of bone marrow transplantation therapy, and search proper salvage regimen after further hike of blood glucose.

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Disclosure of conflict of interest

None.

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