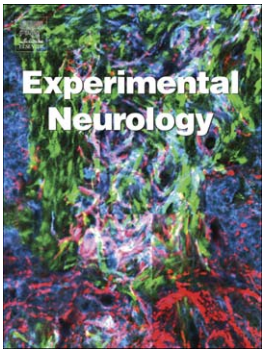

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Therapeutic potential of Mesenchymal Stem Cells for the treatment of diabetic peripheral neuropathy.

Marianna Monfrini[1,2], Elisabetta Donzelli[1], Virginia Rodriguez-Menendez[1], Elisa Ballarini[1], Valentina Alda Carozzi[1], Alessia Chiorazzi[1], Cristina Meregalli[1], Annalisa Canta[1], Norberto Oggioni[1], Luca Crippa[5], Federica Avezza[1], Sara Silvani[3], Barbara Bonandrini[3], Marina Figliuzzi[3], Andrea Remuzzi[3], Carla Porretta-Serapiglia[4], Roberto Bianchi[4], Giuseppe Lauria[4], Giovanni Tredici[1], Guido Cavaletti[1], Arianna Scuteri[1].

1- Experimental Neurology Unit and Milan Center for Neuroscience, School of Medicine and Surgery, University Milano-Bicocca, Via Cadore 48, 20900 Monza, Italy.

2- PhD Neuroscience Program, University Milano-Bicocca, via Cadore 48, 20900, Monza, Italy.

3- Department of Biomedical Engineering, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, 24126 Bergamo, Italy.

4- Neuroalgology and Headache Unit, IRCCS Foundation, Carlo Besta Neurological Institute, Milan, Italy.

5- Istovet, Laboratorio di Analisi Istopatologiche Veterinarie e Servizi per la Ricerca Scientifica, Besana Brianza (MB), Italy.

Corresponding author: Arianna Scuteri,

Experimental Neurology Unit,

School of Medicine and Surgery

University Milano-Bicocca

Via Cadore 48, 20900 Monza, Italy.

Phone: +39-02-64488119

Fax: +39-02-64488250

E-mail address: arianna.scuteri@unimib.it

Abstract

Type-1 Diabetes is generally treated with exogenous insulin administration. Despite treatment, a very common long term consequence of diabetes is the development of a disabling and painful peripheral neuropathy. The transplantation of pancreatic islets is an advanced alternative therapeutic approach, but its clinical application is still very limited, mainly because of the great number of islets required to complete the procedure and of their short-term survival. An intriguing method to improve the performance of pancreatic islets transplantation is the co-transplantation of Mesenchymal Stem Cells (MSCs), adult stem cells already known to support the survival of different cellular populations.

In this proof-of-concept study, we demonstrated using an *in vivo* model of diabetes, the ability of allogenic MSCs to reduce the number of pancreatic islets necessary to achieve glycemic control in diabetic rats, and overall their positive effect on diabetic neuropathy, with the reduction of all the neuropathic signs showed after disease induction.

The cutback of the pancreatic islet number required to control glycemia and the regression of the painful neuropathy make MSC co-transplantation a very promising tool to improve the clinical feasibility of pancreatic islet transplantation for diabetes treatment.

Keywords

Diabetic Neuropathy; Pancreatic Islet Transplantation; Mesenchymal Stem Cell; Diabetes.

Introduction

Diabetes is a metabolic disease with increasing incidence worldwide and with important social and economic effects. The disease is characterized by hyperglycemia, which may be caused by an alteration of insulin production secondary to degeneration of pancreatic beta cells (type-1 diabetes), or by an altered body response to insulin (type-2 diabetes) (Ezquer et al., 2012). The first consequence of the altered insulin production is the inability of the cells to collect and store glucose as energetic substrate, and therefore to produce Adenosine TriPhosphate (ATP). In addition, the high blood glucose concentration produces severe alterations such as protein glycosylation, increased lipid peroxidation, and production of high levels of free radicals (Davey et al., 2014; Kolka and Bergman, 2013; Premkumar and Pabbidi, 2013). These changes are hypothesized to be responsible for long term side effects of diabetes such as microcirculation damage and the onset of peripheral neuropathies (Davey et al., 2014). In fact, at least 1/3 of diabetic patients develop a peripheral neuropathy that even strict glycemic control can only partly prevent (Zenker et al., 2013). Current therapy based on exogenous insulin administration fails to properly adjust blood glucose, often producing peaks of hypo- and hyper-glycemia. Improvements to conventional insulin administration are under investigation, including the use of microinfusion pumps to achieve real time blood glucose, adjustment, but another promising therapeutic approach is based on the replacement of pancreatic islets (Ryan et al., 2005). The first results of pancreatic islets transplantation were encouraging, since this method allowed a better glycemic control with respect to traditional therapies and allowed the reduction of long term side effects (Warnock et al., 2008). However, at least two serious limitations are blocking the path to the diffusion of this method into clinical practice: i) the high number of pancreatic cells required for an effective replacement and ii) the short-term duration of transplanted tissue (Warnock et al., 2008). To obtain a good glycemic control at least 12000-16000 islets/kg are required for each patient, and this implies the use of more than one cadaveric donor (Warnock et al., 2008). Among the causes for the short term survival of transplanted pancreatic islets, immune response against the transplanted tissue and insufficient

vascularization are the most prominent (Tjernberg et al., 2008). Theoretically, the use of immune suppressive drugs could allow to prolong the transplanted tissue survival, but this treatment is potentially associated with severe side effects (Mallet et al., 2009).

A possible strategy to improve the outcome of pancreatic islet transplantation is to partner them with Mesenchymal Stem Cells (MSCs), exploiting their capacity to down-regulate immune cell activation (Waterman et al., 2012) and thus limiting the rejection of transplanted tissue (D'Addio et al., 2014). Moreover, MSCs are able to increase the survival of several cell populations, including neurons, both by direct contact (Scuteri et al., 2006) and by release of trophic factors (Crigler et al., 2006). This ability to increase cellular survival and to provide trophic support might also improve the function of organs and systems typically affected by diabetes, such as the kidney and the peripheral nerves.

So far, we have already demonstrated in a syngenic model of diabetes that 2000 pancreatic islets co-transplanted with MSCs have the same effect of 3000 pancreatic islets, thus reducing the number of pancreatic islets necessary to achieve normoglycemia (Figliuzzi et al., 2009). However, this syngenic model does not reproduce the real clinical setting, where pancreatic islets derive from cadaveric donors, and it could be difficult to obtain a sufficient number of MSCs from the same patients candidates for transplantation (Berman et al., 2010; Wu et al., 2013). Therefore, in order to mimic the most unfavorable clinical situation, it is necessary to explore the potential of an allogenic model co-transplanted with third party MSCs.

For all these reasons, besides checking if allogenic MSCs co-transplantation has the same effect on glycemic control achieved in the syngenic model, the aim of the present proof-of-concept study is to verify their effect on diabetic neuropathy and nephropathy. Moreover, we also verified if MSCs alone, without pancreatic islets, have any effect on diabetic animals. To avoid the use of immune-suppressive drugs, both islets alone and islets with MSCs were encapsulated before the transplantation into a microalginate matrix (Mallet et al., 2009).

Materials and Methods

The study was approved by the Milano-Bicocca University ethics committee (N 0012732/13) and it was performed in conformity with the institutional guidelines, in compliance with national (DL n. 26/2014), international (EEC Council Directive 2010/63/EU, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, US NRC, 1996) laws and policies and with the ARRIVE guidelines. Unless otherwise indicated**,** all reagents and materials were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Animals. Male 8 week-old Lewis rats (175-200 g, Envigo, Udine, Italy) were randomly divided into 5 groups of 8 animals: a) healthy controls, b) diabetic rats, c) diabetic rats transplanted in the peritoneal cavity with microencapsulated allogeneic islets (3000 islets/rat) (Remuzzi et al., 2009), d) diabetic rats transplanted with microencapsulated islets (2000 islets/rat) and 10^6 MSCs/rat, e) diabetic rats intravenously injected with 10^6 MSCs/250µl saline solution/rat. Diabetes was induced by intraperitoneal injection (i.p.) with 60 mg/kg Streptozotocin (STZ). Both body weight and glucose blood level were evaluated weekly. For an automated quantification of blood glucose level a commercial kit was used. In particular, after a 8-hour fast, a drop of blood from the caudal vein was placed on a test stick and inserted in the Accu-Chek® Compact Plus device (Roche Diagnostics, Italy). Blood glucose concentration was immediately calculated as mg glucose /dL blood.

Whole blood from tail vein was also used and serum obtained by the centrifugation of clotted blood at 2.500 g for 15 minutes at 4 °C for determination of the levels of Creatinine and Blood Urea Nitrogen in order to evaluate kidney function with an automatic MIRA PLUS system (Horiba ABX Diagnostic, Montpellier, France, Chiorazzi et al., 2012).

The transplantation with islets alone or islets-MSCs was performed two months after the diabetes induction, after the assessment of an established neuropathy, detectable by a decrease in Nerve Conduction Velocity (NCV) and impaired nociceptive thresholds. Animals were sacrificed 2 months after transplantation (i.e. 4 months after diabetes induction).

Pancreatic Islets. Pancreatic islets were isolated from Wistar rats (Envigo, Udine, Italy, body weight 250-300g), using an automatic procedure already reported (Scuteri et al., 2014). Briefly, the

pancreas of anesthetized rats were distended by injecting a collagenase P solution (Bushranger-Mannheim, Mannheim, Germany), removed and then loaded into a digestion chamber at 37°C. When optimum digestion time was reached, the chamber was flushed with 4°C Hanks' balanced salt solution (HBSS, Gibson Nitrogen Corporation, Paisley, Scotland) and digested tissue was purified by centrifugation on a Histopaque gradient (1.077 g/mL). Islets were cultured at 37°C in an atmosphere of humidified air + 5% CO_2 in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (EuroClone, Pero, Italy).

Mesenchymal Stem Cells (MSCs). MSCs were obtained from the bone marrow of 10 week-old Sprague Dawley rats (Envigo, Udine, Italy) by flushing the femur and tibia diaphysis with 2 ml/bone of α-MEM to which were added 2 mM L-glutamine and antibiotics (Donzelli et al., 2007). MSCs were expanded in α-MEM medium (Lonza Group Ltd, Basel, Switzerland) plus 20% ES cell screened Fetal Bovine Serum (FBS, Hyclone, Logan, UT, USA). Before transplantation, MSCs were stained for 1 hour at 37° C with red fluorescent dye DiI (30µg/ml, Life Technologies).

Islets or islets-MSCs were encapsulated into an alginate matrix, as previously described (Cornolti et al., 2009). Briefly, islets or islets-MSCs were suspended in 1.7% (wt/vol.) Manugel, a high viscosity, pure sodium alginate (Manugel DMB, Monsanto, Cambridge, UK) at a concentration of 1 islet/µl (3000 islets in 3ml) or 0,7 islets+333MSCs/µl (2000 islets + 10^6 MSC in 3 ml). Microbiological tests showed bacteria <5000 cfu/g and yeast and mould <300 cfu/g. We have no data on endotoxin content. The molecular weight cut-off of Manugel was about 560 kDa, as measured in our previous published data (Cornolti et al., 2009) by diffusion of FITC Ficoll (TdB Consultancy AB, Uppsala, Sweden) of graded sizes using high performance liquid chromatography (HPLC) separation (Chem Station LC-Agilent, Palo Alto,CA, USA). The islet–alginate mixture was extruded through an air jet droplet generator into a solution of 100mM $CaCl_2$. The resulting gel beads had diameters ranging from 800 to 950 µm. After complete gelification, the beads we washed in calcium-free Krebs–Ringer, then in Krebs–Ringer–Hepes 25mM, and then cultured overnight in

complete medium at 37 °C. The microcapsules containing 3000 islets or 2000 islets with MSCs were implanted into the rat peritoneal cavity through a Trocar.

Peripheral Nerve Studies

Nerve conduction velocity (NCV). NCV was measured in the tail nerve before and after 2 and 4 months from diabetes induction. Neurophysiologic assessment was determined in the tail of each rat as previously described (Figliuzzi et al., 2013; Renn et al. 2011; Cavaletti et al., 2000). Briefly, the NCV was assessed by placing recording ring electrodes distally in the tail, while the stimulating ring electrodes were placed 5 and 10 cm proximally with respect to the recording point. The latencies of the potentials recorded at the two sites (peak to peak) after nerve stimulation was determined, and NCV was calculated accordingly. All the neurophysiologic determinations were performed under standard conditions in a temperature-controlled room and with vital parameter monitoring of the rats by using the instrument Myto II (EBNeuro Spa, Firenze, Italy).

Nociceptive evaluations. The nociceptive threshold to radiant heat was quantified using the hot-plate paw withdrawal test (Ugo Basile Biological Instruments, Comerio, Italy) as previously described (Bianchi et al., 2004). The mechanical nociceptive threshold was quantified using the Randal-Selitto paw withdrawal test with an Analgesy-meter (Ugo Basile Biological Instruments). Each test was performed at baseline and every four weeks (Figliuzzi et al., 2013).

Myelinated fibers g-ratio evaluation. At the end of the experiment the left sciatic nerve was explanted, fixed by immersion in 3% glutaraldehyde solution for three hours, embedded in epoxy resin and cut to obtain 1 μm-thick sections. These sections were stained with toluidine blue and 60x pictures of four random different fields of each nerve section were taken using a Nikon Eclipse E200 light microscope (Leica Microsystems GmbH, Wetzlar, Germany). One nerve section per animal, three animals per group of treatment, were subjected to a morphometric analysis using an automatic image analyzer compiled by Immagini e Computer SNC (Milano, Italy). Approximately

1500 fibers/group were measured and data were then analyzed with GraphPad Prism statistical package (GraphPad Software, San Diego, CA, USA).

Intraepidermal Nerve Fiber Density (IENFD). At the end of experiment, hind paw skin specimens were collected and plantar glabrous skin was separated from the underlying metatarsal bones. 5-mm round samples were taken, immediately fixed in 2% paraformaldehyde-lysine and periodate sodium for 24 hours at 4 °C and cryoprotected overnight. The skin biopsies were serially cut with a cryostat to obtain 20-μm-thick sections and three sections were randomly selected and immunostained with rabbit polyclonal anti-protein gene product 9.5 (PGP 9.5, Bio-Rad AbD Serotec Ltd., Kidlington, UK) using a free-floating protocol. The total number of PGP 9.5-positive IENF in each section was counted under a light microscope (40X magnification); only the fibers that crossed the dermal-epidermal junction were counted while secondary branching within the epidermis was excluded. The length of the epidermis was measured using a computerized system (Microscience Inc., Seattle, WA, USA) and the density of IENF was calculated as a ratio between the number of fibers and the length in mm (Canta et al., 2016; Lauria et al., 2005).

Histopathology and Oxidative Stress Analysis.

Tissue Collection. At the end of the experiment kidneys and pancreas were explanted from each animal, fixed in Bouin's solution (Bio-Optica, Milan, Italy), and then embedded in paraffin. Kidney sections were stained with the periodic acid–Schiff's (PAS) reagent technique. The presence of Armanni-Epstein lesions, peculiar and significant renal histopathological changes easily detectable in chronic diabetic rats (Greaves 2012; Zhou et al. 2013), was then analyzed under light microscope by a blinded experienced observer. Pancreas sections were processed with alkaline phosphate-Fast Red technique and then stained with mouse-anti insulin as previously reported (Figliuzzi et al., 2013). Briefly, slides were steeped in PBS + 1% bovine serum albumin for 30 minutes at room temperature, and then stained with a mouse anti-insulin primary antibody (1:3000; Sigma-Aldrich) for 2 hours at room temperature. A horse biotinylated anti-mouse antibody (1:200; Vector

Laboratories, Burlingame, CA) was used as secondary antibody. Slides were then incubated with alkaline phosphatase-conjugated streptavidin (Roche, Mannheim, Germany) and counterstained with Harris-type hematoxylin (Bio-Optica, Milan, Italy). Sections were then analyzed under light microscope to study insulin distribution.

β-Cell Morphometric Analysis. Tissue sections were examined under a Zeiss light microscope. For each sampling section, the area was scanned with a 20x objective and the volume density of insulin-positive cells was determined by point counting using an orthogonal grid (20x15 lines) overlaid on the image of the stained section. For each field, grid intersection points coinciding with positive-staining and negative-staining areas were counted. The total number of examined intercepts per animal varied from 8062 to 37973 (mean 21004). Volume density was calculated as the percent ratio between grid points in the positive areas and the total points in the pancreatic tissue.

Thiobarbituric acid-reactive substances (TBARS). Plasma samples and the entire spinal cords were collected at sacrifice and the production of thiobarbituric acid-reactive substances (TBARS) analyzed. To plasma and spinal cord homogenates 1 µl/ml of EDTA and GSH were added (respectively 1.34 and 0.65 mmol/L final concentrations). TBARS were determined by modifications of the methods of Mihara et al. (1980) and Yorek et al (2002). The data was reported as mmol of TBARS produced per ml of serum or mg of spinal cord.

Statistical analysis. Analysis of variance (ANOVA), with the Tukey-Kramer as post-test, was performed using the GraphPad Prism statistical package (GraphPad Software). Data are expressed as mean ± Standard Deviation (SD). A *P*-value below 0.05 was considered statistically significant.

RESULTS

Blood Glucose Level and Body Weight.

Since it has been already demonstrated in a syngenic model that 2000 pancreatic islets have the same effect of 3000 pancreatic islets on blood glucose level if transplanted in presence of MSCs (Figliuzzi et al., 2009), here we verified if the same result was achievable also in a allogenic model.

Control rats showed a progressive gain of weight, while in diabetic rats the growth rate slightly decreased. After transplantation, the group transplanted with islets only or with islets and MSCs started to gain weight, with a similar trend with respect to control rats, whereas the growth rate of the group transplanted only with MSCs remained similar to the diabetic one (Fig. 1a).

Glycemia in diabetic rats was markedly increased with respect to control animals, but following transplantation blood glucose levels quickly decreased to values similar to control normal rats, and the levels remained significantly lower with respect to diabetic rats during all the observation period. No statistically significant differences were observed between rats transplanted with 3000 islets and rats transplanted with 2000 islets and allogenic MSCs (Fig. 1b), thus confirming the result achieved in the syngenic model.

Peripheral Neuropathy Studies

To verify if the presence of MSCs could have a positive effect on diabetic neuropathy, or to give a further improvement with respect to islets alone, we investigated the functionality of the peripheral nerves with a multimodal approach.

Mechanical nociceptive threshold

The analysis of the mechanical nociceptive threshold using the Randall Selitto test evidenced a significant reduction of the paw pressure tolerated by diabetic animals with respect to control rats. On the contrary, the diabetic rats transplanted with islets only or with both islets and MSCs showed a significant improvement, nearly 50% of the nociceptive threshold, with a very similar trend among both groups (Fig. 2a, P value < 0.01).

Thermal nociceptive threshold

With the Hot Plate test we analyzed the latency of animal paw withdraw. As shown in Fig. 2b, after 8 weeks in all diabetic groups there was a significant increase of the withdraw latency with respect to control animals. After 16 weeks the latency values resulted slightly reduced in all diabetic groups, result which may be due to the fluctuations typical of behavioral tests (Whishaw and Kolb, 2004; Le Bars et al., 2001; Gardmark et al., 1998). However, the reduction of latency values was

more marked and more evident only in rats transplanted with both islets and MSCs, the only which showed the same values of healthy animals (Fig. 2b, P value < 0.01)..

Nerve Conduction Velocity (NCV)

The NCV was reduced in diabetic rats, but it was nearly completely restored after transplantation with islets (P value < 0.05), and after transplantation with islets and MSCs (Fig. 2c, P value < 0.01). The NCV of MSCs-transplanted rats was similar to those of diabetic rats, showing no direct effect of MSCs on the functional activity of peripheral nerves.

Intra Epidermal Nerve Fiber Density (IENFD)

In rats with diabetic neuropathy, there was a significant decrease of the number of cutaneous unmyelinated fibers, evaluated by IENFD assessment (P value < 0.05 vs A), but all groups transplanted with MSCs showed an increased IENFD, in particular the group of animals transplanted with MSCs and pancreatic islets (Fig. 3, P value < 0.01 vs B).

g-ratio analysis

To further analyze the effect of MSCs on the nervous system, we performed a morphological and morphometric analysis of sciatic nerve sections. At the pathological level, as shown in Fig. 4a, in the diabetic rats several myelinated fibers (particularly those with the largest size) showed marked myelin irregularities, with evident infoldings of the myelin sheath. These irregularities were very rare in the groups that received islet or islet + MSC transplantation (groups C and D), while they were present in the group E that received MSCs only. When myelin thickness was measured in relationship with the axonal size, significant increase in the g-ratio (suggesting reduction of myelin thickness) was observed in diabetic rats vs. controls (P value < 0.01). This alteration was significantly reduced in the animals treated with islet or islet + MSC transplantation, but also in the animals receiving MSCs only (Fig. 4b).

Plasma and Tissue TBARS.

Since oxidative stress is likely to play an important role in development of diabetes long term side effects, we evaluated and compared both plasma and tissue (spinal cord) TBARS, an index of lipid peroxidation, across the different experimental groups. Both for tissue and plasma samples the TBARS values increased, as expected, in diabetic animals. The transplantation with islets, or islets and MSCs led to a restoration of TBARS values similar to those observed in control untreated rats (Fig. 5a and b).

Kidney Pathology.

An alteration frequently observed in diabetic patients is nephropathy, which is a mirror of the damage occurring to microvessel circulation.

The severity of diabetic STZ-induced nephropathy was evaluated considering only Armanni-Epstein lesion as peculiar and significant renal histopathological changes easily detectable in chronic diabetic rats. Armanni-Epstein lesion is a vacuolar nephropathy associated with poorly controlled diabetes that involves selective renal epithelial cell glycogen accumulation in distal and collecting tubules of diabetic rats. These changes well correlated with blood glucose level and they appear as swollen clear cells with prominent PAS positive cell boundaries and condensed nuclei (Greaves 2012; Zhou et al. 2013). As shown in Fig. 6, these alterations were absent in untreated healthy controls, while they became severe and diffuse in diabetic rats (group B), as well as in diabetic rats treated with MSCs only (group E). On the contrary, in diabetic rats transplanted with islets with or without MSCs (groups C and D), such lesions were strongly reduced at the end of the 4-month period of observation. Similarly, also the index of kidney function, creatinine and blood urea nitrogen, which were increased in diabetic animals (creatinine: 0,73±0,03mg/dL vs 0,48±0,02mg/dL in control; blood urea nitrogen: 61,6±7,25mg/dL vs 33±1,41 mg/dL control), resulted decreased in a statistically significant manner ($p<0.01$) in both the transplanted groups (creatinine: 0.53±0,05 mg/dL in both groups; blood urea nitrogen: 43.5±4,6 mg/dL in group C and 46± 9,45mg/dL in group D).

Endogenous pancreatic islet regeneration.

In order to verify if the transplantation of islets or islets-MSCs can also protect the host islets, we analyzed insulin expression in pancreas sections from each group of animals. Pancreas from diabetic rats showed massive islets degeneration, with the disappearance of insulin-positive cells in pancreas parenchyma (Fig. 7a). After the transplantation of pancreatic islets, with or without MSCs, there was a mild islet regeneration (Fig. 7a), which was however statistically significant at the β-Cell morphometric analysis only for the group co-transplanted with islets and MSCs (Fig.7b). On the contrary, after the administration of only MSCs the insulin distribution was very similar to that observed in diabetic rats (Fig.7a and 7b).

Discussion

In this proof-of-concept study we demonstrated that allogenic MSCs can be useful for the transplantation therapy of type-1 diabetes not only to reduce the number of pancreatic islets necessary to achieve normoglycemia, but also to relief diabetic neuropathy and nephropathy. In fact, the presence of MSCs gave significantly better results with respect to the islet transplantation alone, even if the blood glucose levels were very similar between the two groups.

The main limits for the therapeutic use of pancreatic islet transplantation are the great number of islets required, and the necessity to treat patients with an immune-suppressive therapy to allow long-term survival. In a previous study (Figliuzzi et al., 2009), a sub-optimal number of syngenic pancreatic islets, insufficient to guarantee the normalization or at least the decrease of glycemia values, became effective thanks to the concurrent presence of MSCs. In this paper we added a further piece to MSC knowledge by verifying the ability of these cells: i) to reduce the number of pancreatic islets needed to achieve functional replacement, despite their allogenic origin; ii) to bypass the use of immune-suppressive drugs thanks to their immune-modulating properties and to alginate encapsulation; iii) to lead to an improvement of diabetic neuropathy and nephropathy; iv) to produce their effects without the need for pancreatic islets transplantation, (thus sufficing alone).

The first remark is that MSCs alone, i.e. without pancreatic islets, are unable to improve the course of diabetes in terms of glycemia and body weight, while regarding diabetes complications we

observe an improvement in IENFD and in morphometric analysis (later discussed). This result partly tallied with some literature reports which observed, after the local transplantation of only MSCs in the hind limb muscle, a significant but transient improvement of neuropathic symptoms, such as NCV, probably due to the local release of trophic and angiogenetic factors (Han et al-. 2016; Shibata et al., 2008). These studies, however, also demonstrated that such administration of only MSCs did not have any systemic effects, since their efficacy was limited to the injection site (Shibata et al., 2008). Analyzing the systemic effect of MSCs alone, other authors demonstrated that a single injection of undifferentiated MSCs alone was insufficient to normalize the blood glucose levels, while multiple injections (Banerjee et al., 2005; Lv et al., 2014), as well as the use of engineered MSCs (Dave et al., 2013; Tsai et al., 2014; Van Pham et al., 2014), led to more promising results.

The chance to reduce the needed number of islets and to make effective an islet amount by itself insufficient to reduce the blood glucose level has been already demonstrated in syngenic models (Figliuzzi et al., 2009), but this is the first study confirming the effectiveness of MSC co-encapsulated with pancreatic islets using an allogenic model. The reduction of glycemia, however, was only temporary in our experimental paradigm, since 6 weeks after the transplant the blood glucose started to increase in transplanted animals. This increase had the same trend for islets transplanted alone or in combination with MSCs, thus suggesting a natural decline of islet survival that MSCs, at the selected dose, were unable to counteract, and which can likely explain why the allogenic transplanted tissue had a shorter duration with respect to other syngenic models described in literature (Figliuzzi et al., 2009).

Although MSC direct contact seemed to be ineffective to support pancreatic islet survival, it was however able at least temporarily to improve their functionality. There are several possible mechanisms by which MSCs may affect pancreatic islet functionality. We demonstrated *in vitro* a differentiation of MSCs into insulin-releasing cells after the direct contact with pancreatic islets (Scuteri et al., 2014), however, also the release of anti-inflammatory and anti-oxidant factors,

essential components of MSC positive action and neuro-recovery, can play a pivotal role for islet functionality strengthening as also observed in other models (Ezquer et al., 2012; Yeung et al., 2012; Huang et al., 2014). Another possible mechanism has been suggested by Taneera and coll. (2006), who demonstrated the ability of MSCs to lead to the regeneration of endogenous pancreatic islets (Ezquer et al., 2014; Taneera et al., 2006; Tsai et al., 2014), and also in our model a certain degree of islet regeneration was evident. Remarkably, this effect was evident only in pancreas derived from animals receiving both MSCs and exogenous pancreatic islets, while MSCs alone were unable to promote endogenous islet regeneration.

Lowering of glycemia was paralleled in our study by the relief of common side effects of diabetes, reducing the alterations of kidney morphology, as expected and as already observed in literature (Borg et al., 2014) and improving nerve functionality and pathology. However, our most intriguing result is the observation that the presence of MSCs led to improvement of some neuropathic parameters, particularly those affecting small fibers, as evidenced by the increased intra epidermal small fiber density, by pain relief, in particular the thermal nociceptive threshold. At the pathological level, and in agreement with the NCV results, islet transplantation (both alone or in combination with MSCs) clearly reduced the occurrence of large myelinated fibers with evident myelin sheath irregularities which were present on the contrary in the diabetic as well as in the animal treated with MSC only. However, it is possible that some effect of MSC *per se* is also present, since the g-ratio analysis of sciatic nerve fibers suggested that these cells can directly act on myelination despite the persistence of the previously cited myelin irregularities. Although these results are in line with previous data obtained *in vitro* (Yang et al., 2008; Ravasi et al., 2013), further analysis are needed to clarify the possible functional significance of this observation. Moreover, another possible explanation for this improvement may be ascribed to a direct support to neuronal survival by MSCs, as already reported in different papers (Crigler et al., 2006; Scuteri et al., 2006), as well as, based on our observations in particular on spinal cord TBARS, to the ability

of MSCs to reduce the oxidative stress, which plays a pivotal role into diabetic neuropathy onset and development (Premkumar and Pabbidi, 2013).

Conclusions

In conclusion, based on the results of our proof-of-concept study, it can be hypothesized that MSCs can play a double role in diabetic disease, indirectly by boosting the functionality of the co-transplanted pancreatic islets and promoting endogenous islets regeneration, and directly by protecting the nerve fibers damaged in diabetic neuropathy. Concerning the molecular mechanisms, it is likely that all the manifold positive properties of MSCs are involved, from the direct survival support to the release of trophic, regenerating and anti-inflammatory factors, which all together lead to a widespread benefit, moreover it is yet to explore the possible effect of increasing MSC doses. For all these reasons MSCs should be considered a potential tool to improve the clinical feasibility of pancreatic islet transplantation for the therapy of diabetes mellitus. Provided that optimization of their use would allow to prolong their effect to a longer period than the one achieved in this study.

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Figure Legends

Fig. 1. Body weight and Blood Glucose Level analysis.

a) Body weight were measured weekly, and weights were reported as mean ± SD. **P* value < 0.05 vs A and B. # *P* value < 0.05 vs A.

b) Blood glucose levels was measured weekly, and glucose concentrations were reported as mean ± SD. **P* value < 0.05 vs B. # *P* value < 0.05 vs A.

A: Control rats; B: Diabetic rats; C: Diabetic rats + 3000 Pancreatic Islets; D: Diabetic rats + (2000 Pancreatic Islets+ MSCs); E: Diabetic rats + MSCs.

Fig. 2. Behavioral Tests and Neurophysiological analysis.

a) Mechanical Nociceptive threshold was evaluated by Randal Selitto test before transplantation and at the end of the experiment; results were reported as tolerated grams on the paw (mean ± SD); b) Thermal Nociceptive threshold was evaluated by Hot Plate test before transplantation and at the end of the experiment, results were reported as time of latency of paw withdrawal (mean ± SD); c) Nerve conduction velocity was measured in the caudal nerve before transplantation and at the end of the experiment, velocities were reported as mean ± SD. A: Control rats; B: Diabetic rats; C: Diabetic rats + 3000 Pancreatic Islets; D: Diabetic rats + (2000 Pancreatic Islets+ MSCs); E: Diabetic rats + MSCs. * *P* value < 0.05 vs B and E; ** *P* value < 0.01 vs B and E; $^{\#\#}$ *P* value < 0.001 vs B and E; §§ *P* value < 0.001 vs A.

Fig. 3 Intra Epidermal Nerve Fiber Density analysis.

a) Representative sections of skin punch were obtained from Control (ctrl) rats, Diabetic rats, Diabetic rats receiving 3000 pancreatic islets, Diabetic rats co-transplanted with 2000 pancreatic islets and MSCs, and from Diabetic rats receiving only MSCs. Arrows indicated the unmyelinated axons. Scale bar 50 μm. b) Unmyelinated axons per millimeter of skin were counted (mean ± SD). ** *P* value < 0.01 vs B; $^{\#}$ P value < 0.05 vs A.

Fig 4. Morphological analysis of sciatic nerve.

a) Representative images of sciatic nerve sections from (A) Control rats; (B) Diabetic rats; (C) Diabetic rats receiving 3000 islets; (D) Diabetic rats receiving 2000 islets + MSCs; E) Diabetic rats receiving only MSCs. Scale bar 20μm. b) Frequency distribution of g-ratio values and g-ratio raw data. ** *P* value < 0.01 vs B.

Fig. 5. Oxidative Stress evaluation.

The production of ThioBarbituric Acid Reactive Substance (TBARS), an index of lipid peroxidation, was evaluated in Plasma (a), and in the Spinal cord (b) of CTRL rats (A), Diabetic rats (B), Diabetic rats receiving 3000 pancreatic islets (C), Diabetic rats co-transplanted with 2000 pancreatic islets and MSCs (D), and Diabetic rats receiving only MSCs (E). Data were reported as mean ± SD. * *P* value < 0.05 vs B and E; ** *P* value < 0.01 vs B and E; $^{\#}$ *P* value < 0.01 vs A.

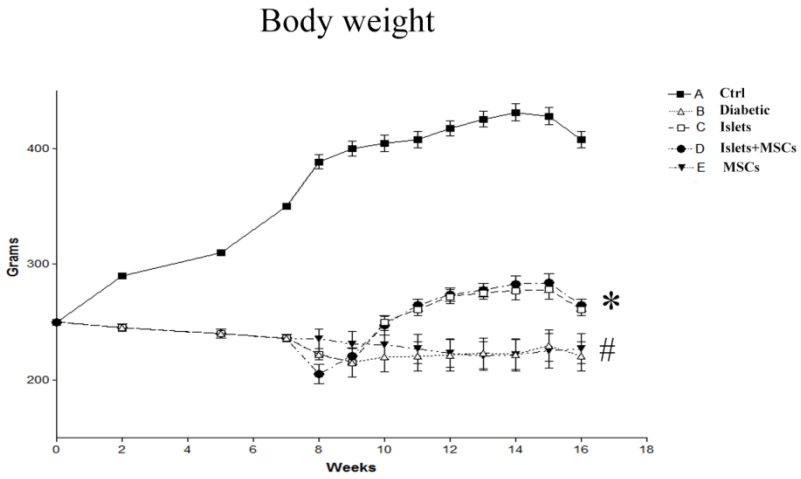
Fig. 6 Kidney Histopathology.

PAS staining of kidney sections from (A) Control rats; (B) Diabetic rats; (C) Diabetic rats receiving 3000 islets; (D) Diabetic rats receiving 2000 islets + MSCs; E) Diabetic rats receiving only MSCs. Scale bar 200μm. Table 1: Evaluation of Armanni-Epstein lesions in distal tubules: Armanni-Epstein lesions of distal and collecting renal tubules were: absent in all the untreated healthy controls (group A); from absent to moderate and diffuse in diabetic rats transplanted with islets with or without MSCs (groups C and D); severe and diffuse in untreated diabetic rats (group B) and in diabetic rats treated with MSCs only (group E). The pathological score was assessed according to literature criteria (Greaves 2012; Zhou et al. 2013).

Fig. 7 Pancreas Histopathology.

a) Insulin staining with alkaline phosphate-Fast red technique evidenced beta cells. (A) Control rats; (B) Diabetic rats; (C) Diabetic rats receiving 3000 islets; (D) Diabetic rats receiving 2000 islets + MSCs (E) Diabetic rats receiving only MSCs. Scale bar 50μm. b) β-Cell morphometric analysis to study the density of insulin-positive cells. §§ *P* value < 0.001 vs A; ** *P* value < 0.01 vs B; $^{\#\#}$ *P* value < 0.01 vs E.

a



b

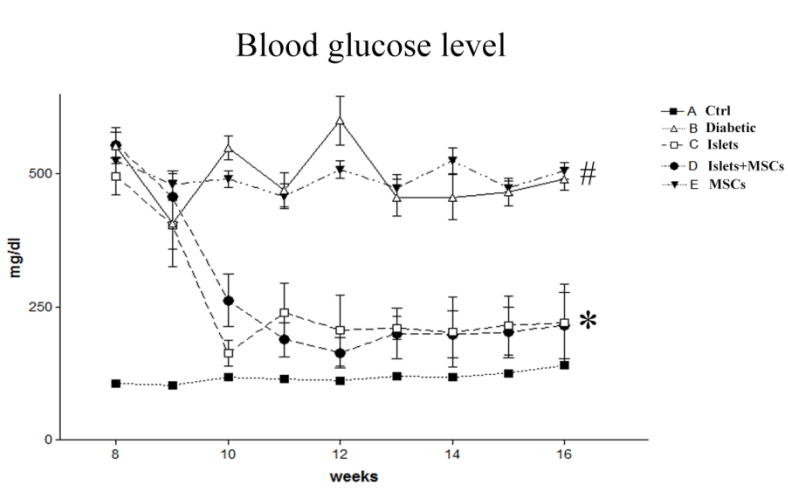


Fig. 1

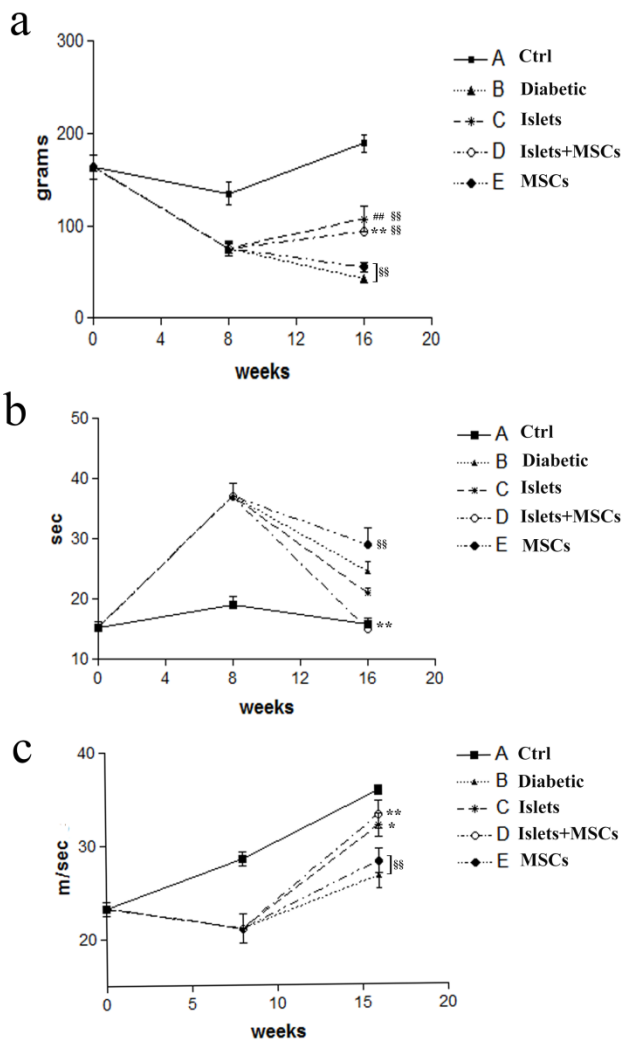


Fig. 2

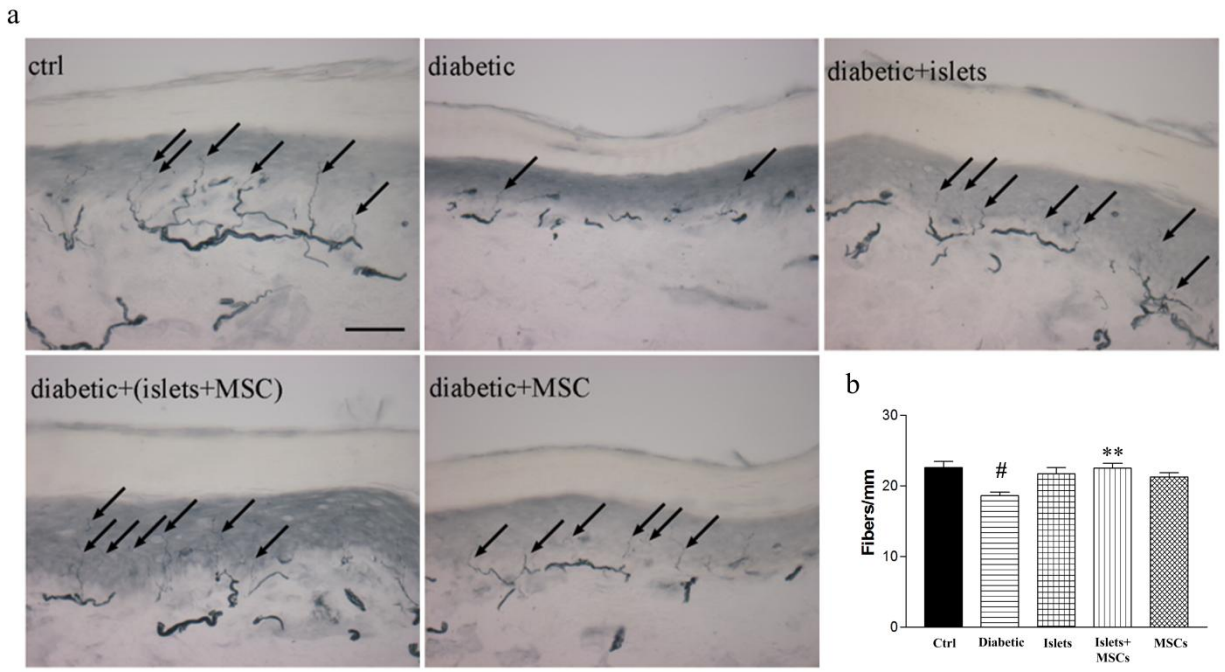
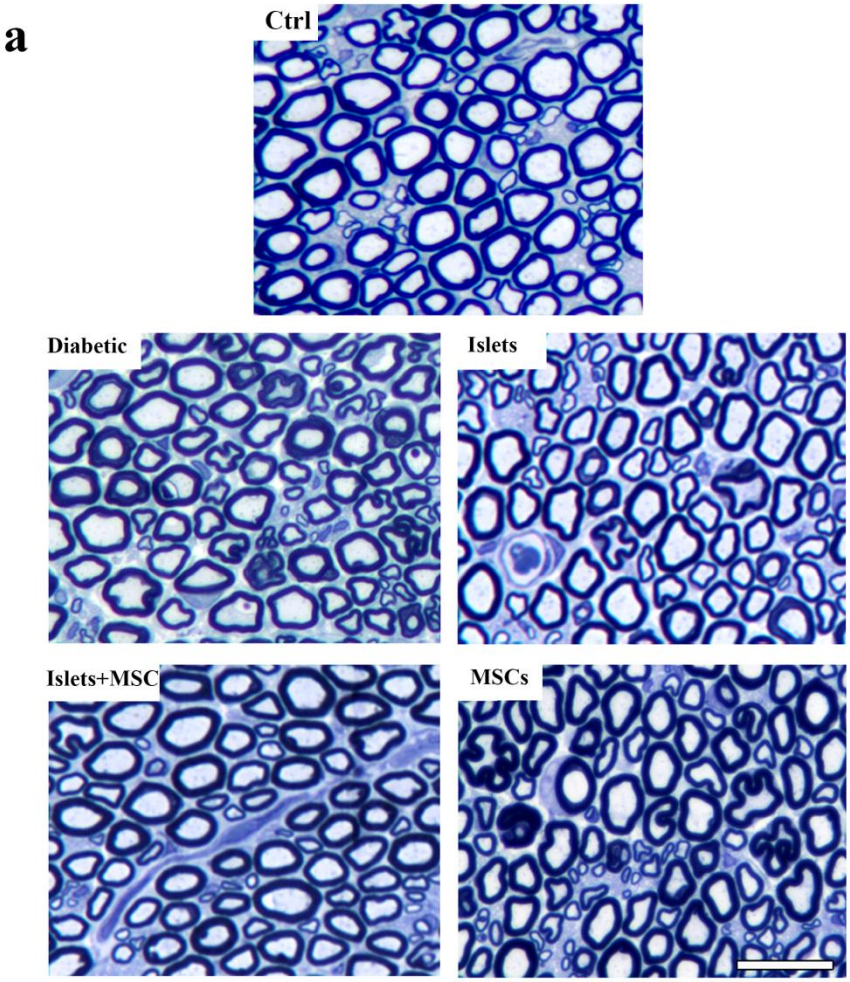


Fig. 3



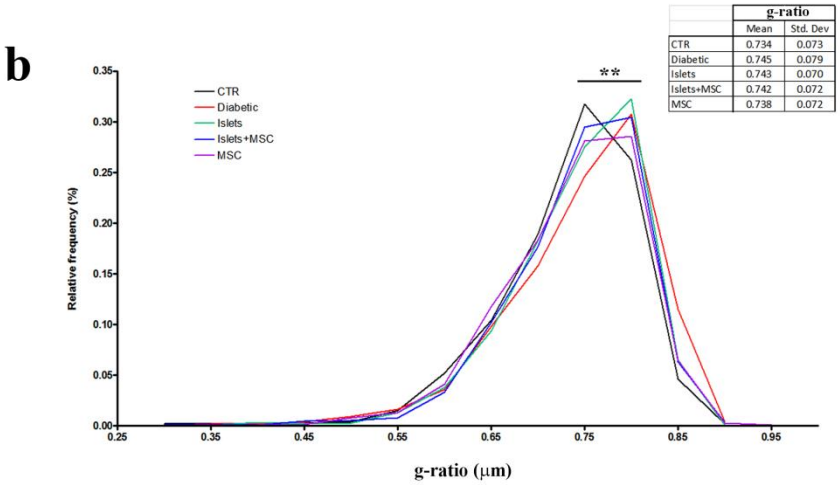


Fig. 4

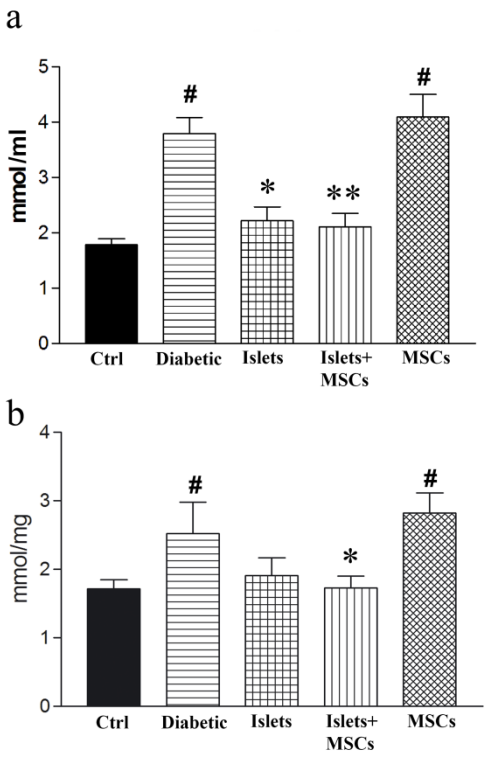


Fig. 5

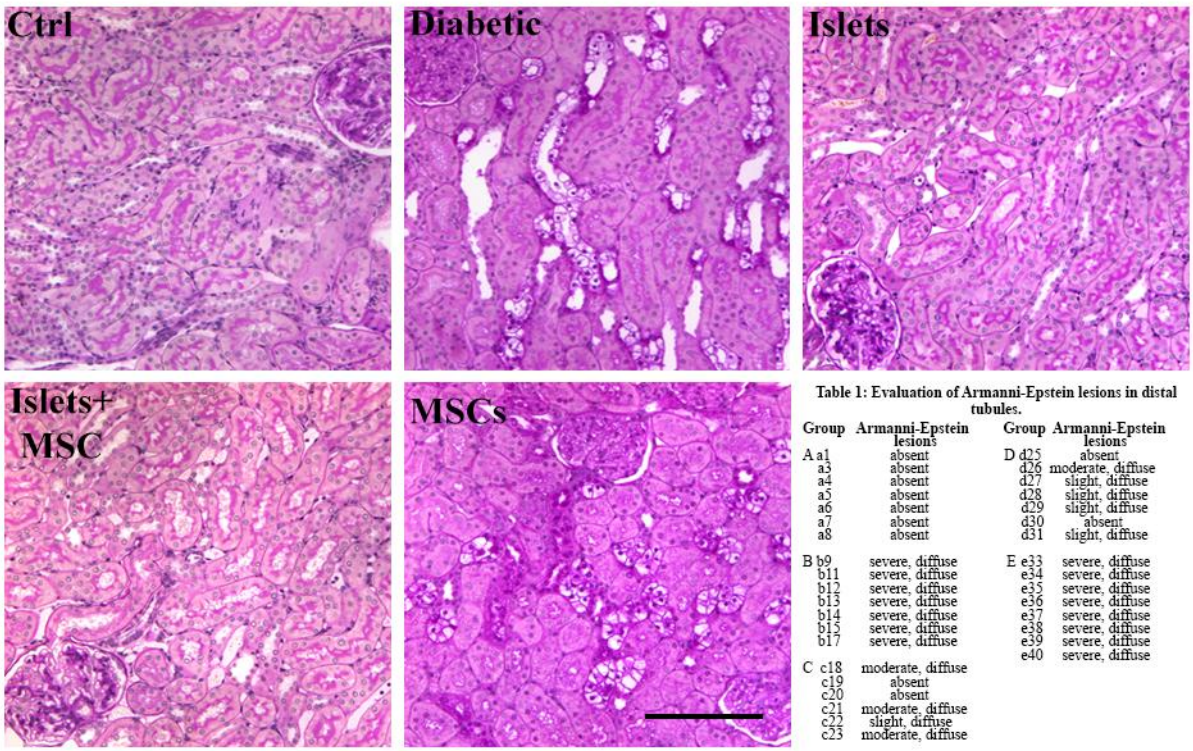


Table 1: Evaluation of Armanni-Epstein lesions in distal tubules.

Group		Armanni-Epstein lesions
A	a1	absent
	a3	absent
	a4	absent
	a5	absent
	a6	absent
	a7	absent
	a8	absent
B	b9	severe, diffuse
	b11	severe, diffuse
	b12	severe, diffuse
	b13	severe, diffuse
	b14	severe, diffuse
	b15	severe, diffuse
	b17	severe, diffuse
C	c18	moderate, diffuse
	c19	absent
	c20	absent
	c21	moderate, diffuse
	c22	slight, diffuse
	c23	moderate, diffuse
D	d25	absent
	d26	moderate, diffuse
	d27	slight, diffuse
	d28	slight, diffuse
	d29	slight, diffuse
	d30	absent
	d31	slight, diffuse
E	e33	severe, diffuse
	e34	severe, diffuse
	e35	severe, diffuse
	e36	severe, diffuse
	e37	severe, diffuse
	e38	severe, diffuse
	e39	severe, diffuse
	e40	severe, diffuse

Fig. 6

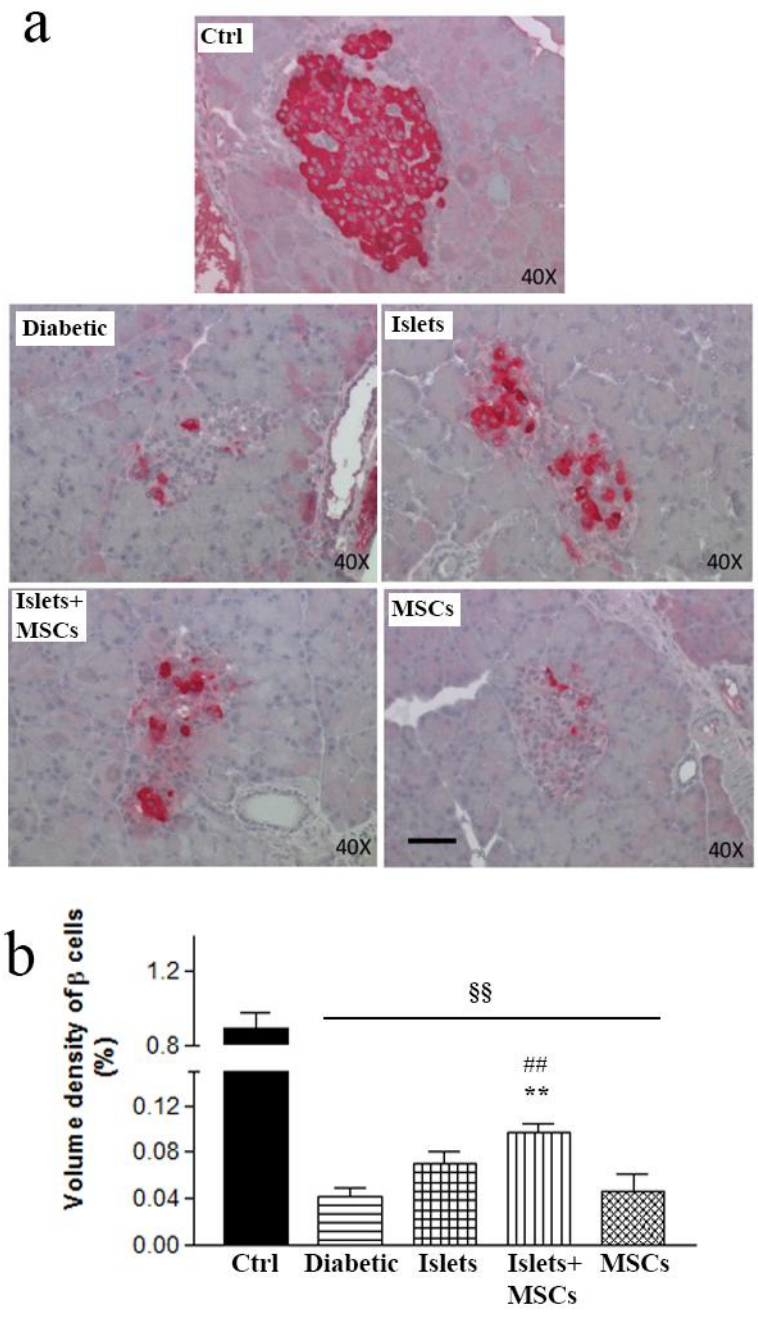


Fig. 7

Highlights (max 85 characters)

1) MSCs reduce the number of Pancreatic Islets necessary to control blood glucose level;

2) MSCs co-transplanted with Pancreatic Islets ameliorate diabetic neuropathy;

3) MSCs co-transplanted with Pancreatic Islets reduce nephrotoxicity.