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STRICT GLYCAEMIC CONTROL BY ISLET TRANSPLANTATION AND INSULIN ADMINISTRATION COULD RELIEVE LONG-TERM COMPLICATIONS AND RESCUE THE RESIDUAL ENDOGENOUS PANCREATIC β CELLS.

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STRICT GLYCAEMIC CONTROL BY ISLET TRANSPLANTATION AND INSULIN ADMINISTRATION COULD RELIEVE LONG-TERM COMPLICATIONS AND RESCUE THE RESIDUAL ENDOGENOUS PANCREATIC β CELLS

Short title: Islet transplantation in long-term diabetes

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ABSTRACT

OBJECTIVE. Islet transplantation is a poorly investigated long-term strategy for insulin replacement in patients with diabetes and for complication treatment. We investigated if islet transplantation and insulin treatment could relieve diabetic neuropathy and rescue the residual endogenous pancreatic β cells.

RESEARCH DESIGN AND METHODS. We investigated 5 groups of Sprague Dawley rats for 8 months: control rats, diabetic rats, insulin-treated diabetic rats with moderate or mild hyperglycaemia and diabetic rats transplanted with microencapsulated islets.

RESULTS. Islet transplantation normalized glycaemia and increased body and muscles weight and reduced proteinuria and altered liver function. Transplantation significantly improved tail nerve conduction velocity, Na+, K+-ATPase and morphological alterations in the sciatic nerve as evidenced by decrease in g-ratio, restored thermal and ameliorate mechanical nociceptive thresholds. Morphometrical analysis of pancreas showed a significant β cell volume increase in transplanted rats as compared to mild and moderate hyperglycaemia rats.

CONCLUSIONS. Our results show that allogeneic islet transplantation has a positive systemic effect in diabetic rats and induces regression of the established neuropathy and restitution of the typical characteristics of the islets. These findings strongly indicate the need for improving glycaemic control in the attempt not only to reverse established diabetic complications but also to improve β cell status in diabetic pancreas.

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INTRODUCTION

Type 1 diabetes is characterized by deficiency of insulin secretion due to the loss of β cell mass by autoimmunity. Both β cell mass and function decline with increasing diabetes duration (1). The consequent chronic hyperglycaemia is believed to be responsible for the development of diabetic complications such as neuropathy, retinopathy, nephropathy and cardiovascular diseases (2). Forty to 50% of diabetic population develop peripheral and autonomic neuropathies (3) and involve a spectrum of functional and structural changes in peripheral nerves including sensory loss and pain. The signs of nerve degeneration consist in an early decrease of nerve conduction velocity (NCV), of nerve action potential amplitude, reduction in Na^+K^+ATPase and changes in nociceptive thermal and mechanical thresholds. The maintenance of normoglycaemia is the only therapy approved for peripheral neuropathy by regulatory bodies in Europe and United States. The DCCT study show that neuropathy severity is related to the duration of disease and that improved glucose control reduces diabetic complications (2). Currently, the only method of providing physiologically regulated control of blood glucose in type 1 diabetic patients is allogeneic islet transplantation.

We have previously reported that transplantation of microencapsulated syngeneic islets after two month streptozotocin (STZ)-induced diabetes in rats produced normoglycaemia, amelioration of impaired nociceptive thresholds, normalization of NCV and Na^+, K^+-ATPase in the sciatic nerve (4). In kidneys of diabetic rats, we observed mild tubular dilatation and tubular cast completely absent in the rats with transplanted islets suggesting regression of these early signs of nephropathy (4).

There are only few studies on the effect of pancreatic islet transplantation in long lasting diabetes and in long-term complications. We then investigated the effect of islet transplantation in animals with a long duration of the disease, when, in addition to functional

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changes, also structural changes are expected to challenge the effectiveness of normoglycaemia conditions.

We used two strategies to control glycaemia, namely microencapsulated allogeneic islet transplantation and daily insulin injections to obtain different levels of glycaemia. To this aim we used two groups of diabetic rats: untreated diabetic rats and diabetic rats maintained in moderate hyperglycaemia (300-400 mg/dl). After 4 months, diabetic rats with insulin-regulated glycaemia were divided in 3 groups; the first group was maintained in the same conditions, the second underwent transplantation with microencapsulated allogeneic rat islets and the last group was treated with insulin to normalize glycaemia. Animals were followed for additional 4 months, with overall duration of the study of 8 months from diabetes induction.

Although it has been reported that normoglycaemia has preventive effects on the maintenance of transplanted islets (4; 5), only a few studies addressed the question whether islet transplantation may exert such an effect also on endogenous pancreatic islets. Thus, we investigated if in STZ-diabetic rats islet transplantation and/or glycaemic control by insulin administration could relieve neuropathic complications and neuropathic pain and could rescue the residual endogenous pancreatic β cells.

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RESEARCH DESIGN AND METHODS

Experimental design. Male Lewis and Sprague-Dawley rats (Harlan Laboratories, Bresso, Italy) 12 weeks of age were used as donors and recipients, respectively. Animal protocol was approved by the Institutional Animal Care and Use Committee of "Mario Negri" Institute, Milan, Italy. Animal care and treatment were conducted in conformity with institutional guidelines, in compliance with national (DL n. 116/1992, Circ. n. 8/1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, 8th edition, US NRC, 2011). Animals were kept on a 12-hour light/dark cycle with free access to water.

Rats were randomly assigned to three experimental groups: healthy rats (control), untreated diabetic rats (hyperglycaemia) and diabetic rats treated subcutaneously with 1 to 15 IU of human recombinant insulin (Lantus®, Sanofi-Aventis SpA, Milano, Italy) to achieve glycaemia between 300 and 400 mg/dl (moderate hyperglycaemia). Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg bw; Sigma, St. Louis, MO). Hyperglycaemia was confirmed 48 h after STZ injection by measurements of tail vein blood glucose levels using a glucometer tester (Ascensia Elite Bayer, Basel, Switzerland). After 4 months from diabetes induction, the moderate hyperglycaemia group was further randomly divided in three groups: a group maintained with the same glycaemic control, a group with insulin treatment to obtain normoglycaemic conditions (mild hyperglycaemia group) and one group that received islet transplantation. The groups of animals were monitored until 8 months from the beginning of the study. Body weight and blood glucose concentration, determined by tail bleeding, were measured daily.

Proteinuria was measured in 24-h urine samples collected in metabolic cages at baseline and every month by the Coomassie method using a Cobas Mira autoanalyzer (Roche

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Diagnostic147 Systems, Basel, Switzerland).

Isolation of pancreatic islets. Islets were isolated from the pancreas of Lewis rats (B.W. 250-300g), using an automatic procedure previously described (4). Briefly, the pancreas of anesthetized rats were distended with collagenase P solution (Boehringer-Mannheim, Mannheim, Germany), removed and 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, Sigma, St. Louis, MO). Islets were cultured at 37°C in an atmosphere of humidified air + 5% CO_2 in RPMI 1640 medium (Life Technologies Italia, Monza, Italy), supplemented with 10% foetal bovine serum (EuroClone, Pero, Italy).

Encapsulation and transplantation of pancreatic islets. Islet encapsulation was performed as previously described (6). Briefly, islets were suspended in 1.7% sodium alginate solution (Manugel DMB, Monsanto plc, Surrey, United Kingdom) at a concentration of 1 islet/µL. The islet-alginate mixture was then extruded through an air jet droplet generator into a 100 mmol/L $CaCl_2$ solution. Microcapsules containing 15,000 islets/kg B.W. were implanted into the rat peritoneal cavity through a small (1–2 cm) midline incision under isofluorane anaesthesia.

Oral glucose tolerance test. An oral glucose tolerance test (OGTT) was performed 4 and 8 months from the beginning of the study by oral administration of 1 g/kg B.W. of glucose after an overnight fast. Blood samples were taken from the tail vein immediately before and 15, 30, 60, 120 and 180 min after glucose administration. Blood glucose

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concentrations were measured using Ascensia elite strips (Bayer Basel, Switzerland). Total area under the curve (AUC) was then calculated.

Behavioural evaluation. Thermal nociceptive threshold to radiant heat was quantified by using the paw withdrawal in a hot plate (Ugo Basile, Comerio, Italy) test (7). Withdrawal latency was defined as time between placement on the hot plate and time of withdrawal and licking of hind paw. The mechanical nociceptive threshold was quantified using the Randal-Selitto paw withdrawal test (7; 8) with an Analgesy-meter (Ugo Basile, Comerio, Italy), which generates a linearly increasing mechanical force. The results represent the maximum pressure tolerated by the animals. The hot-plate tests and the Randall-Selitto test were done every four weeks. For both determinations animals were tested twice, with a 30-min interval and the values were averaged. For mechanical sensitivity the test was done first in the right and then in the left leg.

Nerve conduction velocity in the tail nerve. NCV in the tail was measured by using a Myto EBNeuro electromiograph (EBNeuro, Firenze, Italy) as previously described (9). Briefly, the antidromic NCV in the tail was assessed by placing recording ring electrodes distally on the tail while stimulating ring electrodes were placed 5 and 10 cm proximally from the recording point. The latencies of the potentials recorded at the two sites after nerve stimulation were determined (peak-to-peak stimulus duration 100 ms, filter 1Hz-5MHz) and NCV was calculated. Determinations were done under standard conditions in a temperature-controlled room. Core temperature was maintained at 37°C by using heating pads and lamps.

Plasma and tissue collection. Four and 8 months after the beginning of the study, animals were placed under ketamine/xylazine anaesthesia and blood from abdominal aorta

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collected. At sacrifice sciatic nerves and pancreas were dissected out. Blood specimens and tissues were immediately processed as indicated below. Serum was obtained by centrifugation of clotted blood at 2.500 g for 15 minutes at 4° C and used for urea, creatinine, AST and ALT determination with an automatic system MIRA PLUS (Horiba ABX Diagnostic Montpellier, France) (10).

Plasma Thiobarbituric Acid-Reactive Substances (TBARS). EDTA and glutathione at 1.34 and 0.65 mmol/L final concentrations, respectively, were immediately added to the collected plasma. TBARS levels were determined, as an index of reactive oxygen species production, by previously described protocol (11).

Na^+,K^+-ATPase activity. These enzymatic activity in the sciatic nerve was assessed by spectrophotometric (Ultrospec 2100 pro, Amersham-Biosciences, Cambridge, England) method as described (12) by the coupled-enzyme assay (13). Protein content was determined with DC Protein Microplate Assay Protocol (Bio RAD Italy, Milan, Italy).

Pathological assessment of sciatic nerve myelinated fibers. The left sciatic nerves were removed, fixed by immersion in glutaraldehyde 3% and postfixed in OsO_4, epoxy resin embedded and used for light microscope observations as previously described (14). Toluidine blue stained semithin (1 μ m-thick) sections were prepared from at least two tissue blocks for each animal and examined with a Nikon Coolscope light microscope (Nikon Instruments, Calenzano, Italy). Counting of myelinated fibers was performed semi-automatically with a photomicroscope (Nikon eclipse E200, Leica Microsystem GmbH, Wetzlar, Germany) at a magnification of 60X and the morphometric analysis was performed using a QWin automatic image analyzer (Leica Microsystems GmbH, Wetzlar, Germany). In randomly selected

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sections, all myelinated fibers evaluable in the analyzed space were counted according to previously reported methods on at least 500 myelinated fibers/nerves (15). At the same time, the g-ratio (axonal/myelin diameter) was calculated. Since on the cross sections the shape of the axons was not always round, we calculated mean diameter from measured area, assuming that ideal shape of the axonwould be a circle.

Pancreas processing. After removal, pancreas was dissected from surrounding tissues and fixed by immersion in Bouin's solution (Diapath, Martinengo, Italy) for 4h at 4°C. Samples were dehydrated in ascending concentrations of alcohol and embedded in paraffin. For each sample, 3µm sections were obtained and processed for morphometrical analysis by immunohistochemical detection of insulin using the alkaline phosphate-Fast red technique. Slides were soaked in PBS + 1% BSA for 30 min at room temperature and stained using mouse anti-insulin (diluted 1:3000, Sigma, St. Louis, MO) for 2 h at room temperature. Then, sections were incubated with horse biotinylated anti mouse antibody (diluted 1:200 Vector, Burlingame, CA) for 30 minutes. Slides were then incubated with alkaline phosphatese-conjugated streptavidin (Boehringer-Mannheim, Mannheim, Germany) and counterstained with Harris-type haematoxylin (Bio-Optica, Milano, Italy).

β cells morphometrical analysis. Tissue sections were examined on an Axiovision light microscope (Zeiss, Jena, Germany). For each sampling section, the area was scanned with a 20X objective using a MosaiX software. Measurements were performed on digital images by an image analysis program (ImageJ , National Institutes of Health, Bethesda, MD). The volume density of insulin positive cells was determined by point counting using an orthogonal grid with 17x13 lines digitally overlaid on the stained section image. For each islet, the number of grid points hitting the positive staining and the negative staining islets

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were counted. Volume density was calculated as the percent ratio between grid points in the positive areas over total points in the pancreatic islet. The mean area of insulin positive cells was calculated by multiplying the number of grid points hitting the positive staining by the unit area of the grid. Mean volume of islets was calculated using the formula

$$V_i = (K/\beta) \times (\overline{A})^{3/2} \qquad [\mu m^3]$$

Where $\overline{A}$ is the mean area of insulin positive cells, K = 1.1 is a size distribution coefficient calculated on the basis of islet size distribution (16) and β = 1.5 is a shape coefficient assumed on the basis of minimum and maxim islet axis ratio (17). The density of islets per unit volume (N_V) was obtained by

$$N_V = N_A / (\overline{D}\text{-}t) \qquad [\mu m]$$

Where N_A is the islet numerical density per unit of pancreatic area and t the section thickness, assumed to be 3μm. The mean islet diameter ($\overline{D}$) was obtained from the mean islet volume.

Statistical analysis. Data were analyzed by the analysis of variance (ANOVA) with comparisons between groups made by Bonferroni test using the software StatView (SAS Institute Inc. Cary, NC). Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was assumed for $P<0.05$.

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RESULTS

Glycaemia, body weight and urinary excretion of proteins. All animals were carefully monitored for the entire 8-month period of the study. Weekly non-fasting blood glucose concentration and body weight are reported in Figure 1 A and B. Control rats maintained normal values of blood glucose and progressive body weight gain throughout the study. As expected, untreated diabetic rats consistently maintained serum glucose levels between 500 and 600 mg/dl over the 8 months examined and growth was severely impaired during this period. Glucose control by insulin injection in order to maintain moderate hyperglycaemia was effectively obtained, as shown in Fig. 1A. Improvements in blood glucose control overcame the growth impairment observed in diabetic rats throughout the study (Fig. 1B, P<0. 01). Normoglycaemia was restored 1 or 2 days after islet transplantation, and was maintained for the entire observation period (Fig. 1A). Transplanted rats displayed progressive body weight increase reaching values similar to control rats. In rats with intensive insulin treatment we did not succeed in complete restoration of normoglycaemia (Fig. 1A). Despite mild hyperglycaemia, an improvement in B.W. was recorded also in this group (Fig. 1B). . The time course of urinary protein excretion is shown in Figure 1C. Diabetic animals exhibited significantly higher values of urinary proteins than controls throughout the study. Particularly, proteinuria progressively increased in rats with moderate hyperglycaemia, averaging 547±161 mg/24h at 8 months. Of interest, in hyperglycaemic animals urinary protein excretion did not increase with time and was comparable to that of normal controls at study end. Insulin administration was not associated with lowering of proteinuria that was significantly higher than in control group during the whole treatment period. Islet transplantation exerted an important antiproteinuric effect. Proteinuria in these animals was comparable to normal controls (Figure 1C).

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Blood chemistry. Untreated diabetic rats had a significant increase in BUN, ALT and AST values vs. control animals ($P < 0.05$) at treatment start (data non shown). Insulin administration and islets transplantation allowed to achieve normalization of all these kidney and liver function parameters (Table 1).

Plasma TBARS. STZ-induced diabetes significantly triplicates TBARS levels (from 0.220± 0.022 nmol/mL in controls to 0.731± 0.062 in hyperglycaemic rats; $P < 0.01$). In moderate hyperglycaemia group TBARS levels remained significantly higher as compared to control group. Islet transplant and mild hyperglycaemia significantly blunted plasma TBARS levels, that averaged 0.401 ± 0.025 and 0.367 ± 0.062 ($P< 0.05$ and $P<0.01$, respectively).

OGTT. An OGTT was performed at 4 and 8 months after the STZ injection in all groups. Blood glucose concentration and AUC were significantly higher after the glucose load in hyperglycaemic rats as compared to control animals at 4 and 8 months (Fig.2 and Table 2). Insulin injections to maintain moderate hyperglycaemia lowered the glucose swing both at 4 and 8 months (Fig. 2A and B). A more strict glycaemic control in the mild hyperglycaemic group did not further improve the OGTT curve (Fig. 2B). Conversely, islet transplantation significantly improved both glucose swing and AUC at 8 months ($P<0.01$)(Table 2).

Thermal and mechanical nociceptive thresholds. Figures 3 shows the hind-paw thermal and force withdrawal thresholds, measured at baseline and monthly after induction of diabetes. Both thermal (hyposensitivity) and mechanical (hypersensitivity) nociceptive thresholds were significantly affected by hyperglycaemia starting 2 weeks after STZ injection

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and persisting for the entire 8 month of testing. It is noteworthy that thermal hypo-algesia was highly dependent on glucose level control. In the moderate hyperglycaemia group only a long-term treatment triggered a beneficial effect (Fig. 3A), whereas islets transplantation was more rapid and effective in restoring thermal sensitivity in about one month. Similar results were obtained in mild hyperglycaemia group. Mechanical sensitivity was greatly affected by diabetes with a detection threshold decreased by 50-70% after 4 months in all diabetic groups (Fig. 3B). Starting from a comparable perception threshold, treatments progressively and significantly improved the mechanical perception, islet transplantation being the most effective treatment, reaching a 30% reduction from controls at the end of the observation period.

NCV. Four months after STZ injection NCV was significantly reduced ($P < 0.01$) in the hyperglycaemic group as compared to controls (data not shown). As expected, in the long-term study period NCV increased (from 32.7±1.0 to 39.0±1.6 m/sec) in the control group whereas it was almost unchanged in diabetic animals (from 24.7±0.8 to 26.9±1.3 m/sec). As shown in Figure 3C, at the end of the study islets transplantation triggered a 50% improvement in NCV ($P<0.01$) whereas insulin treatments were less effective with the animals still presenting a 25% reduction in NCV.

Na^+, K^+-ATPase activity. As shown in Figure 3D, diabetes reduced Na^+, K^+-ATPase activity in sciatic nerve from 4.81±0.27 in control rats to 2.93±0.10 µmoles/min/mg protein in the hyperglycaemic group ($P<0.001$). It is of interest that islets or insulin treatments in diabetic rats induced only a slight reduction in Na^+, K^+-ATPase activity (about 10%) that was significantly different from hyperglycaemic group ($P<0.05$).

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Pathological assessment in sciatic nerve. At light microscopy examination, no evident reduction in myelinated fiber density was evident. Very rarely we observed actively degenerating fibers in all diabetic animals. Similarly, we observed only rarely mild changes in myelin sheath structure in the largest myelinated fibres of diabetic rats, suggesting myelinopathy. In view of the overall mild relevance of the pathological changes, the differences among the different groups were assessed morphometrically by means of the g-ratio calculation. Using this quantitative approach, a significant increase in the g-ratio was observed in untreated diabetic rats vs. controls (mean 0.686±0.003 vs. 0.647±0.003, $P < 0.01$). All treatments significantly reduced g-ratio that averaged 0.629±0.002; 0.655±0.003; 0.624±0.003 in islet transplantation, mild hyperglycaemia and moderate hyperglycaemia respectively ($P < 0.01$ for all groups vs. untreated diabetic rats).

β cells morphometrical analysis. Volume density of β cells in the pancreas of control rats averaged 0.83±0.17 and 1.25±0.13% at 4 and 8 months, respectively (Figure 4A). Insulin staining in hyperglycaemic rats revealed that β cell volume density was minimal (0.02±0.00 % positive cells at 4 months and 0.06±0.01% positive cells at 8 months). In contrast, β cells volume density in rats with moderate hyperglycaemia averaged 0.11±0.02% and 0.20±0.03% at 4 and 8 months, respectively. Comparable β cell volume was estimated in mild hyperglycaemic rats as compared to moderate hyperglycaemia. The maximum increase in β cell volume occurred in rats with islet transplantation at 8 months with 0.39±0.09% positively stained, as compared to rats with moderate hyperglycaemia ($P<0.05$).

Similar results were obtained in the mean area of insulin positive cells indicating that, in islet-transplanted group, β cells were organized in clusters resembling islets with almost half of the size (Figure 4B). Representative images of insulin stained cells of the groups studied at 8 months are shown in Figure 5. Islets from the transplanted group were organized

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in a structure similar to native islets in pancreas of the control group.

Estimation of β cell density showed that there were approximately 17.8±2.2 and 21.7±1.5 β cells/μm^3 in islets of control rats at 4 and 8 months, respectively. Hyperglycaemic rats showed a substantial reduction in β cell density of almost 83% at 4 months and 87% at 8 months (Figure 4C). In moderate hyperglycaemic group at 8 months β cell density averaged 6.1±0.72 β cells/μm^3. A statistically significant increase in β cell density was observed in islet treatment group compared with the moderate hyperglycaemic group (9.2±1.3 vs. 6.1±0.72 β cells/μm^3) ($P<0.05$). Furthermore, β cell density was slightly lower at 8 months as compared to mild hyperglycaemic group.

As shown in Figure 6, the volume density of β cells showed a significant linear correlation with log values of glycaemia both at 4 ($R^2=0.78$) and 8 months ($R^2=0.56$) indicating that β cell mass was strongly influenced by metabolic conditions.

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DISCUSSION

In type 1 diabetes immune-mediated reaction induces destruction of pancreatic β cells leading to insulin deficiency and hyperglycaemia. Replacement of β cell mass with islet cells is considered a viable therapeutic option. Several experimental and clinical studies show that pancreatic islet transplantation prevents diabetes complications (18). Peripheral neuropathy is one of the most common long-term complication of diabetes causing severe and prolonged morbidity (19), characterized by lowering in NCV, axonal degeneration, axoglial disjunction, paranodal demyelinization and loss of fiber density (20). In the model of STZ-induced diabetic neuropathy the peripheral nervous system damage is represented by evident and reproducible functional neurophysiological and biochemical changes (21), paralleled by milder pathological changes. We have recently shown that transplantation of syngeneic encapsulated islets in diabetic rats induced regression of diabetic complications. Pancreatic syngeneic transplantation in the STZ-induced diabetes in the rat ameliorated impaired nociceptive thresholds and normalized NCV and Na+, K+-ATPase in the sciatic nerve. In our present investigation we have evaluated the effect of the metabolic control in a disease model of longer duration, lasting 8 months, on the general conditions of the rats and with a specific focus on peripheral nerve damage and islets restoration.

Our present results show that microencapsulated allogeneic islet transplanted in diabetic rats achieved satisfactory glycaemic control, as clearly indicated by a significant reduction in glucose excursion after OGTT and a significant increase in animal growth suggesting an improvement of the overall diabetic status. We also observed a consistent reduction in liver impairment and in proteinuria, as well as a significant antioxidant effects both in islet transplanted and mild hyperglycaemic rats. As it might have been expected, better metabolic control and improvements in NCV, nociception, and Na^+, K^+-ATPase

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activity, were associated with greater reduction in the biomarker of oxidative stress (plasma TBARS levels). In addition, islet transplantation was able to reverse loss of NCV induced by diabetes, while insulin treatment only partially prevented NCV reduction. Although other studies have reported that islet transplantation was not effective in preventing lesions in sciatic nerve of diabetic rats (22), our data are in agreement with those of Sima et al. demonstrating that islet transplantation completely prevent the typical slowing of NCV (23). We also demonstrated that insulin replacement, by both islet transplantation and intensive insulin treatment, counteracts the impairment of Na^+, K^+-ATPase activity. This might be a mechanism that improves neural function, since both enzymatic activity and NCV are mainly related to large myelinated fibers integrity. These results demonstrate that islet transplantation effectively reduce peripheral neuropathy in animals with diabetes of long duration. Of note, also insulin-treated rats with a better metabolic control had a similar improvement in neural function.

The STZ-induced diabetes model has been extensively used to investigate the function of transplanted islets (6;24). These studies were based on the assumption that animals treated with STZ, are unable to recover endogenous β cell function. Our data confirmed that 8 months after diabetes induction by STZ, only residual endogenous β cell mass is present. The volume density and the mean area of β cells were significantly reduced in hyperglycaemic group as compared with those of control rats (0.056±0.015 and 174±26 vs. 1.249±0.135 and 938±68, respectively). Histological examination of the pancreas of these rats showed scattered insulin positive cells mostly dispersed as single cells or in small clusters in spite of well organized and clustered of normal rat pancreas. Also the pancreas of diabetic rats with moderate hyperglycaemia, examined before transplantation, contained only a few β cells (in average 0.11% insulin positive cells). When the blood glucose concentration was normalized by islet transplantation we observed an important increase of insulin positive β cells located

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inside the pre-existing islets. The mean area of clusters of positive cells was also increased in islet-transplanted rats indicating that, in addition to increased number, the regenerated β cells were distributed into islet-like structure recreating the typical islet organization. We observed a milder increased number of regenerated β cells in rats with mild hyperglycaemia, as compared to islet-transplanted group and they had a disorganized distribution. We also demonstrated that there is a linear correlation between volume density of β cells and glycaemia at single animal level. These results indicate that β cell mass is strongly influenced by the metabolic conditions.

The higher β cells regeneration obtained after islet transplantation compared to intensive insulin treatment indicated that re-establishment of normoglycaemia is not the only factor involved in islet regeneration and that other factors must be taken into consideration. The present investigation was not focussed on the mechanism of islet regeneration in transplanted diabetic rats, however we must emphasize that some important issues regarding β cell regeneration following islet transplantation need to be addressed and worth to investigate, in particular the identification of the source of these new β cells is of milestone importance. The question remains as to whether the new β cells originate from undifferentiated precursor cells through neogenesis or from mitotic replication of differentiated β cells. Understanding how β cell regeneration is regulated could lead to the identification of new approaches for enhancing β cell regeneration.

In conclusion, our present results indicate that normalization of blood glucose by islet transplantation improved neural function and induced partial regeneration of β cells with islet-like organization in the pancreas. These findings strongly indicate the need for improving glycaemic control in the attempt not only to reverse established diabetic complications but also to improve β cell status in diabetic pancreas.

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Marina Figliuzzi collaborated in the study design, wrote the manuscript and researcher data. She is the guarantor of the study. Cristina Cavagnini performed animal manipulations, including treatments and researched data; Roberto Bianchi collaborated in the study design, wrote parts of the manuscript and conducted behavioural tests. Raffaella Lombardi and Carla Porretta-Serapiglia conduced the biochemical analysis on sciatic nerves. Giuseppe Lauria collaborated in the study design and coordinated the analysis of behavioural and biochemical and results. Federica Avezza and Barbara Sala performed the haematological determinations. Annalisa Canta, Valentina Carozzi and Paola Marmiroli prepared the specimens and performed the neuropathological and morphometric assessments in sciatic nerves. Alessia Chiorazzi, Cristina Meregalli and Norberto Oggioni performed the neuriophysiological assessments and collaborated in the behavioural testing. Guido Cavaletti collaborated in the study design and coordinated the analysis of all the neurological results. Andrea Remuzzi contributed to study design, manuscript organization and discussion.

All the authors collaborated in the analysis and interpretation of the results and in the manuscript draft and eventually approved the final text.

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FIGURE LEGENDS

Figure 1. (A) Glycaemia and (B) body weight in male SD rats. Values are expressed as mean ± SE.

P<0.001 for control vs. all other groups at all time point;

P<0.001 for hyperglycaemia vs. all other groups at all time point;

† P<0.05 for control vs. islet transplantation;

# P<0.05 for hyperglycaemia vs. mild hyperglycaemia;

••• P<0.001; • p<0.05 for moderate hyperglycaemia vs. islet transplantation;

°°° P<0.001; ° p<0.05 for moderate hyperglycaemia vs. mild hyperglycaemia;

*** P<0.001; * p<0.05 for islet transplantation vs. mild hyperglycaemia.

(C) Time course of urinary protein excretion. Values are expressed as mean ± SE.

††† P<0.001 for control vs. all other groups;

†† P<0.01 for control vs. hyperglycaemia, moderate hyperglycaemia and mild hyperglycaemia;

† P<0.05 for control vs. moderate hyperglycaemia and mild hyperglycaemia;

P<0.001 for hyperglycaemia vs. moderate hyperglycaemia and mild hyperglycaemia;

P<0.01 for hyperglycaemia vs. islet transplantation;

# P<0.05 for hyperglycaemia vs. islet transplantation;

••P<0.01 for moderate hyperglycaemia vs. control;

• P<0.05 for moderate hyperglycaemia vs. hyperglycaemia;

§ P<0.05 for mild hyperglycaemia vs. hyperglycaemia;

** P<0.01 for islet transplantation vs. control;

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*P<0.05 for islet transplantation vs. moderate hyperglycaemia.

Figure 2. Oral glucose tolerance test with blood glucose values in male SD rats at 4 (A) and 8 months (B). Values are expressed as mean ± SE.

Figure 3. (A) Thermal and (B) mechanical nociceptive threshold. (C) Nerve conduction velocity in the tail vein at 8 months and (D) Na^+,K^+-ATPase activity at 8 months. Values are expressed as mean ± SE.

††† P<0.001 for control vs. hyperglycaemia, moderate hyperglycaemia and mild hyperglycaemia;

†† P<0.01 for control vs. moderate hyperglycaemia and islet transplantation;

† P<0.05 for control vs. moderate hyperglycaemia, mild hyperglycaemia and islet transplantation;

P<0.001 for hyperglycaemia vs. mild hyperglycaemia and islet transplantation;

P<0.01 for hyperglycaemia vs. mild hyperglycaemia and islet transplantation;

# P<0.05 for hyperglycaemia vs. moderate hyperglycaemia, mild hyperglycaemia and islet transplantation.

Figure 4. ß cell analysis at 4 and 8 months in SD rats. Parameters investigated are volume density of ß cells (A), mean area of positive insulin cells (B) and ß cell density per unit of volume (C). Results are expressed as mean ±SE.

††† P<0.001 for control vs. all other groups;

†† P<0.01 for control vs. hyperglycaemia and islet transplantation;

P<0.001 for hyperglycaemia vs. moderate hyperglycaemia and islet transplantation;

P<0.01 for hyperglycaemia vs. moderate hyperglycaemia;

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# P<0.05 for hyperglycaemia vs. moderate hyperglycaemia, mild hyperglycaemia and islet transplantation;

• P<0.05 for moderate hyperglycaemia vs. islet transplantation;

* P<0.05 for islet transplantation vs. mild hyperglycaemia.

Figure 5. Representative images of stained insulin cells in all the group of rats at the end of the study.

Figure 6. Correlation blot between volume density of β cells and log value of glycaemia at 4 (A) and 8 months (B). Correlation coefficient R^2=0.78 at 4 months and R^2=0.56 at 8 months.

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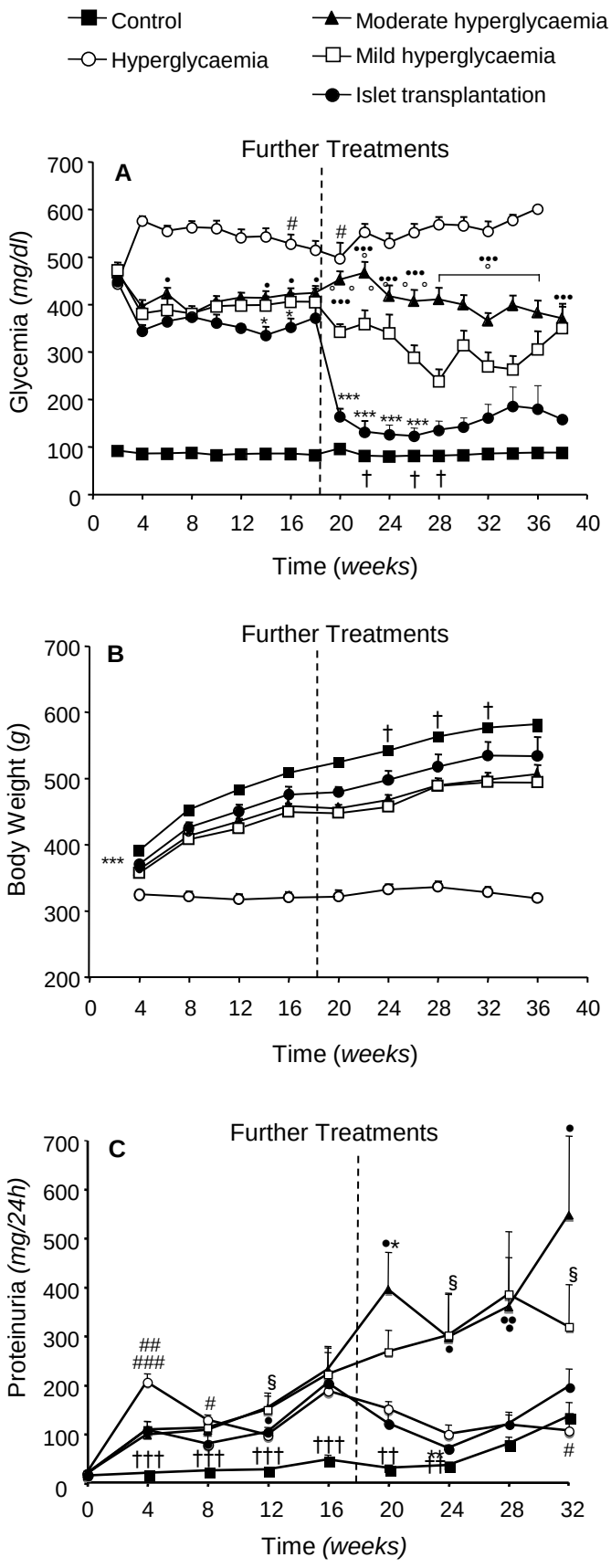

Control
Moderate hyperglycaemia
Hyperglycaemia
Mild hyperglycaemia
Islet transplantation
Further Treatments
A
Glycemia (mg/dl)
Time (weeks)
Further Treatments
B
Body Weight (g)
Time (weeks)
Further Treatments
C
Proteinuria (mg/24h)
Time (weeks)

Figure 1

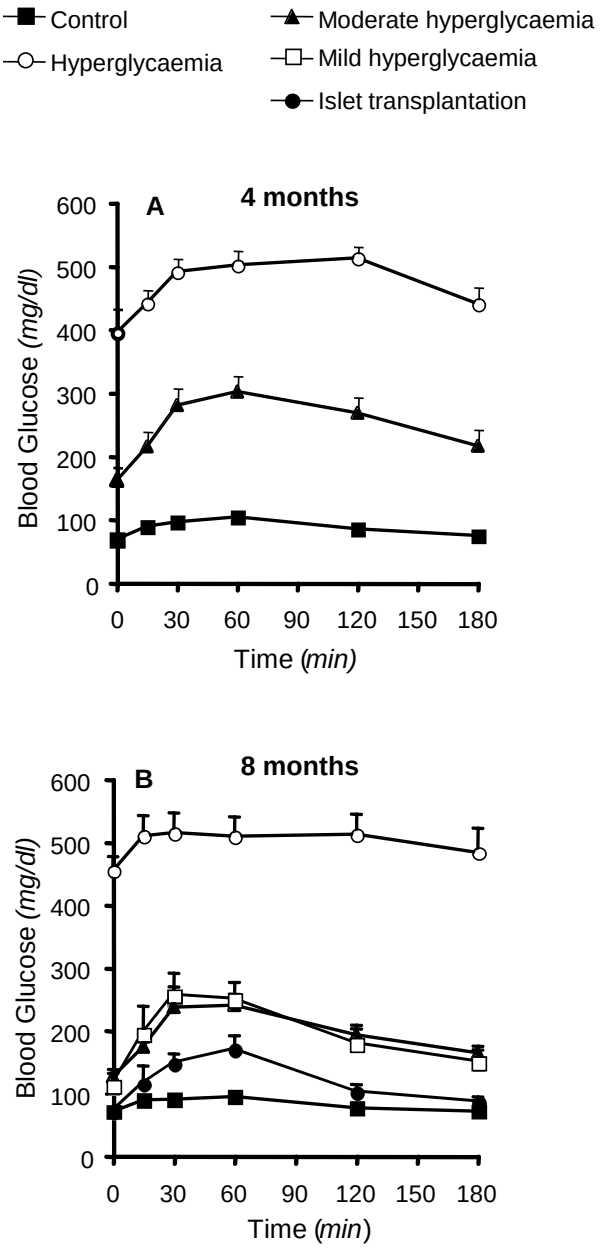

Control
Hyperglycaemia
Moderate hyperglycaemia
Mild hyperglycaemia
Islet transplantation
A
4 months
Blood Glucose (mg/dl)
600
500
400
300
200
100
0
0 30 60 90 120 150 180
Time (min)
B
8 months
Blood Glucose (mg/dl)
600
500
400
300
200
100
0
0 30 60 90 120 150 180
Time (min)

Figure 2

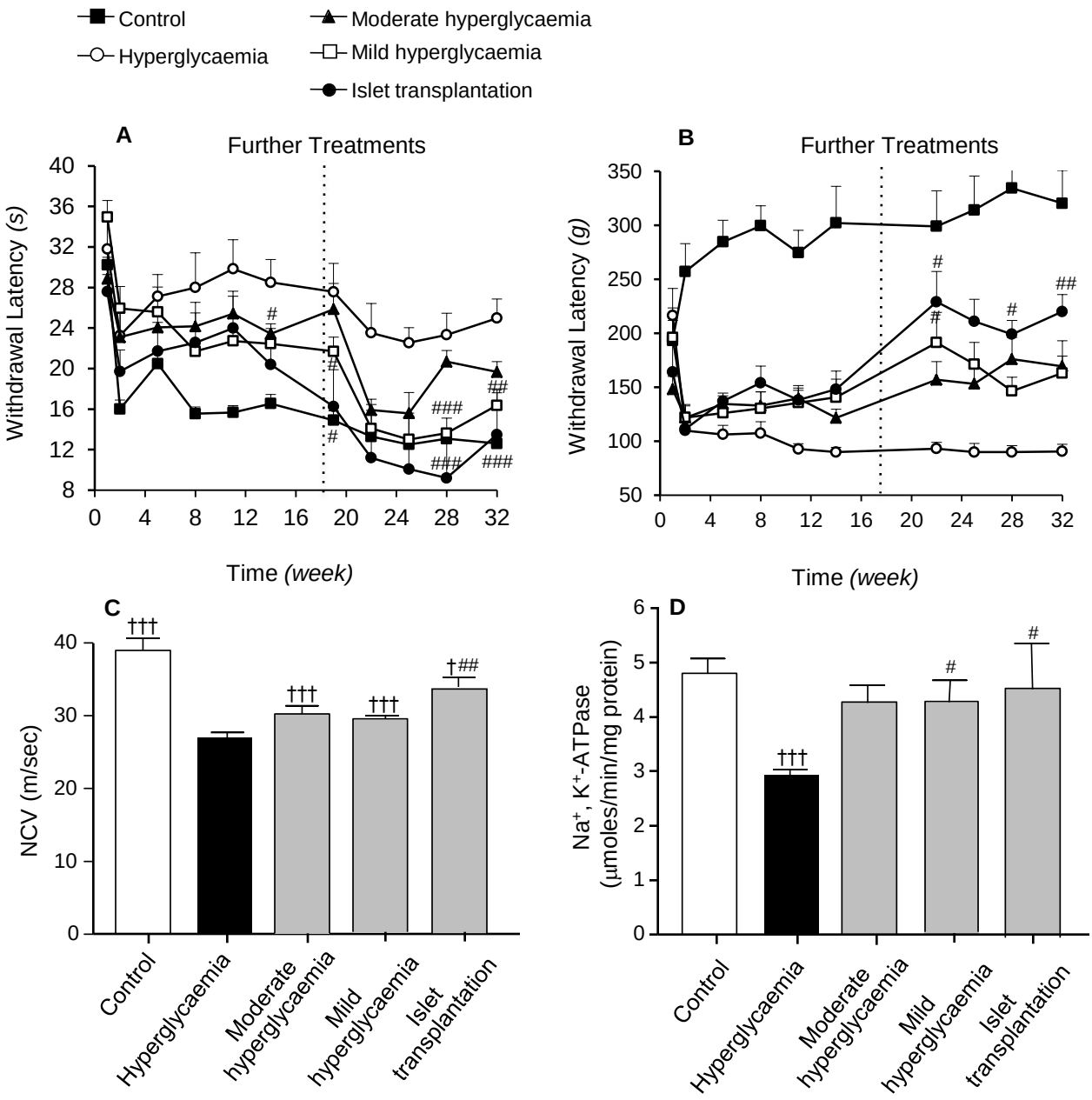

Control
Hyperglycaemia
Moderate hyperglycaemia
Mild hyperglycaemia
Islet transplantation
A
Further Treatments
Withdrawal Latency (s)
Time (week)
B
Further Treatments
Withdrawal Latency (g)
Time (week)
C
NCV (m/sec)
D
Na+, K+-ATPase (μmoles/min/mg protein)
Control
Hyperglycaemia
Moderate hyperglycaemia
Mild hyperglycaemia
Islet transplantation

Figure 3

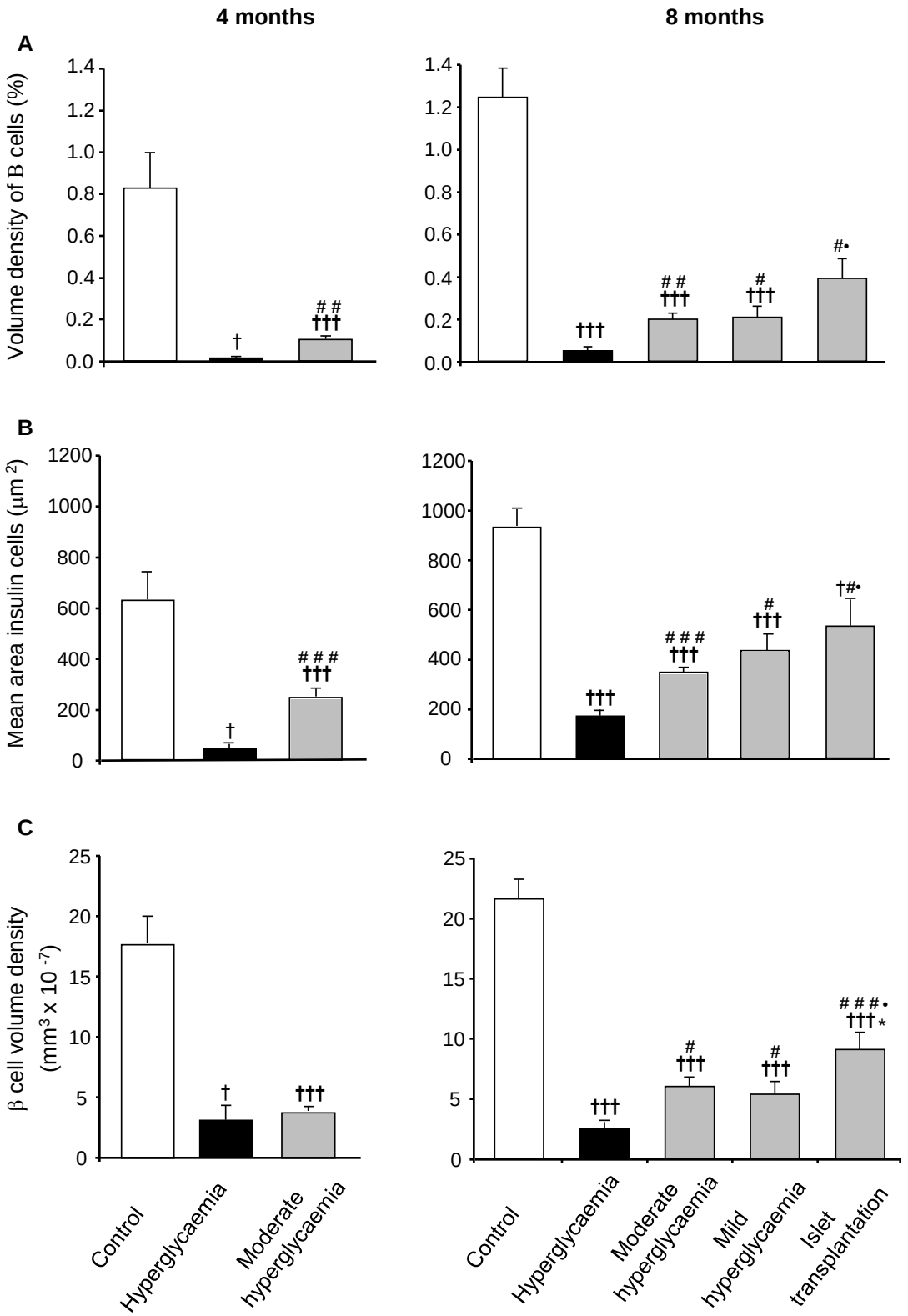

4 months
8 months
A
Volume density of B cells (%)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
†
†††
# #
#•
B
Mean area insulin cells (µm 2)
1200
1000
800
600
400
200
0
# # #
C
β cell volume density
(mm3 x 10 -7)
25
20
15
10
5
0
*
Control
Hyperglycaemia
Moderate hyperglycaemia
Mild hyperglycaemia
Islet transplantation

Figure 4

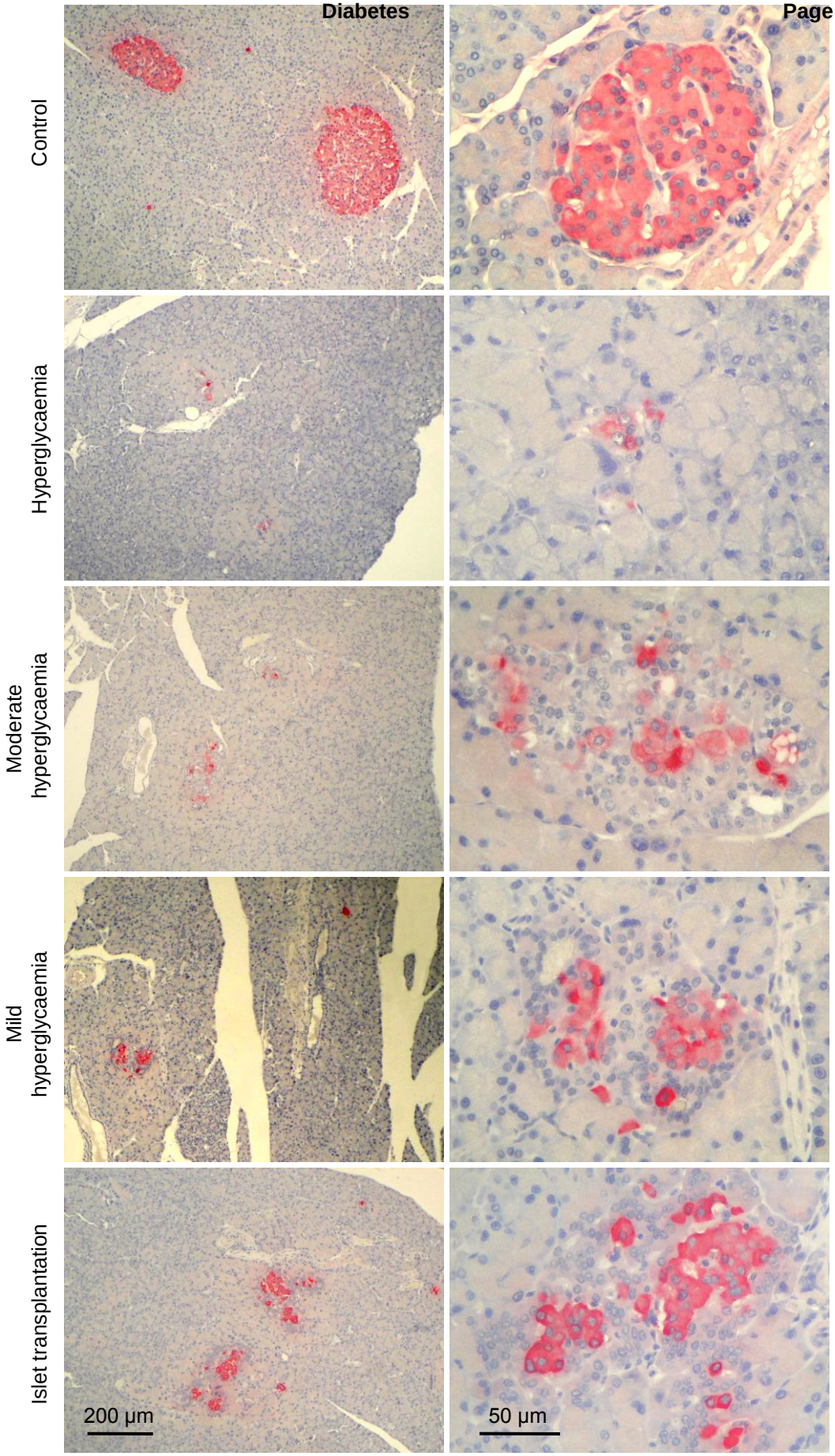
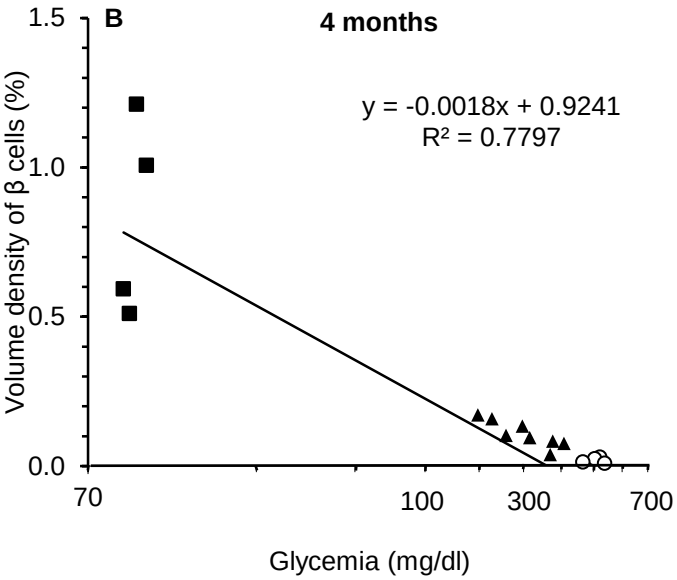

Control
Hyperglycaemia
Moderate hyperglycaemia
Mild hyperglycaemia
Islet transplantation
200 µm
50 µm

Figure 5


B
4 months
y = -0.0018x + 0.9241
R² = 0.7797
Volume density of β cells (%)
1.5
1.0
0.5
0.0
70
100
300
700
Glycemia (mg/dl)

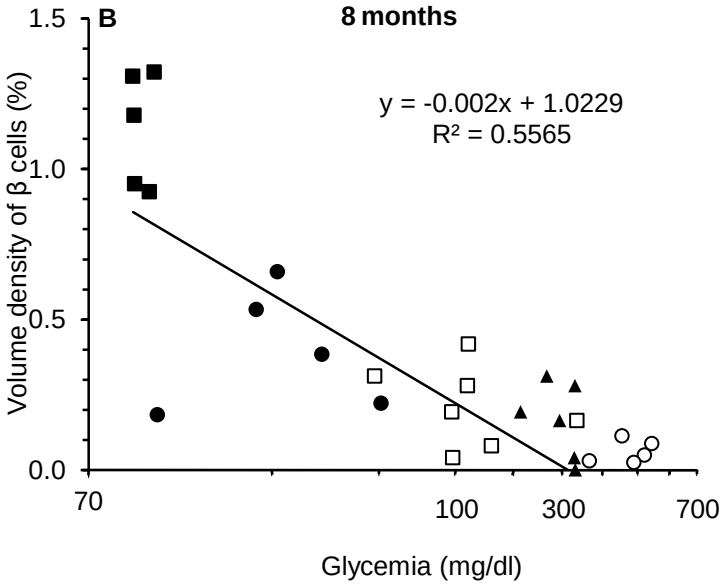

B
8 months
y = -0.002x + 1.0229
R² = 0.5565
Volume density of β cells (%)
1.5
1.0
0.5
0.0
70
100
300
700
Glycemia (mg/dl)

Figure 6

Table 1. Blood chemistry at 8 months from diabetes induction.

	BUN (mmol/l)	*CREATININE (mmol/l)*	*ALT (IU/l)*	*AST (IU/l)*
Control	41.1±3.6†	0.51±0.04	87.3±12.9†	112.8±13.9†
Hyperglycaemia	75.6±0.2	0.58±0.05	137.1±14.3	165.9±17.7
Moderate hyperglycaemia	51.9±4.5	0.60±0.12	88.1±17.0	90.8±12.3
Mild hyperglycaemia	47.5±5.0	0.49±0.06	76.0±19.2	80.9±9.4
Islet transplantation	40.0±4.8	0.55±0.05	62.6±15.8	81.4±20.0

† P<0.05 for control vs. hyperglycaemia

Table 2. Blood glucose area under the curve at 4 and 8 months from diabetes induction.

	AUC (*mg/dl/min*)	
	4 months	**8 months**
Control	16142 ± 347 †††	15085 ± 268 †††, †
Hyperglycaemia	86735 ± 3379 ###	90746 ± 5609 ###
Moderate hyperglycaemia	46974 ± 4406	36329 ± 2524 •••
Mild hyperglycaemia		35851 ± 4692
Islet transplantation		21944 ± 2180 *

††† P<0.001 vs. hyperglycaemia and vs. moderate hyperglycaemia

† P<0.05 vs. islet transplantation and mild hyperglycaemia

P<0.001 vs. all other groups

••• P<0.001 vs. islet transplantation

*P<0.05 vs. mild hyperglycaemia.