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Full Length Research Paper

Protective effect of transplanted bone marrow mononuclearcells (BMMNCs) in organ damage caused due to streptozotocin (STZ) induced diabetes

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Diabetes is a rapidly growing health problem; however, there is currently no effective therapy for diabetes. We know that prolonged diabetes leads to damage of various organs apart from pancreas such as kidney and liver and the role of bone marrow mononuclear cells (BMMNCs) is not clearly understood in regeneration of these organs. In this study, we transplanted chloromethylbenzamido-1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI) labeled BMMNCs in streptozotocin (STZ) induced mice and tracked them in various affected organs. We observed, through histological studies that intravenously transplanted CM-DiI labeled BMMNCs were found in acinar regions of the pancreas and not in islet regions, but regenerated islets in diabetic mice. Interestingly, these cells were not found in the kidney and liver of diabetic mice, but these organs regenerated. Also, there was a significant drop in blood glucose levels after the transplantation. We concluded that transplanted BMMNCs incorporated in the acinar region of the pancreas and not in any other organs, but aided in the regeneration of pancreas, liver and kidney. The transplanted BMMNCs also helped in reduction of blood glucose levels.

Key words: Bone marrow mononuclear cells, diabetes, transplantation, organ regeneration.

INTRODUCTION

Diabetes is known to affect at least 200 million people in the world and this number is expected to double in the near future (Torn et al., 2011; Magliano et al., 2008, Kanaya et al., 2011). Diabetes has severe secondary complications driven by poor glycemic control for which there is currently no cure. Till date, several cellular therapies have been tried like including stem cells, islet cells but these cellular modes of therapies have problems in cell culture and identification. One of the most important studies regarding bone marrow cell plasticity has taken place in the field of diabetes. A number of evidence suggests that bone marrow derived cells have the potential to transdifferentiate into non-hematopoietic cell lineages such as brain (Zhao et al., 2002), liver (Petersen et al., 1999), heart (Orlic et al., 2001), epithelia of gastrointestinal tract (Okamoto et al., 2002) and muscle (Jackson et al., 1999). In type 1 diabetes, the number of β-cells decreases due to the assault by the body's own immune system leading to hyperglycemia. Scientists have shown that β-cells can be repopulated by bone marrow transplantation and that these cells transdifferentiate into insulin producing cells (Ianus et al., 2003). But, other studies contradict the β-cell differentiation capacity of bone marrow cells, as they fail to reproduce similar results (Choi et al., 2003; Lechner et al., 2004; Taneera et al., 2006).

As the diabetes condition progresses, various complications arise such as diabetic nephropathy, neuropathy, cardiomyopathy and retinopathy. Though the

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tissue injury in normal condition is healed by the resident population of progenitor cells in diabetes condition. Due to poor control of glucose level, these cells are unable to restore homeostasis of the affected organs. Hence, one of the most important cell sources, bone marrow cells which are highly rich in progenitor cells having multilineage differentiation capacity comes into play. It is already known that these cells, under the influence of certain factors released from the site of injury, migrate and heal the damaged tissues (Bittner et al., 1999). This potential of bone marrow cells is lost in diabetes condition and the healing capacity is impaired (Tepper et al., 2010). Hence, transplantation of healthy bone marrow cells becomes imperative as both the tissue resident population of progenitor cells and bone marrow cells fail to repair the affected sites.

Not many studies have taken place to track the transplanted bone marrow cells in diabetes condition, and there is a lack of complete understanding about how transplanted bone marrow cells divide itself into groups and migrate to various damaged organs and repair them. Understanding of this phenomena will not only help us to regulate the number of cells required for transplantation, but eventually will help bring down the complications that arise due to transplantation. In this study, we try to understand the homing patterns of bone marrow cells in diabetes condition by tracking them using fluorescent dye in various organs.

MATERIALS AND METHODS

Experimental animals

All animal protocols were approved by Institutional Animal Ethics Committee of Vellore Institute of Technology University, Vellore. Laboratory bred 6 to 8 weeks old male mice weighing around 25 to 30 gms were obtained from animal house of VIT university and used in the experiments. The standards set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) were maintained. Mice were caged in a well ventilated room with the temperature around 20 to 25°C, humidity around 60 to 70% with 12 h light/dark cycle. Mice were fed *ad libitum* with standard diet pellets purchased from commercial manufacturer and water.

Induction of diabetes

Streptozotocin (STZ) was purchased from Sigma chemicals, St. Louis, MO, USA. Modified protocol to induce diabetes was followed (Arora et al., 2009). Mice were fasted for 16 h prior to the administration of STZ. STZ was freshly prepared by dissolving in 0.01 M sodium citrate buffer solution, pH 4.5 and was used within 15 min. Low multiple doses at 40 mg/kg STZ were intraperitoneally administered for 5 consecutive days. The glucose levels were measured using a glucometer (One Touch Horizon) and strips (Lifescan) from the blood samples collected from the tail vein of the mice. Control mice were injected with equal amount of buffer.

Isolation of bone marrow mononuclear cells (BMMNCS)

Tibias and femurs were removed after sacrificing mice by cervical dislocation procedure. Bone marrow was isolated by flushing the bones with phosphate buffered saline (PBS), pH 7.4 with 10 mM ethylene diamine tetraacetic acid (EDTA) solution. BMMNCs were then separated by density centrifugation using Histopaque-1083 (Sigma-Aldrich, St. Louis, MO, USA). Remaining erythrocyte contamination was removed by using red blood corpuscles (RBC) lysis buffer solution. The total number of viable cells was counted using trypan blue dye exclusion assay (Sigma Aldrich, St. Louis, MO, USA).

Chloromethylbenzamido-1,1'-dioctadecyl-3,3,3',3 tetramethylindocarbocyanine perchlorate (CM-DiI) labeling of BMMNCs

CM-DiI (Molecular Probes, USA) labeling of BMMNCs was done using the modified manufacturer's protocol. Briefly, the stock solution of 1 mg/mL was prepared in ethanol and the isolated cells were incubated at 37°C for 15 min followed by incubation at 4°C for 15 min in PBS with the final concentration of 4 µM of CM-DiI, as this concentration does not inhibit the replicative processes of cells (Weir et al., 2008).

Transplantation of BMMNCs

STZ mice were subjected to total body irradiation with a dose of 9Gy from a ^{60}Co source (Banerjee et al., 2005). Around $2x10^6$ million unfractionated allogeneic CM-DiI labeled BMMNCs suspended in PBS pH 7.4 were intravenously injected in each of 6 mice, 35 days after STZ induction. Six (6) control mice also received the same number of cells and blood glucose levels were measured every week for 50 days. The number of animal used are: 6 control mice, 6 diabetic/STZ mice, 6 control + transplanted mice and 6 diabetic + transplanted mice.

Hematoxylin and eosin (H&E) staining

Tissues were chopped and placed in 10% buffered formalin for 2 days in the dark. These tissues were then sequentially dehydrated in increasing concentrations of ethanol with 1 h in each concentration of 70, 75, 80, 90, 95 and 100% followed by 30 min incubation in chloroform twice at room temperature. Tissues were then incubated in paraffin wax at 58 to 60°C for 4 h and blocks were made. 5 µm sections were placed on slide and stained with H&E stains.

Microscopy

The fluorescence microscopy as well as the H&E stained imaging has been done after sacrificing the animal on fixed tissue.

Statistical analysis

To calculate the extent of damage in the pancreas and kidney, the area of cells in the islets and glomerular regions of the organs were counted, respectively and were compared to their controls and relative percentage of damage was calculated. To calculate the damage in the liver, several fields in the section were chosen and the total area of cells present in each field was counted and compared with control. Calculation of the area of the islet/glomerulus /liver nuclei was done using Infinity capture and analyze software (Infinity 2.1, Luminera, Corp, Ottawa, Ontario, Canada). All the results are presented as the mean ± standard error of the mean. Statistical analysis was performed by Student's t-test

for comparisons between two groups and by analysis of variance for more than two groups and $p < 0.05$ was considered to be statistically significant.

RESULTS

Induction of diabetes

After the administration of multiple doses of STZ in 12 mice, the average blood glucose level after a month was found to be 303 ± 6.87 mg/dL, and the average blood glucose level of the 12 control mice was found to be 109 ± 7.27 mg/dL (Figure 1A). It was also found from the histological study that the average area of islets of control mice in the imaging regions was found to be 307814 ± 22156 μm^2 and that of diabetic mice was found to be 57511 ± 10346 μm^2 (Figure 1B). STZ majorly affected the islet region of the pancreas but did not affect the acinar region of the pancreas (Figure 1C).

Isolation, CM-DiI labeling and transplantation of BMMNCs

The total number of cells isolated from the procedure were counted by trypan blue dye exclusion assay and around 2×10^6 live cells were labeled with CM-DiI at the final concentration of 4 µM. CM-DiI labeled cells were excited at 553 nm and they emitted light at 570 nm wavelength. The cells appeared to be orange/red in color (Figure 1D). Allogeneic CM-DiI labeled BMMNCs were then injected in 6 STZ induced mice and 6 control mice. These cells were tracked in various organs such as the pancreas, kidney and liver.

Organ damage and regeneration post diabetic induction and bone marrow transplantation

After the induction of STZ, the islet region of the pancreas was severely damaged. Prolonged diabetes, as in our case, led to the damage of various organs such as the kidney and liver. The damage is a measure of the decrease in area of islet, glomerulus and hepatocyte nucleus radius decrease and regeneration is also the measure of the increase in all the same values. In Figure 2A, we observed damage in diabetic pancreas, kidney and liver as compared to the control, which regenerated after bone marrow transplantation in diabetic condition. Upon BMMNCs transplantation in control condition, no such regeneration was observed. Hence, all the three affected organs were regenerated after the transplantation of BMMNCs (Fig**ure** 2A and B) in diabetic mice.

Tracking of BMMNCs in damaged organs

After 55 days of allogeneic transplantation of CM-DiI labeled BMMNCs in STZ induced mice, it was observed that no fluorescence was detected in the tissue sections of the islet region of diabetic pancreas but there was regeneration of the organ. It was also found that CM-DiI labeled BMMNCs engrafted in the acinar region of exocrine pancreas and heavy fluorescence was detected (Figure 3A). Statistically, CM-DiI labeled BMMNCs were not found in the kidney and liver section as there was no fluorescence detected (Figure 3B) but BMMNCS transplantation regenerated the organs. Also, there was hardly any fluorescence detected in BMMNC transplanted control mice.

Effect of transplanted CM-DiI BMMNCs on blood glucose level

After transplantation, the blood glucose levels were monitored every week for 8 weeks and it was observed that there was a significant drop in the blood glucose level (Figure 4A). The average blood glucose level dropped from 302.5 ± 6.86 to 257.167 ± 18.17 mg/dL after the first week of transplantation and to 120.66 ± 3.88 mg/dL after 8 weeks of transplantation. It was also observed that the STZ induced mice did not spontaneously recover and the STZ induced diabetic control mice had blood glucose levels consistently around 300 mg/dL. The BMMNC transplanted diabetic mice had almost become normoglycemic. We have further investigated the mechanism of regeneration in the damaged organs and found that upregulation of vascular endothelial growth factor (VEGF) can be a potential cause for regeneration as shown in Figures 4B and C. In Figure 4B, we observed that VEGF is downregulated in diabetic BMMNCs as compared to the control with and without transplantation. Although we observed significant increase in VEGF expression after BMMNCs transplantation in both diabetic and control condition but the increase in diabetic BMMNCs is always less than the control. In Figure 4C, we found that like BMMNCs, even the expression of VEGF in all three organs is downregulated in diabetic mice, which gets upregulated upon BMMNCs transplantation in diabetic condition. Therefore, we infer that transplantation of VEGF signals to the cells in proximity to regenerate by increasing the VEGF in the BMMNCs as well as in organs.

DISCUSSION

In the present study, we have observed that administration of STZ results in the damage of islet cells but does not significantly affect the exocrine region of the pancreas containing acinar cells hence, increasing the blood glucose levels. CM-DiI labels BMMNCs by entering inside the cells; at higher concentrations, it inhibits the replication of cells (Weir et al., 2008). Transplantation of allogeneic unfractionated CM-DiI labeled BMMNCs in

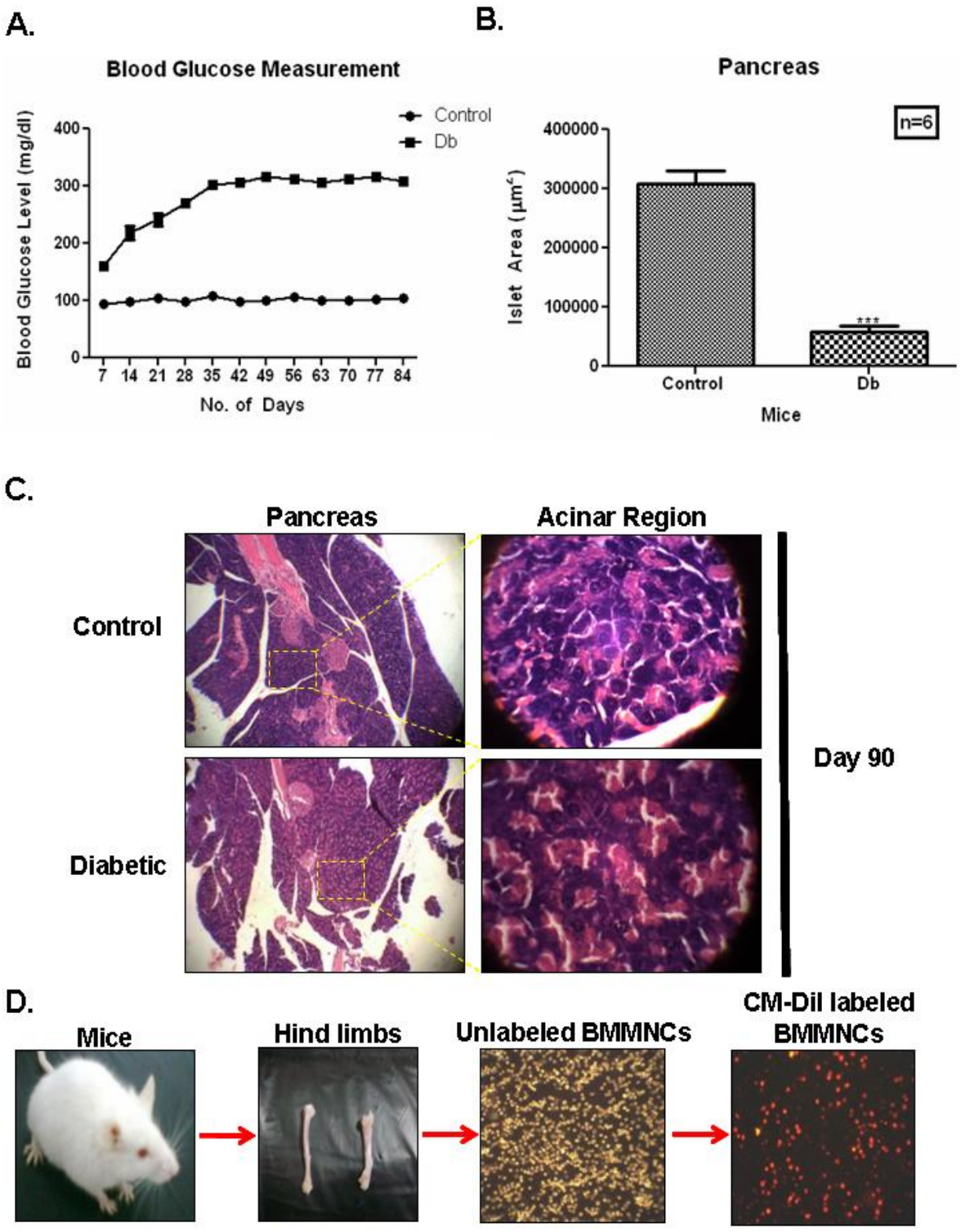

Figure 1. Induction of diabetes and BMMNC isolation: A, Blood glucose levels of STZ administered mice and vehicle injected (control) mice. Data are presented as mean ± SEM, with mean of STZ treated mice significantly differing from control mice having p value < 0.05; B, area of islets of control mice and STZ treated mice, data are presented as mean ± SEM, with mean of STZ treated mice significantly differing from control mice having p value < 0.05; C, H&E staining of pancreas tissue section of STZ treated mice and control mice showing that the acinar region of STZ treated mice has not been significantly damaged when compared to control mice; D, overall methodology of isolating and fluorescently labeling BMMNCs from tibia and femur of Swiss albino with fluorescent CM-DiI stain.

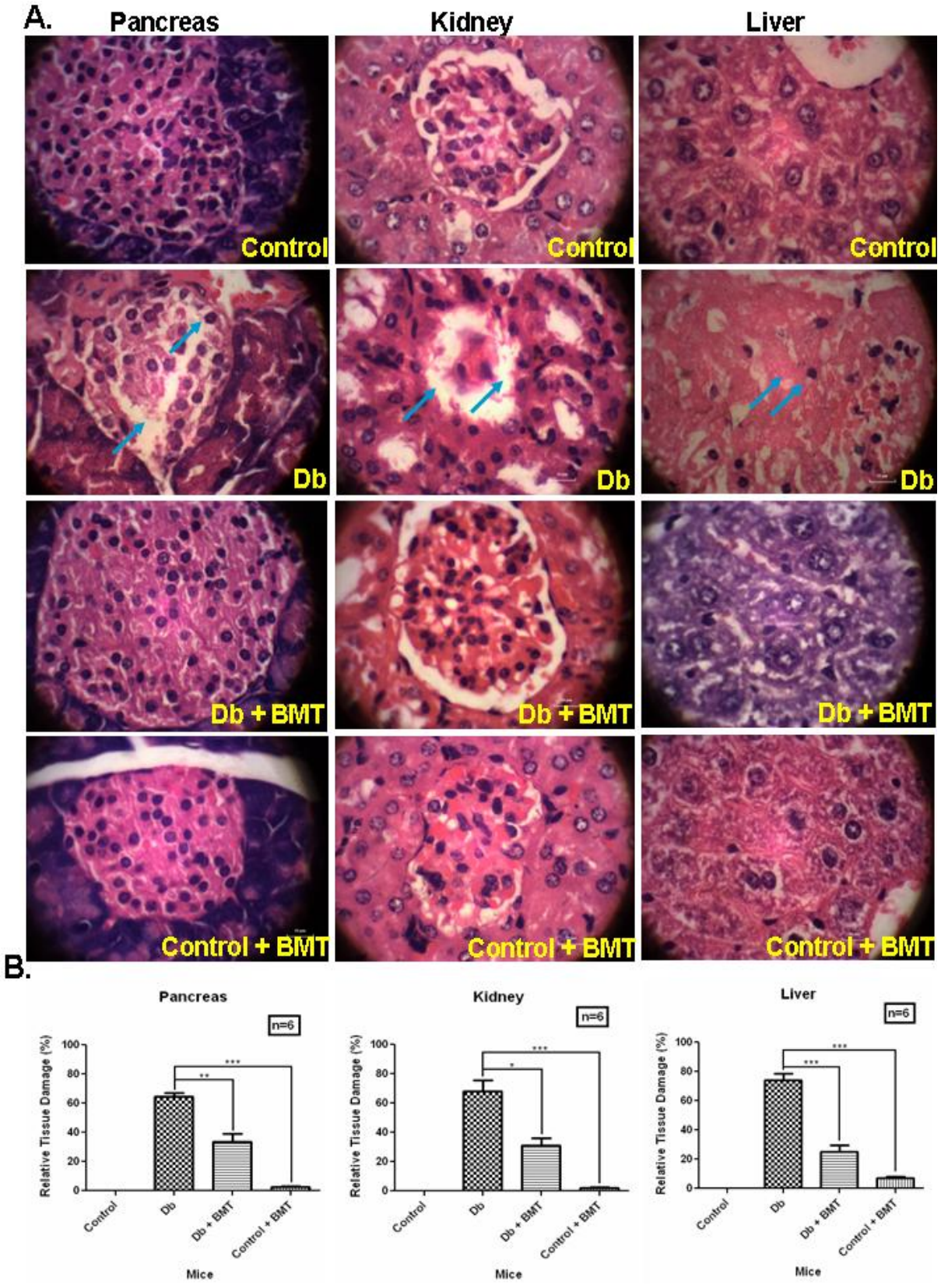

Figure 2. Effect of BMMNC transplantation in different organs: (A) H&E staining of pancreas, liver and kidney of control, diabetic, BMMNC injected diabetic and BMMNC injected control is shown; (B) the statistical analysis of the area of islet, glomerulus and hepatocyte nucleus radius in various organs is shown. Data are mean ± SEM with mean from each group significantly different from each other with p value < 0.05.

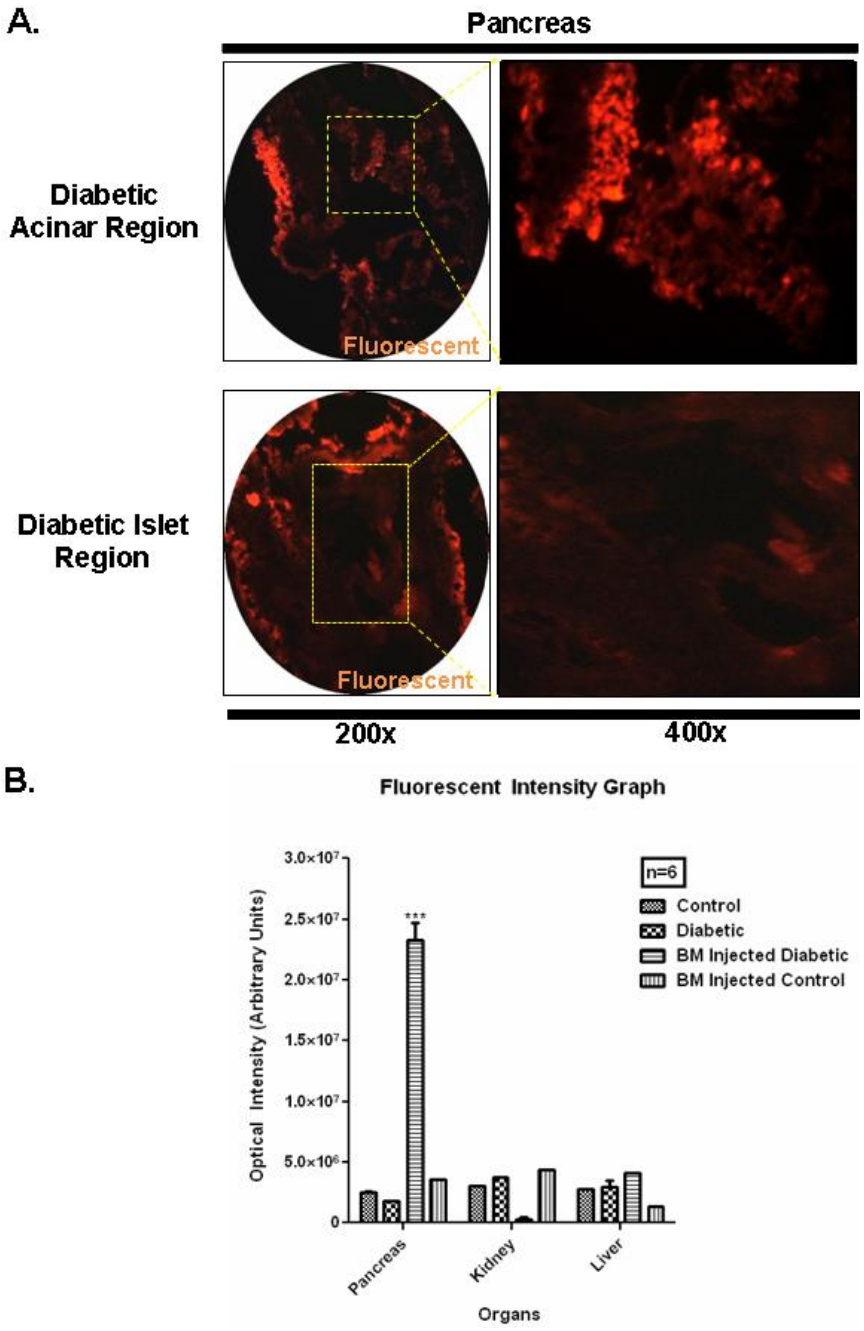

Figure 3. Tracking of CM-DiI labeled BMMNCs in different organs: (A) Fluorescent image of pancreatic acinar cells and pancreatic islet cells at 200x magnification and 400x magnification of the selected area; (B) the graph shows the overall fluorescent intensity of transplanted CM-DiI labeled BMMNCs engraftment in pancreas, kidney and liver; Data are represented as mean ± SEM with mean of fluorescence of pancreas significantly differing from mean of other organs with p value < 0.05.

irradiated STZ induced mice resulted in the regeneration of various organs which were damaged due to hyperglycemia. It was seen that irradiation did not have any effect on blood glucose levels of STZ induced mice (Data not shown). Incorporation of the transplanted cells in the acinar region of the pancreas and not in the islet regions could have helped in stimulating endogenous pancreatic precursor cells present in this region through various signaling mechanisms. This could have led to the process of transdifferentiation replenishing β-cells, as it

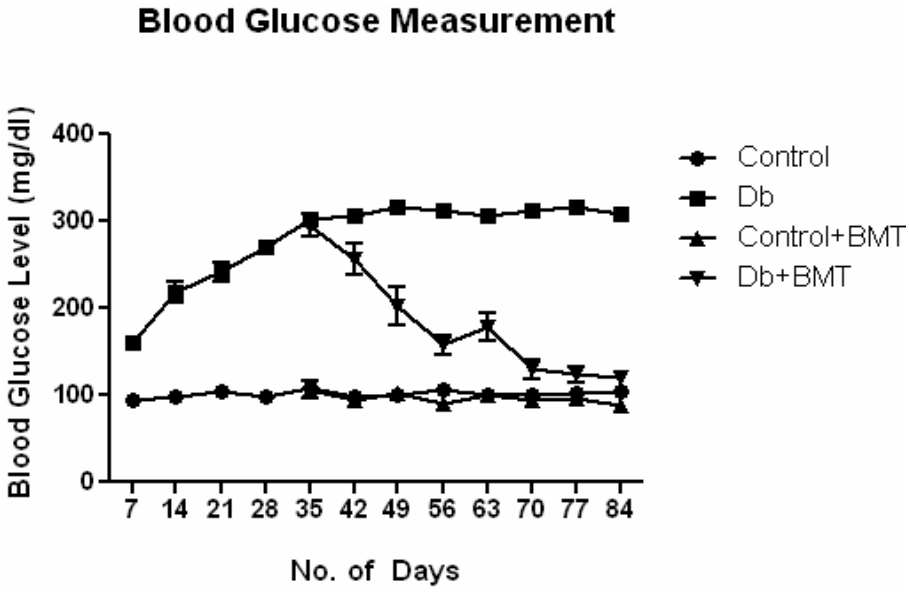

Figure 4. Blood glucose level before and after BMMNC transplantation in diabetic and control mice. The graph shows the weekly measurement of random blood glucose levels throughout the period of study. At day 36, STZ induced mice and buffer injected control mice were transplanted with CM-DiI labeled BMMNCs. Data are mean ± SEM with *p* value < 0.05.

has already been reported that acinar cells can transdifferentiate to insulin producing cells (Okuno et al., 2007) and regenerate pancreatic islets subsequently reverting to normoglycemic condition. In this study, we also obserde that the damage to other organs was caused due to poor glycemic control ameliorated after the transplantation of fluorescently labeled BMMNCs helping them to regenerate. Interestingly, BMMNCs did not incorporate at the site of injury or in its proximity in these organs but regenerated them. This could be due to engraftment of these cells in the distal site of affected organs which could have triggered the precursors cells to repair the damaged site, which in diabetes condition is impaired. It could also be well due to restoration of blood glucose levels reverting the system back to its normal pathophysiological condition helping the body to maintain homeostasis with repair mechanisms taking over their routine functions. Though, certain reports show that BMMNC transplantation fails to induce regeneration of islets (Akashi et al., 2008; Hamamoto et al., 2010). Our study supports the hypothesis that BMMNC transplantation aids in islet regeneration (Huang et al., 2010). However, islet regeneration could be due to bone marrow derived from pancreatic stem cell which may mediate the regeneration process through endothelial progenitor cells as VEGF is impaired in diabetic condition, which gets upregulated in the BMMNCs transplanted condition as shown in Figures 4B and C. We speculate that organ regeneration gets facilitated via VEGF through a paracrine mediated manner. Few other studies report that the regeneration of kidney and liver could be due to the use of stem cells (Iskovich et al., 2007; Levicar et al., 2007; Ezquer et al., 2008, Ezquer et al., 2009). Also, further analysis on the resulting phenotype of transplanted BMMNCs after the engraftment and lineage tracing of regenerated islets in pancreatic tissue would give us a very clear picture on regenerative capacities of BMMNCs and the potential of resident precursor cells to transdifferentiate. We reported that allogeneic unfractionated fluorescently labeled BMMNC transplantation in diabetes can regenerate damaged kidney and liver apart from the pancreas. This study does not answer or reason the regeneration of kidney and liver in absence of CM-DiI labeled BMNCs but it certainly opens new avenues for further research in the field of diabetes induced organ damage and regeneration potential of BMMNCs.

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Abbreviations: BMMNCs, Bone marrow mononuclear cells; **CM-DiI,** chloromethylbenzamido-1, 1'-dioctadecyl-3,3,3',3 tetramethylindocarbocyanine perchlorate; **STZ,**

streptozotocin; **PBS,** phosphate buffered saline; **CPCSEA,** Committee for the purpose of control and supervision of experiments on animals; **EDTA,** ethylenediaminetetraacetic acid; **RBC,** red blood corpuscles.

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