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CHARACTERIZATION OF MESENCHYMAL STEM CELLS EFFECT ON PANCREATIC ISLETS: A TOOL FOR TYPE I DIABETES THERAPY

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Summary

Diabetes Type 1 is a metabolic syndrome characterized by a progressive degeneration of β-cells, due to an autoimmune mechanism; it follows that diabetic patients show low insulin level and consequently they need for an insulin replacement therapy. Even if in the last decades many improvements have been made especially from the pharmacological point of view, new strategies have been investigated, in order to improve glycemic control; among these, a very promising approach is represented by pancreatic islet transplantation. Compared to whole pancreas transplantation, this approach is more minimally invasive and even a milder immunosuppressive regimen is needed. The clinical use is however limited by the large number of islets needed for the transplant and by their short survival after transplantation, so new approaches have been proposed; one of these is the use of Mesenchymal Stem Cells (MSCs); MSCs have been proposed for the management of many degenerative diseases for their immune-modulatory, differentiation and survival-supporting properties. Aim of this study was to investigate the putative positive effect of MSCs on pancreatic islets in vitro, and to verify the actual improvement in diabetes animal model. We set up direct and indirect co-cultures in order to analyze the role of soluble factors and mixed co-cultures in which both previous conditions coexisted. We observed that MSCs can prolong islet survival by soluble factors, while when directly co-cultured with MSCs changed their expression profile and differentiated in insulin-releasing cells. In mixed co-cultures both the results were present therefore we moved to in vivo models to verify the effect of mixed co-cultures. In our model, diabetes

was induced in rats by Streptozotocin intraperitoneal injection. The animals were divided into five groups: healthy rats; diabetic rats; diabetic rats transplanted with pancreatic islets; diabetic rats transplanted with pancreatic islets and MSCs; diabetic rats transplanted only with MSCs. As reported in literature, 2,000 pancreatic islets transplanted alone, in diabetic rats, were not able to improve clinical case. For this reason we decided to transplant 3,000 islets alone, while only 2,000 if co-transplanted with MSCs. In both these groups we observed an improvement of clinical case, indeed a good glycemic profile was reached and behavioral test confirmed the positive effect of transplants. Moreover we can assert that very similar results were obtained from the group receiving 3,000 islets alone and those receiving only 2,000 pancreatic islets and 1,000,000 MSCs, indicating that MSCs were able to support pancreatic islets survival and functionality. These data are very promising and made the co-transplantation of pancreatic islets and MSCs a concrete alternative approach for Type 1 Diabetes therapy, however further studies may clarify all molecular aspects involved in MSCs positive effect on pancreatic islets.

Introduction

1.1 Definition and classification

Diabetes Mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyper glycaemia with disturbances of carbohydrates, lipids and proteins metabolism resulting from defect in insulin secretion, insulin action or both. Diabetes Mellitus may present with characteristic symptoms such as polydipsia, polyuria, blurring vision and weight loss.

The first accepted classification of diabetes mellitus was published by WHO in 1980 and, modified form, in 1985 (1,2).

- **Type 1** (Beta cells destruction, usually leading to absolute insulin deficiency)

  * Immune mediated diabetes: this form of diabetes, representing 5-10%, results from a cellular-mediated autoimmune destruction of the beta cells. Markers of the immune destruction of beta cells includes islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65) and autoantibodies to the Tyrosine phosphatases IA-2 and IA-2β. Also, the disease has strong HLA associations, with linkage to DQA and DQB genes, and it is influenced by the DRB genes.

  * Idiopathic diabetes: some forms of type 1 diabetes have no known etiologies; some of these patients have permanent insulinopenia and are more prone to ketoacidosis, but

have no evidence of autoimmune. This form of diabetes is strongly inherited, lacks immunological evidence for β-cells autoimmunity, and is not HLA associated.

- **Type 2** (ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance)

  This form, which accounts for 90-95% of those with diabetes, includes individuals who have insulin resistance and usually have a relative (rather than absolute) insulin deficiency. At least initially, and often throughout they lifetime, these individuals do not need insulin treatment to survive. Most patients with this form of diabetes are obese and obesity itself causes some degree of insulin resistance.

- **Other specific types** of diabetes

  * Genetic defect of β-cell: some forms are associated with monogenic defects in β-cell function and are frequently characterized by onset of hyperglycemia, due to an impaired insulin secretion with minimal or no defects in insulin action, at an early age (generally before age 25 years); therefore are named MODY, Maturity-onset Diabetes in Young.

  * Genetic defects in insulin action: the metabolic abnormalities associated with mutations of the insulin receptors may range from modest hyperglycemia to severe diabetes. Leprechaunism and the Rabson-Mendenhall syndrome are two pediatric

syndromes that have mutations in the insulin receptor gene with subsequent alterations in insulin receptors function and extreme insulin resistance.

* Diseases of the exocrine pancreas: any process that injures the pancreas can cause diabetes including pancreatitis, trauma, infection, pancreactomy and pancreatic carcinoma

* Endocrinopathies: several hormones (growth hormone, cortisol, glucagon, and epinephrine) antagonize insulin action. Excess of these hormones can cause diabetes (acromegaly, Cushing's syndrome, glucagonoma) and hyperglycemia resolves when the hormone excess is resolved.

*Drug- or chemical-induced diabetes: there are drugs and hormones that can impair insulin action, for example nicotine acid and glucocorticoids. Patients receiving α-interferon have been reported to develop diabetes associated with islet cell antibodies.

* Infections: certain viruses have been associated with β-cell destruction; for example in patients with congenital rubella, although frequently HLA and immune markers, characteristic of type 1, are present. Also Coxsackie virus B, cytomegalovirus, adenovirus and mumps have been implicated in inducing certain cases of diabetes.

*Uncommon forms of immune-mediated diabetes in this category are included the stiff-man syndrome, an autoimmune disorder of central nervous system, patients usually have high titers of GAD autoantibodies. Anti-insulin receptor antibodies are occasionally found in patients with systemic Lupus Erythematosus and other autoimmune diseases.

*Other genetic syndrome sometimes associated with diabetes: many genetic syndromes are accompanied by an increased incidence of diabetes such as Down, Klinefelter, Turner and Wolfram syndromes.

- **Gestational Diabetes Mellitus**: is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. The definition applies whether insulin or only diet modification is used for treatment and whether or not the condition persist after pregnancy (3)

1.2 Epidemiology of Type 1 Diabetes

Type 1 Diabetes may occur at any age, but most typically presents in childhood. The World Health Organization DIAMOND Project group (DIAbets MONDiale) estimates 4.5% of the Childhood population is affected by T1 Diabetes with a variable incidence in Europe from 57.4 to 3.9/100,000 per year and in U.S.A. from 25.9 to 13.1/100,000 per year.

According to data publish by Health Ministry in Italy the incidence is 7.6/100,000 per year.

1.3 Pathogenesis of Type 1 Diabetes

This type of diabetes derives from a several or absolute loss of insulin, due to a reduction of beta cell mass. T1DM usually arises in childhood and becomes evident and severe during puberty. Patients with T1DM depend on exogenous insulin for survival and without replacement therapy they develop severe metabolic complications such as ketoacidosis and coma. Three different interrelate mechanisms cause the T1DM: genetic susceptibility, autoimmunity and environmental damages.

It is retains that genetic susceptibility to an altered immune-regulation, linked to specific MHC II alleles, predispose specific persons to develop an autoimmunity against β cells. In turn autoimmunity can spontaneously establish oneself or can be triggered by environmental factors, which induce β cells damage which expose beta cells themselves to an autoimmune reaction. Since the majority of these cells are destroyed, diabetes mellitus becomes clear.

1.3.1 Acute complication T1DM

Diabetic ketoacidosis (DKA) was formerly considered a hallmark of T1DM, it is associated with absolute or relative insulin deficiency, volume depletion and acid-base abnormalities.

The manifestation of DKA includes nausea/vomiting, thirst/polyuria, abdominal pain; DKA may be the initial symptom complex that leads to a diagnosis of T1DM, but more frequently it occurs in individuals with establish diabetes.

1.3.2 Chronic complications of T1DM

The chronic complications of T1DM affect many organs and are responsible for the majority of morbidity and mortality associated with the disease. Chronic complications can be divided into vascular and non-vascular. The vascular complications are further divided into microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular complications (coronary artery disease, peripheral arterial disease, cerebrovascular disease). The microvascular complications result from chronic hyperglycemia. Large, randomized clinical trials of individuals with T1DM have conclusively demonstrated that a reduction in chronic hyper glycaemia prevents of delays retinopathy, neuropathy and nephropathy. Some individuals never develop nephropathy or retinopathy; many of these patients have glycemic control that is indistinguishable from those who develop microvascular complications, suggesting that there is a genetic susceptibility for developing particular complications. Although chronic hyperglycemia is an important etiologic factor leading to DM complications, the mechanisms by which it leads to such diverse cellular and organs dysfunctions is unknown. Four prominent theories, which are not mutually exclusive, have been proposed to explain how hyperglycemia might leads to the chronic complications.

- One theory is that increased intracellular glucose levels have leads to the formation of advanced glycosylation end products (AGEs) via the non-enzymatic glycosylation of intra- and extracellular proteins. Non-enzymatic glycosylation results from the interaction of glucose with the amino groups on proteins. AGEs have been shown to cross-link proteins, accelerate atherosclerosis, promote glomerular dysfunction, reduce nitric oxide synthesis, induce endothelial dysfunction and alter extracellular matrix composition and structure.
- A second theory is based on the observation that hyperglycemia increases glucose metabolism via the sorbitol pathway. Intracellular glucose is predominantly metabolized by phosphorylation and subsequent glycolysis, but when increased, some glucose is converted to sorbitol by the enzyme aldose reductase. Increased sorbitol concentration alters redox potential, increase cellular osmolality and generates reactive oxygen species.
- A third hypothesis proposed that hyperglycemia increases the formation of diacylglycerol leading the activation of protein kinase C (PKC), which in turn alters the transcription of genes for fibronectin, type IV collagen, contractile protein and extracellular matrix proteins in endothelial cells and neurons
- A fourth theory proposed that hyperglycemia increases the flux through the hexosamine pathway, which generates fructose-6-phosphate, a substrate for O-linked glycosylation and proteoglycan production. The hexosamine pathway may alter function by glycosylation of proteins such as endothelial nitric oxide

synthase or by changes in gene expression of transforming growth factor β or plasminogen activator inhibitor-1.

1.3.2.1Ophthalmologic complications

Blindness is primarily the result of progressive retinopathy and clinically significant macular edema. Diabetic retinopathy is classified into two stages: non-proliferative and proliferative. Non-proliferative diabetic retinopathy usually appears late in the first decade or early in the second decade of the disease and is marked by retinal vascular micro aneurysm, bolt hemorrhages and cotton wool spots. The pathophysiologic mechanisms invoked in non-proliferative retinopathy include loss of retinal pericytes, increased retinal vascular permeability, alterations in retinal blood flow and abnormal retinal microvasculature, all of which lead to retinal ischemia.

1.3.2.2 Renal complication of DM

Like other microvascular complications the pathogenesis of diabetic nephropathy is related to chronic hyperglycemia; the mechanism by which chronic hyperglycemia leads to end-stage renal disease involve the effect of soluble factors hemodynamic alterations in the renal microcirculation (glomerular hyper filtration or hyper perfusion, increased glomerular capillary pressure) and structural changes in the glomerulus (increased extracellular matrix, basement membrane thickness, mesangial expansion, fibrosis). The natural history if diabetic nephropathy is characterized by a predictable sequence of events: glomerular hyper perfusion and renal hypertrophy occur in the first years after

DM onset and are associated with an increase of the glomerular filtration rate. Microalbuminuria appearance is an important risk factor for progression to overt proteinuria. Once macroalbumunuria develops, blood pressure rises slightly and the pathologic changes are likely irreversible.

1.3.2.3 Diabetic neuropathy

Diabetic neuropathy occurs in 50% of individuals with long-standing T1DM. It may manifest as polyneuropathy, mononeuropathy and/or autonomic neuropathy. The most common form of diabetic neuropathy is distal symmetric polyneuropathy; it most frequently presents with distal sensory loss but half of patients do not have symptoms. Hyperesthesia, paresthesia and dysesthesia also may occur. Symptoms may include a sensation of numbness, tingling, sharpness or burning that begins in the feet and spreads proximally. Pain typically involves the lower extremities is usually; both an acute and chronic form of painful diabetic neuropathy have been described. As diabetic neuropathy progresses, the pain subsides and eventually disappears, but sensory deficit in the lower extremities persists. Diabetic polyradiculopathy is a syndrome characterized by severe disabling pain distribution of one or more nerve roots, fortunately is usually self-limited and resolve over 6-12 months. Mononeuropathy, dysfunction of isolated cranial or peripheral nerves, is less common and presents with pain and motor weakness in the distribution of a single nerve. Individuals with long-lasting T1DM may develop signs of autonomic dysfunction involving the cholinergic, noradrenergic and peptidergic systems.

DM-related autonomic neuropathy can involve multiple systems, such as the cardiovascular, gastrointestinal, genitourinary and metabolic systems.

1.4 Anatomy

The pancreas is an elongated structure that lies in the epigastrum and the left upper quadrant. It is soft and lobulated and located on the posterior abdominal wall behind the peritoneum. It crosses the transpyloric plane. The pancreas is divided into a head, neck, body and tail. The head is disc shaped and is located into the cavity of the duodenum. A part of the head extends to the left behind the superior mesenteric vessels and is called uncinate process. The neck is the constricted portion of the pancreas and connect the head to the body; the neck is situated in front of the portal vein and the origin of the superior mesenteric artery from the aorta. The body runs upward and to the left across section and it has a triangular section. The tail passes forward in the spleno-renal ligament and comes in contact with the hilum of the spleen (fig.1a).

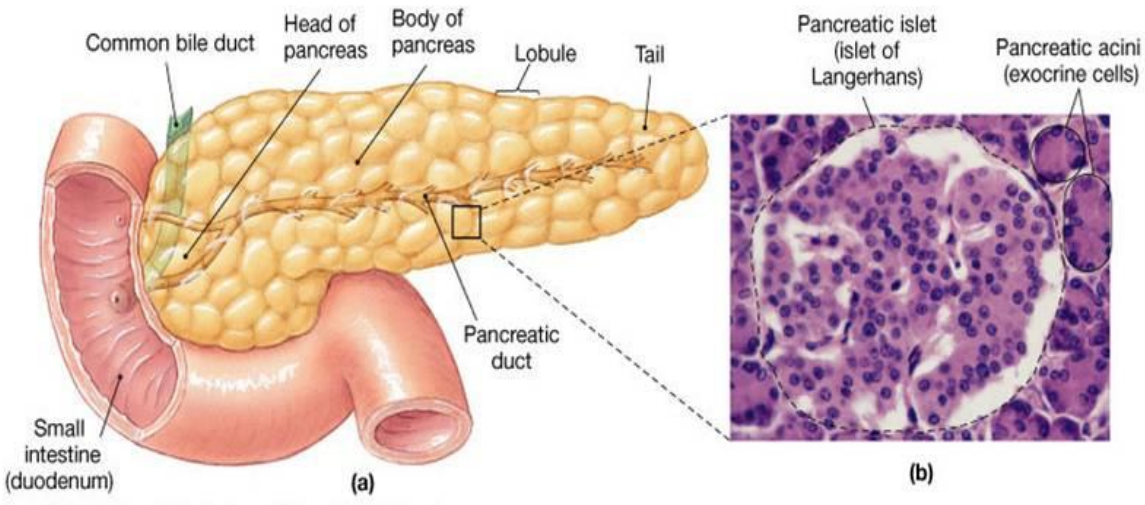


Fig 1 Pancreas anatomy; (a) macro-anatomy; (b) micro-anatomy

Anteriorly, from right to left, is in relation with transverse colon and the attachment of transverse mesocolon, the lesser sac and the stomach; posteriorly, from right to left, the bile duct, the portal and splenic veins, the inferior vena cava, the aorta, the origin of the superior mesenteric artery, the left psoas muscle, the left suprarenal gland, the left kidney and the hilum of the spleen.

The main duct of the pancreas begins in the tail and runs the length of the gland, receiving numerous tributaries on the way. It opens into the second part of the duodenum at about its middle with the bile duct on the major duodenal papilla. Sometimes the main duct drains separately into the duodenum. The accessory duct of the pancreas, if present, drains the upper part of the head and then opens into the duodenum a short distance to the main duct on the minor duodenal papilla; often the accessory duct is in communication with the main duct. The splenic artery and the superior and inferior pancreatic-duodenal arteries supply the pancreas; the corresponding veins drain into the portal system. Lymph nodes are situated along the arteries that supply the gland; the efferent vessels ultimately train into the celiac and superior mesenteric lymph nodes. Sympathetic (derived from the celiac ganglionic plexus, the superior mesenteric plexus and the hepatic plexus) and parasympathetic (vagal) nerve fibers supply this area.

Pancreas is a mixed exocrine-endocrine gland that produces digestive enzymes and hormones (fig 1b). The exocrine portion of the pancreas is a compound acinar gland, the acinus is composed of several serous cells surrounding a lumen. These cells are highly

polarized, with a spherical nucleus and are typical protein-secreting cells. The human exocrine pancreas secrets several proteases (trypsinogens 1, 2, 3; chymotrypsinogen, proelastases 1, 2; protease E, kallikreinogen, procarboxipeptidases A1, A2, B1, B2), amylase, lipases (triglycerides lipase, colipase, carboxyl ester hydrolase), phospholipase A2 and nucleases (deoxyribonuclease ribonuclease). The majority of the enzymes are stored as pro-enzymes in secretory granules of acinar cells, being activated in the lumen of the small intestine after secretion. This is very important for the protection of the pancreas. Pancreatic secretion is controlled mainly through two hormones: secretin and cholecystokinin, which are produced by enteroendocrine cells of the duodenal mucosa. Stimulation of vagus nerve also produces pancreatic secretion. Secretin promotes secretion of an abundant fluid, poor in enzyme activity and rich in bicarbonate; it is secreted by the small interlobular duct cells and serves to neutralize the acidic chyme. Cholecystokinin promotes secretion of a less abundant but enzyme-rich fluid; this hormone acts mainly in the extrusion of zymogen granules. The integrated action of both these hormones provides for a heavy secretion of enzyme-rich pancreatic juice.

The endocrine portion, organized in cluster of endocrine epithelial cells known as islets of Langerhans, produces the hormones insulin and glucagon, fundamental for carbohydrate metabolism. In sections, each islet consists of polygonal or round cells, arranged in cords separated by a network of blood capillaries. The islets of Langerhans contain several cell types, that secrete hormones that increase or decrease blood glucose. Alpha cells, representing about 20%, produce Glucagon that acts on several tissues to make energy, stored in glycogen and fat, available through glycogenolysis and lipolysis,

so as to increase blood glucose level. Beta cells, representing about 70%, act on several tissues to cause entry of glucose into cells, promoting decrease of blood glucose content. Delta cells, representing about 5%, secrete somatostatin that inhibits the release of other islet cells hormones through paracrine action. Rare are F (PP) cells, they secrete pancreatic polypeptide, that acts on gastrointestinal tract promoting gastric secretion and inhibiting gastrointestinal motility. There are two different sub-populations: D1 and enterochromaffin cells. The D1 cells secrete a hormone that induce glycogenolysis and stimulate gastric juices secretion; the enterochromaffin cells synthesize serotonin.

1.5 Insulin structure and physiology

Insulin gene is located on the short arm of chromosome 11 and it encodes a 1430 nucleotide insulin messenger precursor that contains two sequences of 179 and 786 nucleotides, that are excised from the precursor to generate the insulin messenger RNA. The insulin mRNA directs the synthesis of the precursor pre-pro-insulin composed of 110 amino-acids (Fig.2).

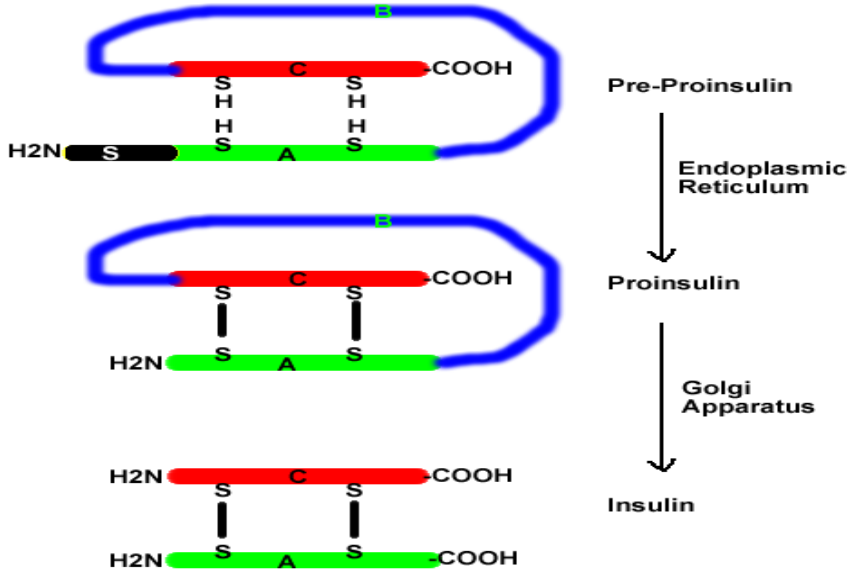


Fig.2 Schematic representation of insulin post-translational modifications

After it has passed through the endoplasmic reticulum, 24 amino-acids, named signal peptide, are removed, leaving another form, pro-insulin; this one passes into vesicles budded off from Golgi body, here C-peptide (33 aa) is removed with two other amino-acids converting pro-insulin into the final structure with two chains α-chain (21 aa) and β-chain (30 aa). (Fig.3)

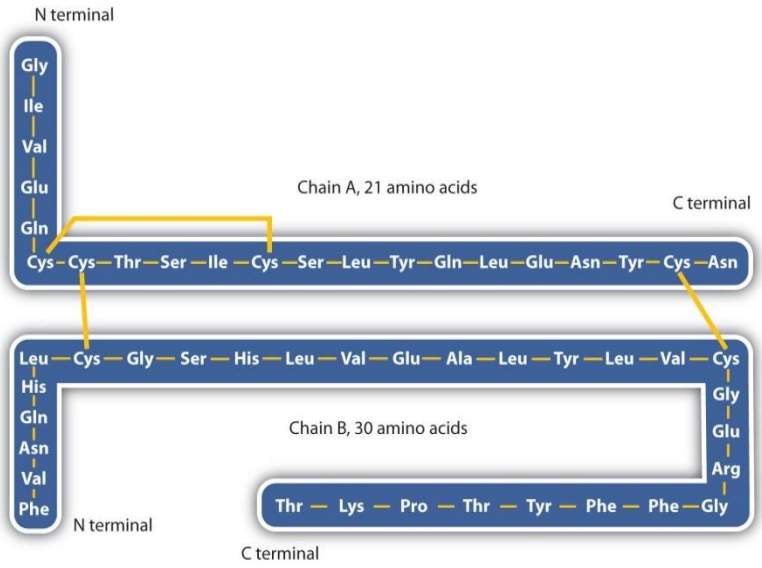


Fig.3 Amino acid sequence of human insulin

About secondary structure within α-chain two alpha helix delimit a flat ribbon section, which enables the helices to lie alongside one other; β-chain appears to wrap around α-chain; it contains a large section of α helix and glycine 20 and 23 allow it to fold into a V shape. About tertiary structure, is further stabilized by disulphide bridges: two between α and β chain (between thiol groups,-SH, of A7Cys-B7Cys and A20Cys-B19Cys) and one within the α-chain (A6Cys-A11Cys) (Fig.4: α-chain in blue, β-chain in green, disulphide bridges in red)

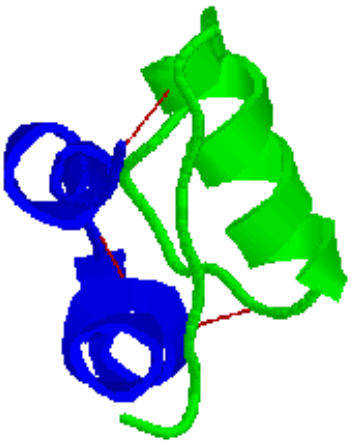


Fig.4 Human insulin tertiary structure

Regarding quaternary structure insulin can form into granules consisting of hexamers (6 insulin molecules grouped around two zinc ions) due to interactions between hydrophobic surfaces (Fig 5).

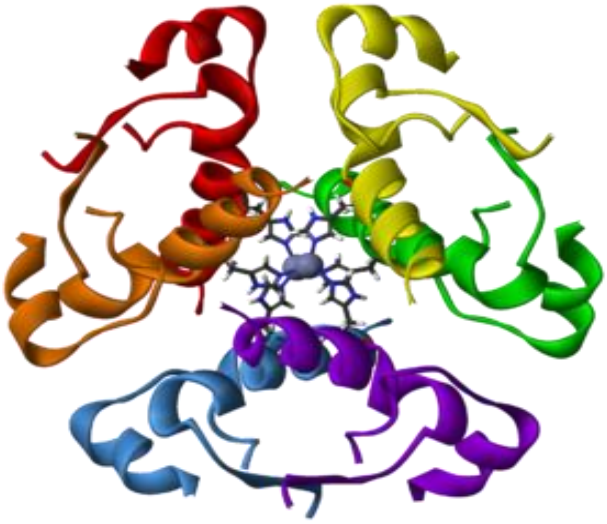


Fig.5 Human insulin quaternary structure

This toroidal form is the one in which insulin is stored in beta cells and secreted into the blood stream; insulin may also form into dimers, however the active form is apparently a single unit (monomer).

The actions of insulin are initiated by binding to its membrane receptors, which are virtually present on all tissues, even if the concentration varies from as few as 40 receptors on erythrocytes to more than 200,000 receptors on hepatocytes and adipocytes. First of all the insulin receptor (IR) binds the insulin with high affinity and a high degree of specificity, then produces the transmembrane signal that alters intracellular metabolism and mediates the insulin action. The insulin receptor is a heterodimeric membrane protein, composed of two subunits: α and β (Fig.6).

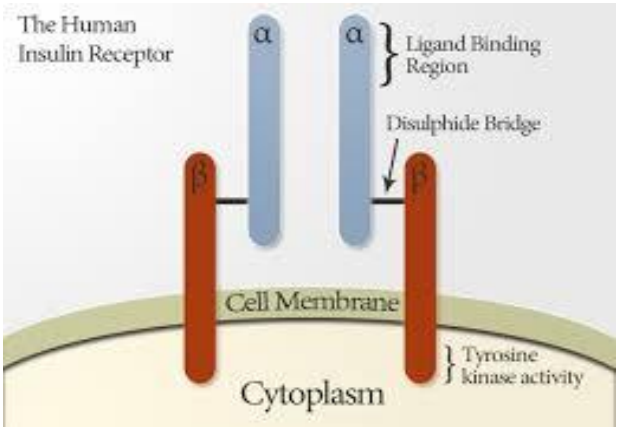


Fig.6 Schematic representation of insulin receptor

Like alpha and beta chains of insulin, alpha and beta sub-units of insulin receptor are derived from a single-chain precursor. The α subunit is totally extracellular, it contains the insulin-binding site and is linked to the extracellular part of the β unit. The β unit is composed of three compartmentalized regions: the extracellular, transmembrane and cytosolic domains. Since 1982 it has been known that β-subunit of the receptor was an insulin-stimulated protein kinase capable of phosphorylating itself and other substrates on tyrosine residues (ref Kasuga et al). More in detail two surfaces of insulin are understood to interact with the insulin receptor: the first consists of hormone-dimerizing residues and contacts the primary binding site on the receptor; the second consists predominantly of hormone-dimerizing residues and is proposed to interact with a secondary insulin receptor site. This binding to α-subunit transduces signals to β-subunit to activate the tyrosine kinase activity; the most important domain for the auto-phosphorylation and exogenous tyrosine kinase activity is the tri-tyrosine domain (aa1146/1151/1152), which lies in the tyrosine kinase catalytic domain. The kinase activity would initiate a phosphorylation cascade, but insulin receptor differs from the other receptor/tyrosine kinases in that it does not directly bind effector molecules, but

rather it phosphorylates its major substrate, IRS1, on tyrosine residues, and IRS1, in turn, bind effector molecules. The insulin pathway is very complex, indeed it involves many cellular activities, represented in the figure below (Fig.7)

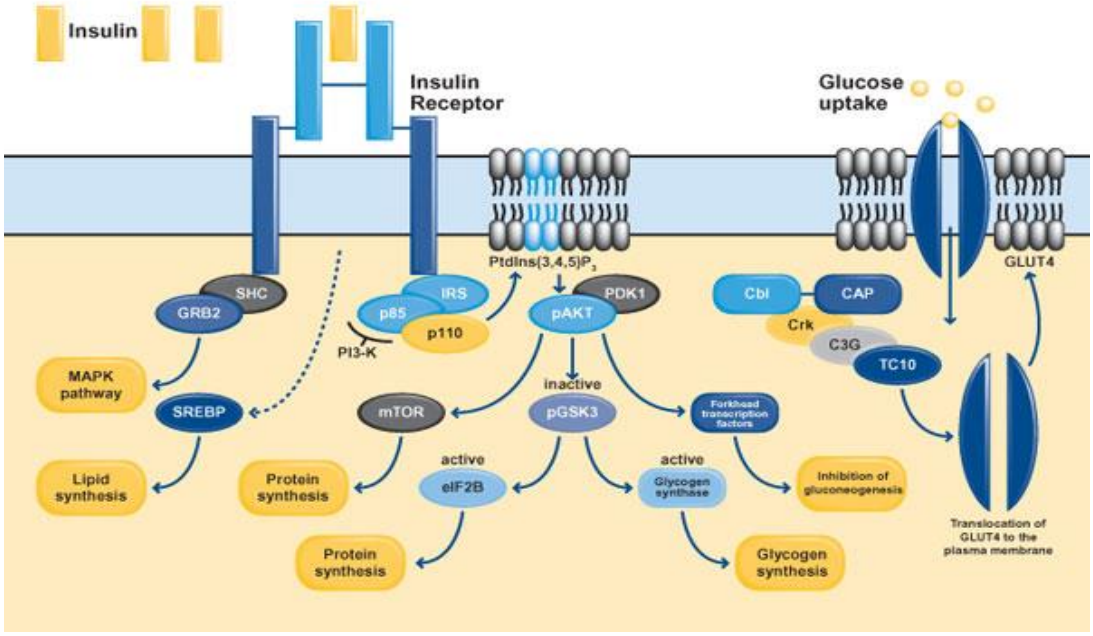


Fig 7 Schematic representation of molecular insulin pathways

1. Glucose storage and uptake

   Receptor activation leads to the phosphorylation of the key tyrosine residues on IRS proteins, some of which are recognized by Src homology 2 (SH2) domains on the p58 regulatory subunit of PI3-kinase. The catalytic subunit of PI3-kinase, p110, then phosphorylates phosphatidylinositol (4,5) bisphosphate PI(4,5)P_2 leading to the formation of phosphatidylinositol (3,4,5) triphosphate PI(3,4,5)P_3. A key downstream effector of PI(3,4,5)P_3 is ATK, which is recruited to the cytoplasm, where its leads to the phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3), a major substrate of GSK3 in glycogen synthase, the enzyme of

the last step in glycogen synthesis. In addition to promoting glucose storage, insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis. Insulin directly controls the activities of a set of metabolic enzymes by phosphorylation and de-phosphorylation events and also regulates the expression of genes encoding hepatic enzymes involved in gluconeogenesis.

A key action of insulin is to stimulate glucose uptake into cells by inducing translocation of glucose transporter, GLUT 4, from intracellular storage to plasma membrane. PI3K-kinase and AKT are known to play a role in GLUT4 translocation (Lizcano and Alessi 2002), but also a PI3-kinase independent pathway provides for GLUT4 recruitment to the plasma membrane. In particular, in this pathway, insulin receptor activation leads to the phosphorylation of Cbl, which is associated with the adaptor protein CAP, then, Cbl-CAP complex phosphorylated translocates to lipid rafts in the plasma membrane. Cbl then interacts with the adapter protein Crk, which is constitutively associated with the Rho-family guanine nucleotide exchange factor, C3G. In turn C3G activates members of the GTP-binding protein family, TC10 which promote GLUT4 translocation to the plasma membrane.

2. Protein synthesis

Insulin stimulates amino acid uptake into cells, inhibits protein degradation and promotes protein synthesis. Under basal conditions the constitutive activity of GSK3 leads to the phosphorylation and inhibition of a guanine nucleotide exchange factor eIF2B, which regulates the initiation of protein translation.

Therefore, upon receipt of an insulin signal, inactivation of GSK3 by AKT leads to the de-phosphorylation of eIF2B thereby promoting protein synthesis and the storage of amino acids. AKT also actives mammalian target of rapamycin (mTOR), which promotes protein synthesis through p70 ribosomal S6 kinase and inhibition of eIF-4E binding protein.

3. Regulation of lipid synthesis

   Insulin leads the uptake of fatty acids and synthesis of lipids and at the same time it inhibits lipolysis, through decreasing cytosolic concentration of cAMP by activating a cAMP phosphodiesterase in adipocytes.

4. Mitogenic responses

   Other signal transduction proteins interact with IRS including GRB2, an adaptor protein that contains SH3 domains, which in turn associates with the guanine nucleotide exchange factor son-of sevenless (SOS) and elicits activation of the MAPK cascade leading the mitogenic responses. SHC is another substrate of the insulin receptor. Upon phosphorylation SHC associated with GRB2 can activate the MAPK pathway independently of IRS.

Insulin: past, present, future.

The history of insulin of insulin begins at turn of the twentieth. Since 1890the link between the pancreas and diabetes was identified by Mering and Minkowski; in the early 1900s Sharpey-Schäfer hypothesized that pancreatic islets might release a secretion involved in glucose homeostasis. In the first two decades many investigators isolated this

secretion and demonstrated its transient hypoglycemic effect. In 1921 Dr. Banting, assisted by Best in the laboratory of MacLeod, University of Toronto; demonstrated that pancreatic islet extracts consistently reduced hyperglycemia in diabetic dogs. Later that year the biochemist Collip developed a protocol to purify what they later named “insulin” [Latin: insula]. For the first time in 1922 a 14-year-old diabetic patient was treated with pancreas extract of bovine pancreas: Diabetes became from an inevitably fatal condition to a treatable metabolic disease. In 1923 Nobel Prize in Medicine and Physiology was awarded to Banting and MacLeod. Insulin therapy has significantly evolved since 1920s, with improvements in insulin purification, production, formulation and delivery system. Until 1980s the commercially available insulin was extracted from either bovine and porcine pancreas. In latest 1970s the advent of recombinant DNA technique gave rise to recombinant human insulin, this milestone marked a new era in the evolution of insulin therapy. Since early 1980s insulin replacement improvements have been reached through the development of insulin analogues and alternative delivery routes. Several chemically or genetically modified insulins have been developed that tune the pharmacokinetics of insulin activity for personalized therapy. Chou and colleges have newly published a paper, in which a glucose-responsive insulin activity is demonstrated in diabetic animal model; so even if many alternative strategies for T1DM have been investigated, many progress in pharmacological field are underway.

“ *in using insulin it would of course be ideal if it could be supplied so as to imitate the natural process*” J.J.R. MacLeod and W.R. Campell, 1925

1.6 Diabetes type 1 Therapy

1.6.1 Pharmacological treatment

Diabetes type 1 is characterized by a partial or complete deficit of insulin secretion, which inevitably involves in a fasting and postprandial hyperglycemia, but also in an alteration of lipids and proteins metabolism; therefore an insulin replacement therapy is needful in order to reproduce and mimic physiological insulin concentrations both during interprandial and night-time phases, and during postprandial phases, as it occurs in healthy subjects. In the past decade new commercially available recombinant insulin became purest and had better pharmacokinetic profiles, implying a decrease of chronic complications and so a better quality of life and life expectancy for diabetic patients. The goal of a correct insulin replacement therapy is glycaemia normalization with the least possible incidence of hypoglycemic episodes. To achieve this it is necessary a therapeutic scheme that reproduce physiologic insulin profile, characterized by insulin peaks after meals and adequate insulin concentration during interprandial phases.

The therapeutic scheme, named “basal-bolus”, is achievable both with multi injective treatment and with insulin pump. The Multi Dose Injection (MDI) therapy consist of administration of rapid and ultra-rapid insulin just before every meal (or at mealtime) to respond to insulin prandial needs and single or multiple injections of intermediate/short-acting insulin to respond to basal insulin needs. View the table

TYPE	ONSET	PEAK	DURATION
Rapid-acting LisPro, Aspart, Glulysine	10’-25’	30-90’	3-5h

Short-acting			
Novolin	30'-1h	2-4h	5-8h
Intermediate-acting NPH	2-3h	6-10h	18-24h
Long-acting Lantus, Detemir	2-4h	8-14h/no peak	20-24h
Premixed Combinations of specific proportion of intermediate-acting and short-acting insulin	10'-30'	2-5h	14h up to 24h

Tab.1 commercial available insulins for Multi Dose Injection therapy

The insulin infusion pump is actually the therapeutic scheme that mimics closest physiologically secretion. The pump releases exclusively rapid-acting insulin both in continuously 24 hours (basal insulin), while bolus doses are administered at mealtime or when a hyperglycemic peak occurs.

1.6.2 Whole pancreas transplantation

In 1966 W. Kelly and R. Lillehei performed the first human pancreas transplant simultaneously with a kidney transplant at the University of Minnesota, but unfortunately after only two months recipient's death occurred because of many complications. The morbidity and mortality associated with early transplantations was high and in a

publication in 1970 was reported that only 20% of the patient lived through pancreas transplantation (Lillehei 1970). Fortunately the morbidity and mortality associated with pancreas transplantation has improved greatly and the rate of success has increased. In 2002 the American Diabetes Association recommended simultaneous kidney-pancreas transplantation as an acceptable treatment for advanced insulin-dependent diabetes associated with imminent or established end-stage renal failure (Clinical Trial: American Diabetes Association S111). Immunosuppression in pancreas transplantation follows a similar pattern to other solid transplants. Thymoglobulin, alemtuzumab, basiliximab, are used to achieve profound immune depletion lasting for the first three months if the risk of rejection is greatest. The maintenance combination of tacrolimus and mycophenolatemofetil to block T-cell activation and expansion, respectively. Good glycaemic control has been shown to reduce the decline in renal function (SMail et al 2012; Goel et al 2012). The effect of pancreas transplantation on renal function is particularly important since most transplant patients are treated with nephrotoxic calcineurin inhibitors, which may exacerbate any diabetes-associated glomerular disease. This is of particular relevance in pancreas-only recipients, where nephrotoxic immunosuppression may advance the patients' need for dialysis. Fioretto and colleges studied non-uremic patients who underwent pancreas transplantation alone (Fioretto et al 1998). Although there was a decline in renal function at 5 years, this remained stable at 10 years. Diabetic glomerular lesions were not significantly changed at 5 years but were dramatically improved after 10 years, with most patients' glomerular structure returning to normal at the 10-year follow-up. These studies also showed that tubulointerstitial

remodeling was possible (Mauer 2013). Indeed, Boggi and colleges noted a reduction in proteinuria in pancreas-only recipients post-transplant (Boggi et al 2011). Pancreas transplantation has also been shown to prevent the development of diabetic nephropathy within kidney allografts (Bohman et al 1985). Evidence for improvement in diabetic neuropathy after pancreas transplantation is limited. Improvements in neurological function after 24 months of stable pancreatic function are reported with improvements in motor, sensory and autonomic neuropathy, which persisted up to 10 years post-transplant (Navarro, Kennedy 1990). These and other studies suggest some degree of reversibility of diabetic neuropathy (Allen 1997 Martinenghi 1997); however, larger more robust studies have been hampered by the lack of a 'gold standard' outcome measure and by the invasive nature of current investigations. The recent establishment of confocal corneal microscopy as a reliable, non-invasive, readily repeatable investigation for detecting new nerve growth may address this difficulty, and early evidence after pancreas transplantation has been promising (Shtein 2013 Tavakoli 2013). Giannarelli and colleges studied patients with Type 1 diabetes who underwent pancreas-only transplantation beneficial effects on retinopathy were reported, with a significant improvement in the incidence of proliferative retinopathy, the need for laser treatment, and also in non-proliferative retinopathy (Giannarelli et al. 2006). Although the results of this and other small studies have been encouraging, a high proportion of patients already have advanced retinopathy at the time of transplantation. Koznarova and colleges suggest that improvement may be observed even in advanced retinopathy (Koznarova et al. 2000), but stabilization rather than improvement is a more realistic goal. Indeed stabilization of

disease progression has been observed in a number of studies. It has been suggested that rejection episodes may have a deleterious effect on retinopathy progression and the potential for sudden deterioration in vision either early after transplantation or after graft failure (Chow et al 1999; Sosna et al 1998).

Data suggest that pancreas–kidney transplantation reduces cardiovascular death [80] and functional studies have shown improvement in blood pressure and dyslipidaemia compared with kidney transplant alone improvements in systolic and diastolic ventricular function (Luan et al 2007, Fiorina et al 2000) and beneficial effects on endothelial dysfunction (Stadler et al 2009; Fiorina et al 2003) as well as a reduction in the progression of atherosclerosis; however, low cardiac event rates after pancreas transplantation may also reflect stringent cardiac screening and preoperative optimization in the pancreas transplant group. Pancreas transplantation is a recognized treatment for selected people with diabetes, offering insulin independence and improved quality of life, with graft and patient survival rates comparable to other solid organ transplantation. Simultaneous pancreas–kidney transplantation offers benefits in terms of graft and patient survival compared with kidney transplant alone for people with diabetes and end-stage renal disease. At the present time, whole-organ pancreas transplantation alone should always be considered as an alternative for people being referred for islet cell transplantation as, although it is a more invasive procedure, it is more likely to lead to insulin independence with established long-term outcome benefits. Pancreas transplantation has traditionally been restricted to young and fit patients, but as the morbidity of pancreas transplantation diminishes and the longer-term outcomes

improve, the potential indications for this procedure are increasing. There remains a need for improved graft monitoring by means of immunological or biochemical biomarkers to improve outcomes, and to help target appropriate intervention therapies for pancreas grafts showing suboptimal function.

1.6.3 New approach for T1DM

1.6.3.1 Pancreatic islets transplantation

As islets of Langerhans only comprise 2% of the pancreas, it could be argued that 98% of the whole pancreas is surplus to requirements. The aim of islets transplantation is to "reverse" diabetes by transplanting the endocrine component of the pancreas only. Islets transplantation involves four principal components: pancreas donation and retrieval, islets isolation, islets culture and islet transplantation. Advantages of islets transplantation, in respect of whole pancreas transplantation, are the minimal invasive procedure, the mild immunosuppressive therapy required. On the other hand a disadvantage that must be consider is that islets isolation is a challenging procedure requiring considerable expertise, and among critical aspects of islets transplantation there is the high number of islets needed in order to reach normoglycaemia, that means that donor/recipient ration is between 2:1 and 4:1. Furthermore insulin independence, compared with one after whole pancreas transplantation, is equivalent after one year, but is dramatically decreased after 5 years (Johnson et al 2012). Therefore even if many are the advantages of islets transplantation, few limitations exist, so new strategies have been proposed in order to improve islets transplantation.

1.6.3.2 Pancreatic islets and tissue engineering

During the last years new cellular biological and biotechnological knowledge have permitted the development of targeted technologies for the tissue and organ restoration, marking a new biomedical branch defined as tissue engineering. This technique permit to expand cells ex vivo and use them for tissue regeneration, using three-dimensional biocompatible polymeric matrixes; chemical, mechanical and physic characteristics of the matrix can be modulated to be adaptable to different cell types, in order to regenerate different tissues. These bio-artificial structures represent the second generation of systems for organs and tissue replacement; the first one is representing by traditional artificial organs, such as pacemakers, cardiac valves, joint prosthesis. The tissue engineering associate the viable cells transplant potentially to the artificial organs technology in order to achieve functional structures. The principal applications of tissue engineering are:

- Ulcer and burn treatment: structural compounds for tissue substitution, such as artificial skin (Tonello C et. 2003 ; Brun P et al. 2000; Galassi G et al. 2000; Zacchi V, et al. 1998)
- Cartilage and bone regeneration (Brun P, et al 1999; Pavesio A, et al 2003);
- Implants for the modulation of metabolic systems, such as artificial liver and artificial pancreas (Zavan et al. 2003).

1.6.3.3 Biomaterials:

The biomaterial is a three-dimensional support that permit to the cells a three dimensional distribution, but also the possibility to deposit extra-cellular matrix. The

biomaterials can be composed by one or a combination of compounds and must have three characteristics:

- Tolerability: they must be immunologically inert; to minimize negative response the biomaterial should have constant surface properties in order to prevent the absorption, a good blood-compatibility and should preserve its composition
- Biodegradability: the biomaterial should be a provisional support, after the integration should be substituted by tissue
- Interaction: the biomaterial should actively interacts with host cells, exchanging physical and chemical signals (Caplan AI. 2000)

The composition of biomaterials is inspired to extra-cellular matrix, principally composed by the ground substance, an amorphous gelatinous material, that fills the space between fibers and cells. It actually consists of large molecules called glycosaminoglycans (GAGs), which link together to form even larger molecules called proteoglycans. These molecules are very good at absorbing water, indeed 90% of the extracellular matrix is made up of water, giving extracellular matrix very good at resisting compressive forces.

There are agents found in nature, such as collagen, gelatin, hyaluronate, fibrin, alginate, agarose, and chitosan (Lee et al 2001, Nicodemus et al 2008, Peppas et al 2006) as well as synthetic products such as poly acrylic acid, polyethylene oxide, poly vinyl alcohol, polyphosphazene, and Teflon (PTFE). Of these agents, alginate has been one of the most popular. It is bioneutral, evoking little response from the host immune system, it is resistant to oxidative damage, and forms a permselective barrier (Zimmermann et al 2007). Apart from materials used, there are dimensions of the capsule that need to be

considered. Encapsulation devices range from 'microscale' devices like alginate microcapsules to the 'macroscale' PTFE Encaptra macrocapsule. Microcapsules are small cell-containing droplets ranging from 100 nm to 1 mm in size (Tuch et al 2009, Elliott et al 2007), while macrocapsules can be as large as 3 cm by 8 cm, and hold up to 250μl of tissue (Tarantal et al 2009). They both have their advantages; microcapsules, by virtue of their size, have a shorter diffusion distance for oxygen and other nutrients, while macrocapsules have rough surfaces that can promote neovascularization (Cornolti et al 2009, Lambert et al 2005). While it would be ideal to place capsules in the blood stream to optimize oxygen and nutrient delivery as well as waste removal, intravascular devices have been largely abandoned due to risks of thrombosis and hemorrhage (Vaithilingam et al 2011). Overall, the choice of capsule size, material, and implant site must be balanced to obtain the previously stated goals of transplantation.

Obtaining a large source of cells for cellular therapy is a major challenge. Stem cell-based strategies represent significant therapeutic potential owing to both the intrinsic regenerative capacity and the immunomodulatory potential of stem cells. While the capacity of stem cells to self-renew and to differentiate into specialized cell types can be harnessed to make available a self-replenishing supply of glucose-responsive insulin-producing cells for transplantation, the immunomodulatory properties of stem cells, such as MSCs, can be used to help arrest cell destruction, preserve residual cell mass, facilitate endogenous cell regeneration, and ameliorate islet graft rejection (Fiorina et al 2011; Barcala et al 2013;Fa¨ndrich et al 2010; Sims et al 2012). Thus, stem cells with immunomodulatory properties can potentially be used, both alone and in combination

with cell replacement strategies, to reverse hyperglycemia in T1D (Madec et al. 2009; Jurewicz et al. 2010; Rackham et al 2011). Stem cells obtained from a variety of sources have been tested for their cell-regenerative potential and for their ability to restore immune homeostasis or promote longitudinal islet graft survival. In recent study, Figliuzzi and collaborators analyzed an approach via which MSCs may help the success of pancreatic islets transplantation and address dome of the current problems associated to islets transplantation (Figliuzzi et al 2014). MSCs may exert pro-angiogenic and immunomodulatory effects if they are transplanted with pancreatic islets. The immunomodulatory possibilities of MSCs may help to reduce inflammatory damage to islets. MSCs may also reduce the autoimmunity through their capability to inhibit T cell proliferation and suppress the maturation and the maturation of dendritic cells. Several other approach have been attempting to identify immune privileged sites, in order to minimize the role of autoimmunity. (Ezquer et al 2008; Faldini et al 2014; Kutuchova et al 2015).

1.7 Stem Cells

Stem Cells (SCs) are undifferentiated cells characterized by two peculiar properties: self-renewal, in order to maintain a stem cell pool and differentiation towards mature cells with highly specific functions (Weissman et al 2001). The differentiation is a process through which an undifferentiated cell acquires specific characteristics. During the differentiation many complex mechanisms finely regulated are involved, among these gene expression is involved. SCs are located in particular micro-environments called niches, in which proliferative and differentiation properties are controlled by complex signaling pathways. These signals could arrive from niche itself, such as cytokines and growth factors, or could be released after the direct contact between stem cells and supporting cells. In niche stem cells are maintained at G0 phase so that a specific signal could promote differentiation process. Three different ways of cellular division are describes for stem cells, Fig.8 (Cai et al 2004):

- Symmetric proliferative division: the mitosis generates two identical and undifferentiated daughter cells;
- Asymmetric division: the mitosis generates on daughter cell identical to the mother cell and one progenitor cell;
- Symmetric differentiation division: the mitosis generates two lineage-specific progenitor cells.

Asymmetric division maintained a stable pool of stem cells and at the same time progenitors cells are generated.

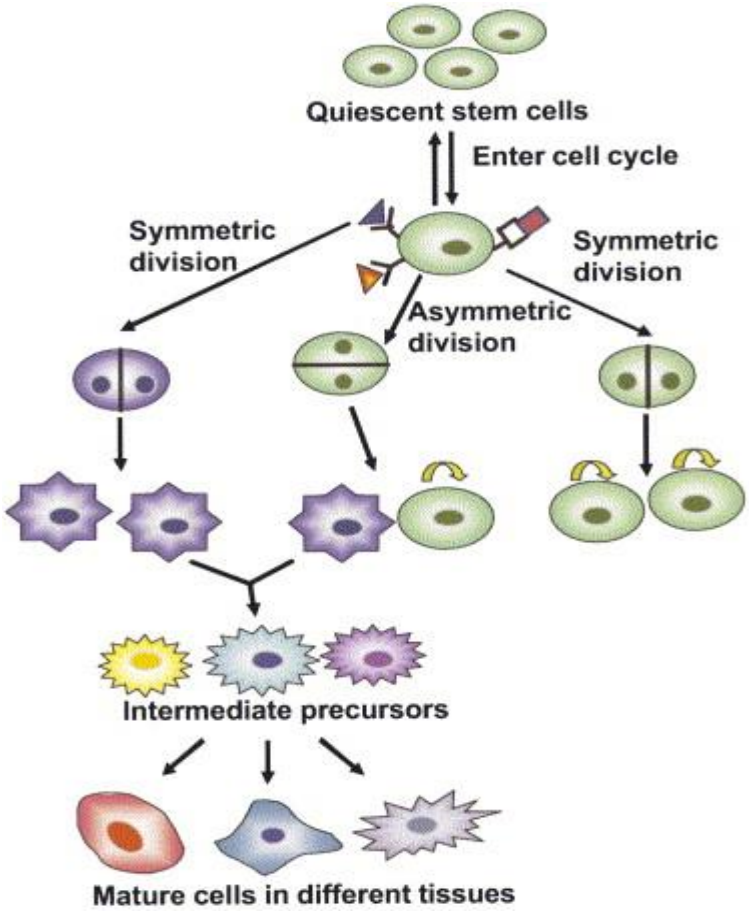


Fig.8 Schematic representation of stem cells division (Cai et al 2004)

Currently the mechanisms at the base of the switch form quiescent stem cells to differentiating cells remain partially unclear, however three possible mechanisms have been proposed:

- Removal of growth factors responsible of the maintenance of staminal pool
- Presence of differentiation factors and activation of their pathways
- Removal differentiation inhibitors or inactivation of their receptors and pathways.

Stem cells must have these requisites: self-renewal, clonality and differentiation potential.

Self-renewal, as mentioned before, is the peculiar process through which a stem cell indefinitely generates two identical daughter cells, which in turn maintain an undifferentiated stage guaranteeing a stem cell pool. Self-renewal program depended by finely balanced action of proto-oncogenes and onco-suppressor genes, that respectively promote and inhibit the process. Many cyclins and Cyclin-dependent Kinases (CdKs) control cellular cycle through target gene phosphorylation, among these p53 and Rb2 (onco-suppressors) (Morgan, 1995). In vitro only embryonic stem cells are able to indefinitely proliferate, while adult stem cells have a restricted self-renewal capability and after some passages they reach senescence replicative. This event is correlated to a progressive telomeres shortening occurs in in vitro cultures (Stenderup et al 2003) or to a lacking telomeres activity (Zimmermann et al 2003).

Clonality: a clonal population is defined as generated by a single cell and so presenting identical genetic elements to the mother cells (Melton et al 2004).

Differentiation potential is defined as the capability to differentiate towards mature cells with highly specific functions; depending on which cellular lineages could be generated stem cells can be classified as:

- Totipotent stem cells are able to generate all cellular types and tissue, including extra-embryonal ones; the zygote is the unique cell with this potential.
- Pluripotent stem cells are able to generate all cellular type and tissue derived from three embryonic sheets (endoderm, mesoderm, and ectoderm), inner cell mass cells are pluripotent cells.

- Multipotent stem cells are able to generate a reduced type of cells and tissue belonging to only one embryonic sheet.
- Unipotent cells are able to generate only one cell line

This classification, based on differentiation potential is not so strict, indeed some adult stem cells have demonstrated an extender differentiation capability. (Vast et al 2002).

Even if stem cells are located in different micro-environmental and also have different physiological requirements, some characteristic are shared by all stem cells (Melton 2004):

- TGFβ, Notch, Wnt e Jak/Stat signal-transduction pathways (Ivanova et al;

2002)

- express molecules involved in cell cycle regulation, that arrest the cell cycle at G0 phase or permit a fast progression in cell cycle (Burdon et al. 1999)(Savatier 2002)

-chromatin remodeling due to HDAC inhibitors (Melton et al 2004)

-post-translational regulation due to type Vasa RNA-helicases (Zimmermann et al 2003)

Plasticity

Many studies reported that Adult Stem Cells (ASCs) are able to differentiate in lineages owing to an embryonic sheet different from the original one: this capability is named plasticity. Three are the possible differentiation mechanisms involved:

*Trans-differentiation: tissue-specific stem cells could reprogram itself in other tissue-specific stem cells

*De-differentiation: tissue-specific stem cells could regress to a premature stage and change differentiation lineage

*Cellular fusion: tissue-specific stem cells could fuse with other cellular type, forming heterokaryon that switch the expression profile from the one characteristic of original stem cells to the one of the partner cells.

The ASC once differentiated in a lineage-specific cell change its morphology and express peculiar markers typical of that cellular line.

Bone-marrow derived ASCs have been widely studied; among these non-hematopoietic stem cells located in stroma: the Mesenchymal Stem Cells.

Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs) must have three peculiar characteristics:

§ Specific Markers expression: in particular in MSCs the expression of CD105, CD73, CD90, CD166, CD54, CD55, CD13, and CD44 must be positive, while the expression of CD45, CD14, CD34, MCHII, CD11b, CD79, CD19and CD31 must be negative (Dominici et al 2006).

§ Multi-potential differentiation: MSCs are able to differentiate towards mesengenic lineages generating osteoblasts, chondrocytes and adipocytes. (Kassem1993, Johnstone1998, Justesen 2002).

§ Plastic adherent property: due to this capability MSCs can be isolated from hematopoietic cells.

Furthermore MSCs have a characteristic fibroblast-like morphology and a big round nucleus. MSCs are often extract from bone-marrow, in which they represent 0.01%-0.001% on the total amount of nuclear cells, through in vitro cultures is possible to expand this cell population. MSCs growth is divided into three phases: a Lag phase, a latency phase,

that last three or four days; a Log phase, in which a rapid growth occurs, and a stationary phase (Colter alt al Prockop 2001).

MSCs are also characterized by immune-modulatory properties Fig.9, this characteristic makes this population a potential tool for therapeutic approach for many diseases; moreover MSCs express MHCI while they are negative for the expression of MCHII, indicating an hypo-immunogenic profile. MSCs can inhibit the majority of cells involved in immune response. In particular they inhibit the function, the proliferation and activation of mature T cells (Di Nicola 2002); at the same time they down-regulate Naïve T cells and memory T-cells (Krampera 2003). It has been demonstrate that MSCs up-regulate antigen presenting cells (APC) and they also modulate dendritic cells activity, mediating a decrease of pro-inflammation cytokines, IFN-γ, IL-12 and TNF-α and an increase of anti-inflammatory, IL-10 (Beyth 2005, Jiang 2005).

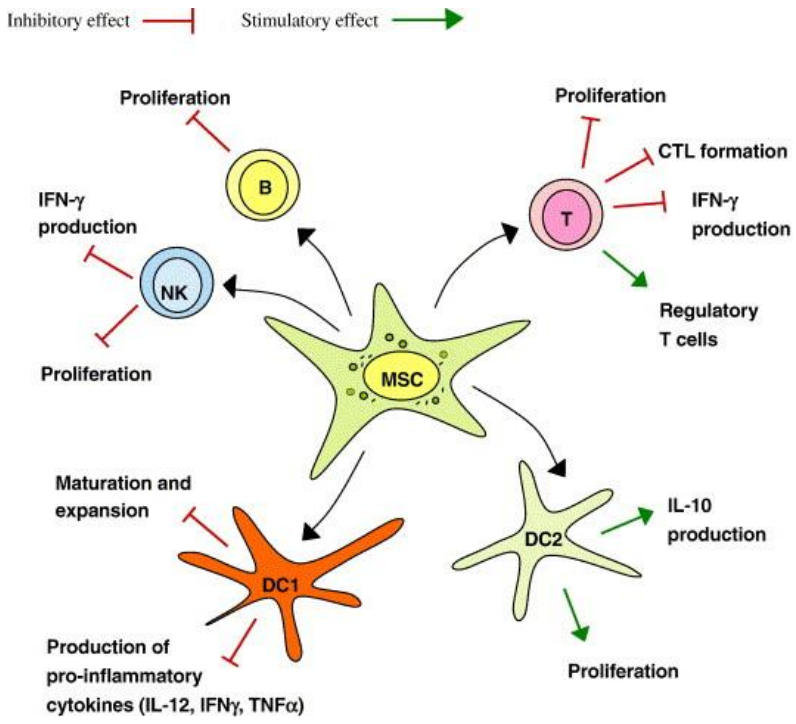


Fig 9. Schematic illustration of the effect of MSCs on the immune system.

1.8 Animal Models of T1DM

Surgical and toxin-mediated pancreatic damage are valuable tools in the study of the consequences of hyperglyceamia and the development of diabetic complications. However is almost impossible to restore normoglyceamia in pancreatectomised animals. (Harder et al 2003). Non-surgical methods of inducing hyperglyceamia by damaging the pancreas include administration of toxin such as Streptozotocin and Alloxan.

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a nitrosurea derivate isolated from *Streptomyces achromogenes* with broad-spectrum antibiotic and antineoplastic activity. It is a powerful alkylating drug, that has been demonstrated with glucose transport, glucokinase function, and also induce multiple DNA strand breaks. In particular STZ is taken up by pancreatic β-cells *via* glucose transporter GLUT2, the diabetogenic action of STZ is to reduce expression of GLUT2. Intracellular action of STZ consists in fragmentation of β-cells DNA; it has been demonstrated that STZ-induced β-cell death is alkylation of DNA, this activity is related to nitrosurea moiety, especially ate the O^6 position of guanine. Furthermore STZ is a nitric oxide donor and NO, so even this aspect contributes to STZ-induced DNA damage. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation. In addition STZ inhibits the Krebs cycle, binding to the iron-containing aconitase inhibiting enzyme activity (Szkudelski 2001).

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) is synthesized by uric acid oxidation; it is a hydrophilic and unstable substance; its half-life at neutral pH and 37˚C is about 1.5 min. The action of alloxan in the pancreas is preceded by the rapid uptake by the β-cells

via GLUT2; the rapid uptake has been proposed to be the one of the important features determining alloxan diabetogenicity. The formation of reactive oxygen species is preceded by alloxan reduction, through which dialuric acid is formed; it is the re-oxidized back to alloxan establishing a redox cycle for the generation of superoxide radicals (Munday 1988). One of the targets of the reactive oxygen species in DNA of pancreatic islets.

The non-obese diabetic (NOD) mouse and bio breeding (BB) rat are the two most commonly used animals that spontaneously develop diseases with similarities to human T1 Diabetes.

Furthermore many transgenic animals are also available see table

GENE	COPY NUMBER	PHENOTYPE	REFERENCE
Insulin Receptor	0	Severe hyperglycaemia	Joshi 1996
Insulin Receptor	1(in muscle)	Mild insulin resistance	Chang 1994
IRS1	0	Mild insulin resistance, growth retardation	Tamemoto 1994
IRS2	0	Hyperglycaemia, reduced β-cell mass	Withers 1998, Kubota 2000
Glucokinase	0	Severe hyperglycaemia	Grupe 1995

Glucokinase	1	Non-progressive glucose intolerance		Grupe1995
GLUT4	>2	Increased insulin sensitivity		Liu 1993

Tab.2 Transegenic animal model for T1DM

The animal models for T1DM have provide to understand and clarify many aspect of the pathogenesis of the human disease and patients have directly benefited from the use of animals, however now a day is not possible to reproduce the paradigm of human diabetic complications and disappointed results have been reached from the studies aimed to prevent T1DM based on strategies that are successful in rodents. (Rees and Alcolado 2005).

Materials and Methods

Animal care and treatment were conformed with institutional guidelines, in compliance with national (DL116/1992, Circ n. 8/1994) and international (EEC Council Directive Directive 2010/63, OJL 358, Dec 1987; NIH Guide for the Care and Use of Animals, US NRC 1996) laws and policies.

2.1 In Vitro experiments

2.1.1 Pancreatic Islet Isolation

Pancreatic islets were isolated from 12 weeks aged male healthy Lewis rats, body weight 250-300gr (Harlan Laboratories, Italy). After anesthesia pancreatic glands were distended with collagenase P solution (Boheringer-Mannhein, Germany), removed and loaded into a digestive chamber at 37°C. When optimum digestion was reached, the chamber was flushed with 4°C Hank's Balanced Salt Solution (HBSS, Gibson Nitrogen Corporation, Scotland) and digested tissue was purified by Hystopaque gradient (1.077g/ml Sigma, St. Louis, MO). Islets were cultured in RPMI 1640 Medium (Lifetechnologies, Italia) supplemented with 10% fetal bovine serum (Euroclone, Italy) and incubated at 37°C, 5% CO_2. Pancreatic islet were maintained as floating cultures in RPMI 1640 medium (Euroclone, Pero, Italy) supplemented with 2mM L-Glutamine, 100mM Penicillin/Streptomycin and 0.25 mM Fungizone. Before co-cultures setting-up islets were stained with a vital fluorescent dye Calcein (BD Bioscience, Franklin Lakes, NJ, USA) dissolved in DMSO for 30 minutes at 37°C.

2.1.2 rMSCs isolation

rMSCs were obtained from 10 week-old Wistar rats (Harlan, Italy) bone marrow by flushing the femur and tibia diaphysis with 2ml/bone of α-MEM supplemented with 2mM L-Glutamine and 100 mM Penicillin /Streptomycin (Euroclone, Pero, Italy). The cell suspension was seeded in flask with α-MEM supplemented with 20% ES cell screened serum, 2mM L-Glutamine and 100 mM Penicillin/Streptomycin and 0.25 mM Fungizone. When 80% of confluence was reached, cells were detached with 0.05% Trypsin/EDTA, seeded at the density of 4,000 cells/cm^2 and incubated at 37°C, 5%CO_2. Before co-cultures setting-up, rMSC were stained with 30µm/mg red fluorescent dye DiI (Molecular Probes Inc., OR,USA) dissolved in EtOH 100% for 1 hour at 37°C.

2.1.3 Cultures and co-cultures set up

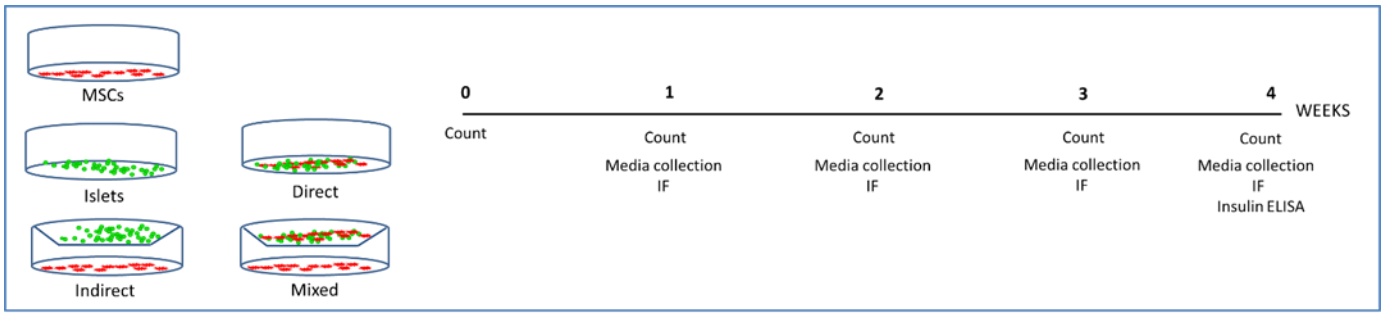


Fig 10. Schematic representation of in vitro experiments

-MSCs: 500.000 MSCs alone were seeded on culture dish

-pancreatic islets: 500 pancreatic islets were located in a culture dish and they were cultured free-floating.

-direct co-cultures: in order to obtained a direct contact between MSC and pancreatic islets, MSC were directly seeded on floating pancreatic islets, after 24 hours the co-cultures were moved in a new flask

-indirect co-cultures: in order to avoid a direct contact and to let MSC and pancreatic islet to share the medium, MSC were seeded on the bottom of the dish, while pancreatic islets were floating in the cell-strainer

-mixed co-cultures: this co-cultures were obtained combining direct and indirect co-cultures in order to evaluate a possible synergism between two conditions.

2.1.4 Islet counting

In order to assess islet survival, islets were counting every week for three weeks. We considered viable islets those presenting bordi ben definiti and with a diameter between 100µm and 300µm, the data were shown as survival percentage.

2.1.5 Static test

In order to evaluate pancreatic functionality every week for three weeks static test was performed. All cultures were exposed to different glucose concentrations: High glucose concentration 20mM (HG) and Low glucose concentration 1,67mM (LG) in order to respectively mimic hyperglycemia and hypoglycemia.

As first step all cultures were pre-conditioned with LG medium for 2 hours, subsequently they were exposed to LG medium (collected) for 1 hour, then to HG medium (collected)

for 1 hour and then re-exposed to LG medium (collected). At the end of the static test all cultures were maintained in respective maintenance medium.

2.1.6 Insulin ELISA test

insulin sandwich ELISA assay (DRG Instruments GmbH, Germany) was performed on the media collected each week for three weeks. Absorbance was detected at 450 µm (FluoSTART omega,Labtech). Concentrations of insulin were obtain by computerized data reduction of the absorbance for the Calibrators, versus the concentration using cubic spline regression.

2.1.7 Immunofluorescence

*Pancreatic Islets were pelletted, then washed with PBS and fixed for 1h at RT in Parformaldehyde 4%. Sequential infiltrations with sucrose 5%, 10% were performed, 30' each, the last one with sucrose 20% was left ON; the day after sucrose was eliminated and OCT (Optimal Cutting Temperature) medium was added. The samples were frozen in isopentan previously cooled in liquid nitrogen. Section of 20µm were obtained at cryostat.

*Mesenchymal stem cells: MSC seeded on glass slide were washed with PBS and fixed with paraformaldehyde 4%.

The samples were washed twice with PBS, then a wash with Glycine 0,1M was performed in order to decrease basal auto-fluorescence. We used two different Blocking buffer, for section of pancreatic islets non-specific sites were blocked with 5%BSA in PB, while for

MSC we used 1%BSA, 0,5%Triton X-100 in PBS. Then we incubated primary antibody ON at 4°C and the day after secondary antibodies were added for 1 hour at RT. After washing with PBS, coverslips were mounted and cultures were examined using confocal microscope equipped with krypton/argon laser. Noise reduction was achieved by Kalman filtering.

2.2 In Vivo experiments

Diabetes was induced by a single intraperitoneal injection of Streptozotocin 60mg/kg in fasting male Lewis rats, 12 weeks aged, body weight 200-250 grams. After few days glycemic levels were checked and we considered diabetic rats those presenting glycemic levels over 300mg/dl.

Rats were randomly assigned to five experimental groups: healthy rat (GROUP A), diabetic rats (GROUP B), diabetic rat receiving 3000 pancreatic islets (GROUP C), diabetic rats receiving 2000 pancreatic islets + MSCs (GROUP D), diabetic rats receiving MSC (GROUP E). Diabetes was induced by a single intra-peritoneal injection of Streptozotocin (Sigma Aldrich, St. Louis, MO) 60mg/kg. Blood glucose levels were measured after 48 hours using a glucometer (Ascensia Elite; Bayer, Basel, Switzerland): rats presenting a glucose blood level between 300 and 400 mg/dL were included in the study.

Group C received 3000 pancreatic islets, by literature is known that 3000 pancreatic islets are able to restore glucose levels in diabetic rats. (Remuzzi et al 2009)

Group D received 2000 pancreatic islets in direct contact with 10^6 MSCs and 10^6 MSCs were also injected in caudal vein, resuspended in 250 µl physiological solution.

Group E received 10^6 MSCs resuspended in 250 µl physiological solution.

Encapsulated Pancreatic islets and encapsulated pancreatic islets and MSCs transplantation was effected after 8 weeks from diabetes induction.

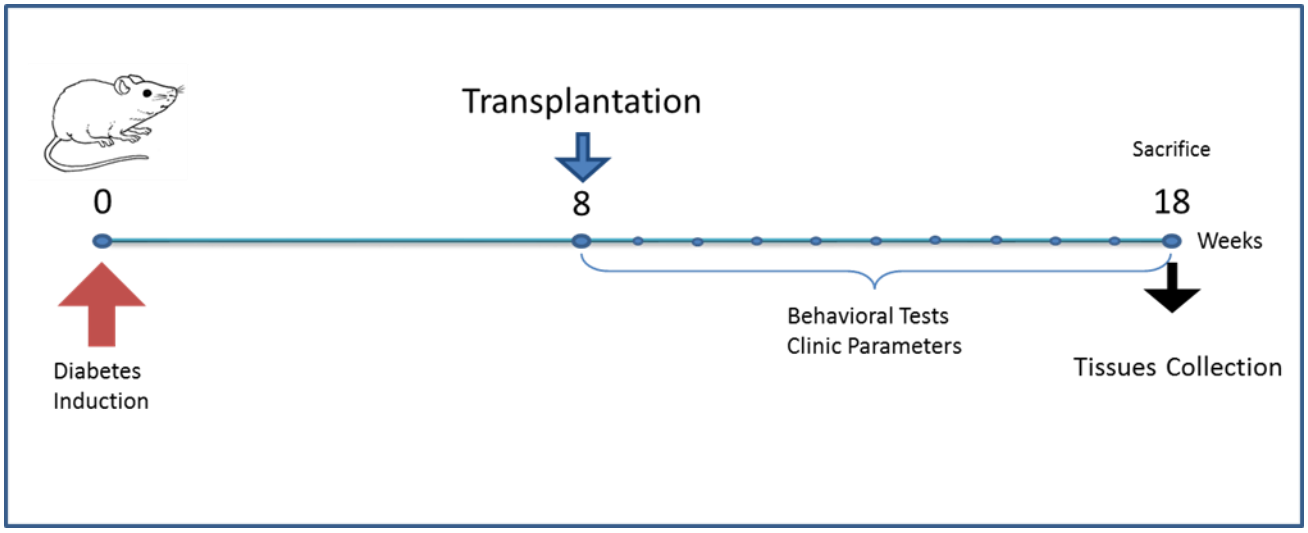


Fig.11 Schematic representation of in vivo experiments

2.2.1 Pancreatic islets encapsulation

After O.N. culture, pancreatic islets were suspended in solution of 1.7% sodium alginate (Manugel DMB, Monsanto plc, Surrey, UK) at a concentration of 3islets equivalent, the molecular weight of alginate ranged between 450 kDa and 560 kDa; the islet-alginate mixture was extruded through an air droplet generator into a solution of 100mM CaCl2 solution. The resulting gel beads had diameters ranging from 800 and 950 µm.

2.2.2 Pancreatic islets transplantation

Encapsulated islets were transplanted into intraperitoneal cavity using a trocar after 8 weeks of diabetes induction.

2.2.3 Body Weight

The body weight was monitored once a week for all 18 weeks.

2.2.4 Glycemia

Blood glucose levels were measured once a week for all 18 weeks, using a glucometer (Ascensia Elite; Bayer, Basel, Switzerland).

2.2.5 Behavioral Test

*Hot plate test: thermal nociceptive threshold to radiant heat was quantify by using the paw withdrawal in a hot plate. Withdrawal latency was defined as time between placement on the hot plate and time of withdrawal and licking of hind paw. Cut-off period was 60 seconds.

*Randall-Selitto paw withdrawal test: mechanical nociceptive threshold was quantified by using the Randall-Selitto paw withdrawal test with an analgesy meter (Ugo Basile, Comerio Italy), which generates a linearly increasing mechanical force. The results indicate the maximum pressure tolerated by the animal.

2.2.6 NCV in tail

NCV was measured using Myto EBNeuro electromyography (EBNeuro, Florence, Italy). Briefly the antidromic NCV in the tail was assessed by positioning recording ring electrodes distally on the tail and stimulating ring electrodes at 5 and 10 centimeters proximally from the recording site. The latencies of the potentials recorded at the two points after nerve stimulation were determined (100ms. duration stimulus peak-to-peak) and NCV was calculated. Determinations were performed under standard conditions in a temperature-controlled room. Core temperature was maintained at 37°C by using heating pads and lamps. Animals were anesthetized with isoflurane.

After 18 weeks all animals were scarified with CO_2.

2.2.7 T-BARS

Blood was collected in order to performed T-BAR test, EDTA and gluthatione 1.34 mmol/L 0.65 mmol/L (final concentrations) were immediately added to the collected plasma. Levels of thiobarbituirc acid-reactive substances was determined, as an index of reactive species production.

2.2.8 Intraepidermal Nerve Fiber Density

A skin biopsy was immediately fixed in 2% paraformaldehyde-lysine-periodate for 24 h at 4°C, then cut serially with a cryostat and immune-labelled with anti-PGP 9.5. The total number of PGP 9.5-positive intraepidermal nerve fibers in each section was counted (40×). The length of the epidermis was measured (Microscience Inc.), and the linear density of intraepidermal nerve fiber was obtained.

2.2.9 Tissue Collection:

- Kidneys were explanted from each animals, fixed in Bouin's solution (Bio-Optica, Milan, Italy), and then embedded in paraffin. Sections were stained with the periodic acid–Schiff's (PAS) reagent technique. The degree of glomerulo-sclerosis and tubular dilatation were evaluated.
- Pancreas we also explanted, fixed and embedded in paraffin; sections were stained with Dithizone in order to evaluate morphological changes in pancreatic islets.
- Sciatic nerves and Dorsal Root Ganglia (DRGs) morphometric analysis: in order to evaluated morphological alterations in peripheral nervous system, sciatic nerves and DRGs were extracted, then fixed in Glutaraldehyde 3% for three hours, after three washes in Phosphate buffer 0,12M samples were included in epossidic resin, then were cut in sections of 1 µm and stained with toluidine blue; analysis was performed on slides every 25 µm. Morphometric analysis was performed using ImageJ software.

Results

3.1 In vitro experiments

In order to investigate the mechanism(s) at the base of the putative positive effect of MSCs on pancreatic islets, we set up different cultures and co-cultures: MSCs alone, cultured in adhesion; islets alone, cultured free-floating; direct co-cultures, in which MSCs and pancreatic were in direct contact; indirect co-cultures, in which islets and MSCs shared the same medium, without any type of contact, prevented by the use of a semi-permeable strainer; and mixed co-culture, in which both direct and indirect conditions coexisted. In direct co-cultures we evaluated the effect of a direct contact; since MSCs are able to release many soluble factors, such as trophic factors and cytokines in indirect co-cultures we investigate their possible participation to MSCs effect on pancreatic isles. In mixed co-cultures we evaluated the possible synergism between direct and indirect conditions.

3.1.1 Direct co-culture setting up

In order to obtain a direct contact between pancreatic isles and MSCs since pancreatic islets were cultured free-floating in the medium while MSCs are cultured in adhesion, we firstly verified the feasibility of the direct co-culture. MSC were previously stained with vital fluorescent dye DiI (red) and pancreatic islets with calcein (green); MSCs were detached and added directly in the flask containing pancreatic islets, 24h later pancreatic islets are moved to another flask.

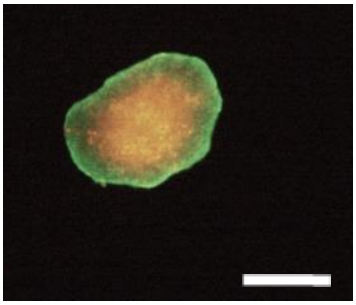


Fig 12.Direct co-culture of pancreatic islet stained in green and MSCs stained in red; yellow represent the merge of the two signals. Scale bar 100 µm

As shown in Fig. 12, MSCs were able to adhere to pancreatic islet surface, as shown in Fig the yellow spots represented the MSCs adhered to pancreatic islets surface, due to the overlapping of green, pancreatic islets, and red, MSC, demonstrating that the direct contact has been reached between them.

In order to identify the best ratio between pancreatic islets and MSCs, we set up direct cultures: 500 islets/500.000 MSCs and 500 islets/10^6 MSCs; since we did not observe any differences in terms of adhesion between them we decided to use first ratio, and we used this ratio for the other co-culture conditions.

3.1.2 Evaluation of pancreatic islets vitality

As mentioned before, pancreatic islets were stained with calcein, that is a vital fluorescent dye, so through the simple observation at inverted microscopy the distribution of calcein gave us information about pancreatic islets vitality: after one week,

the vital dye is equally distributed in the pancreatic islet, both in islets cultured alone as shown in fig 13.a, and in islets in direct co-culture fig 13.b.

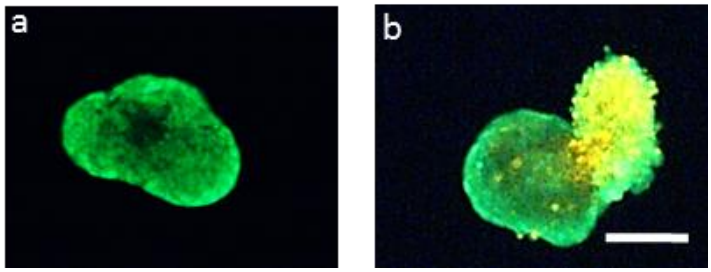


Fig 13. Evaluation of calcein dye distribution after one week of culture in (a) pancreatic islets, in green, alone and (b) pancreatic islets in direct co-culture with MCS, in red, red. Scale bar 100 µm.

After three weeks, fig.14a, in pancreatic islet cultured alone the dye wasn't detectable in the core, but it was still present in pancreatic islet borders, indicating a progressive degeneration of pancreatic islets. On the contrary fig14.b calcein dye was still equally distributed, indicating that pancreatic islets cultured with MSCs were undamaged with respect to pancreatic islets cultured alone

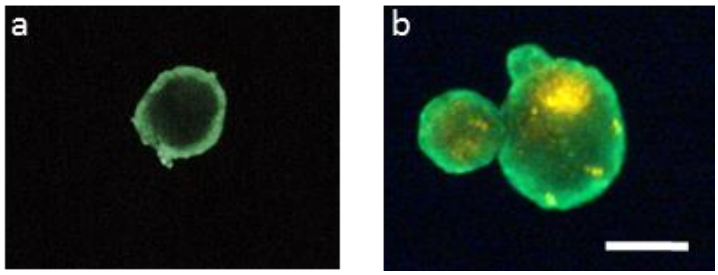
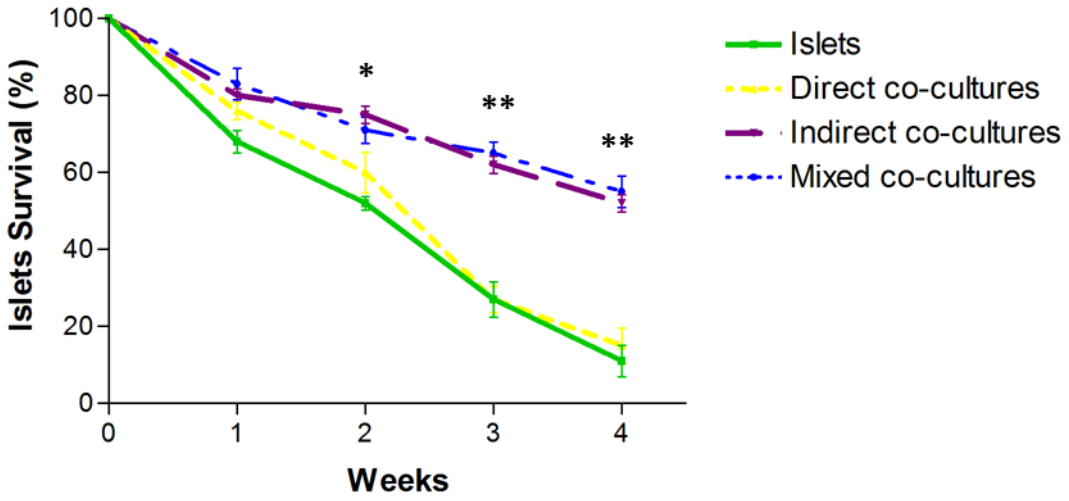


Fig 14. Evaluation of calcein dye distribution after three weeks of culture in (a) pancreatic islets, in green, alone and (b) pancreatic islets in direct co-culture with MCS, in red, red. Scale bar 100 µm.

3.1.3 Pancreatic islets count

Pancreatic islets in all different culture conditions were weekly counted for all four weeks at optical microscope; we counted the islet presenting at least 150 µm of diameter and well defined borders.



Percentage of pancreatic islets survival are expressed as mean± SD;

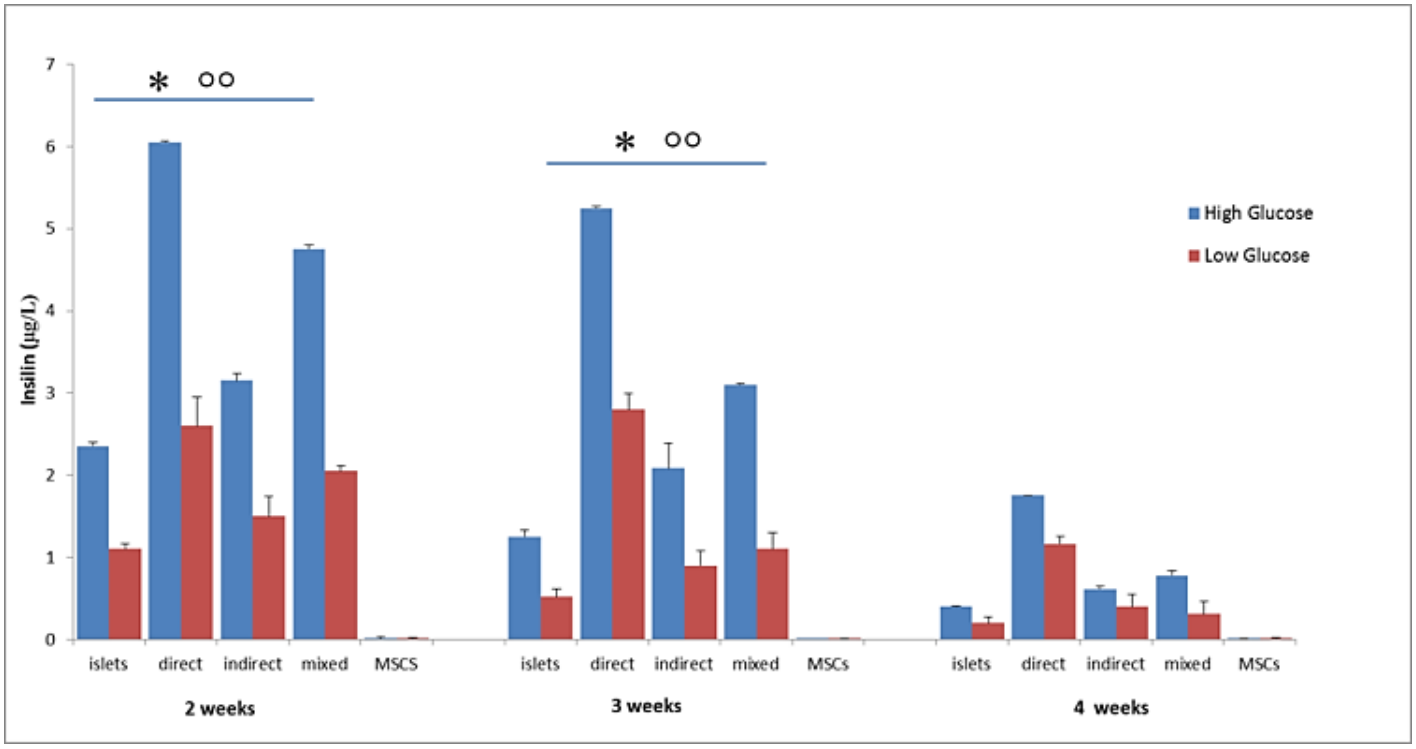
* p<0.05 islets alone vs mixed; ** p<0.001 islets vs mixed, mixed vs direct.

As shown in the graph (Fig.15), after one week values of islets count were heterogeneous; after two weeks differences between four co-culture conditions emerged: survival percentage between islets cultures alone and those in direct co-culture was not statistically significant, while it was significant the difference between islets cultured alone and indirect and mixed co-cultures(* p<0.05 islets alone vs mixed). After three and four weeks no differences were observed between pancreatic islets cultured alone and those in direct contact with MSCs and the survival was around 13%. On the contrary a statistically significant increase was observed for both indirect and mixed co-cultures: the survival percentage was around 52%, suggesting that MSCs effect should change in

relation with culture condition and so that different mechanisms may be involved (**p<0.001 islets alone vs indirect and mixed, and direct vs indirect and mixed).

3.1.4 Evaluation of pancreatic islet functionality

To analyze the islet functionality and its modifications when co-cultured with MScs, we firstly performed the Static Test, that consists to expose to different glucose concentration all cultures, that is High Glucose Medium 20mM and Low Glucose Medium 1,67mM; these concentrations mimed respectively hyper- and hypoglycemia. After glucose stimulation all medium were collected and Insulin Elisa Assay was performed. In graph (Fig.16) insulin concentrations are reported µg/L.



3, 4 weeks. Percentage of pancreatic islets survival are expressed as mean± SD.

(* p<0.05; °° p<0.01 Direct culture vs MSCs).

As attended pancreatic islets alone were able to correctly respond to glucose variations, indeed high levels of insulin corresponded to high glucose levels in the medium and vice versa low insulin concentrations corresponded to low glucose levels. In the medium of pancreatic islets in direct co-culture with MSCs higher levels of insulin were detected, especially after two and three weeks, while this difference was less evident after four weeks, due to the degeneration of pancreatic islets. Similarly in indirect co-culture medium higher levels of insulin were detected with respect of pancreatic islets cultured alone, but in any case insulin levels were lowest with respect to direct-cultures. Even in mixed co-cultures we detected higher insulin level with respect to islets cultured alone. These data demonstrate that the presence of MSCs leads to an increase of insulin released (* $p<0.05$ Islets vs Direct, Indirect and Mixed co-cultures; °° $p<0.01$ Direct culture vs MSCs)

3.1.5 Immunofluorescence Analysis

In order to deeply investigate the reasons by which an increase of insulin levels was observed in pancreatic co-cultures medium, we performed immunofluorescence for insulin both on pancreatic islets and MSCs, in all culture conditions. As mentioned before, pancreatic islets were stained with calcein, in green, while MSCs with DiI, in red; insulin resulted blue. As attended pancreatic islets, cultured alone (Fig. 17a), expressed insulin, while MSCs did not, appearing only red (Fig.17b); the MSCs fibroblast-like morphology was well evidenced since DiI bound actin filaments.

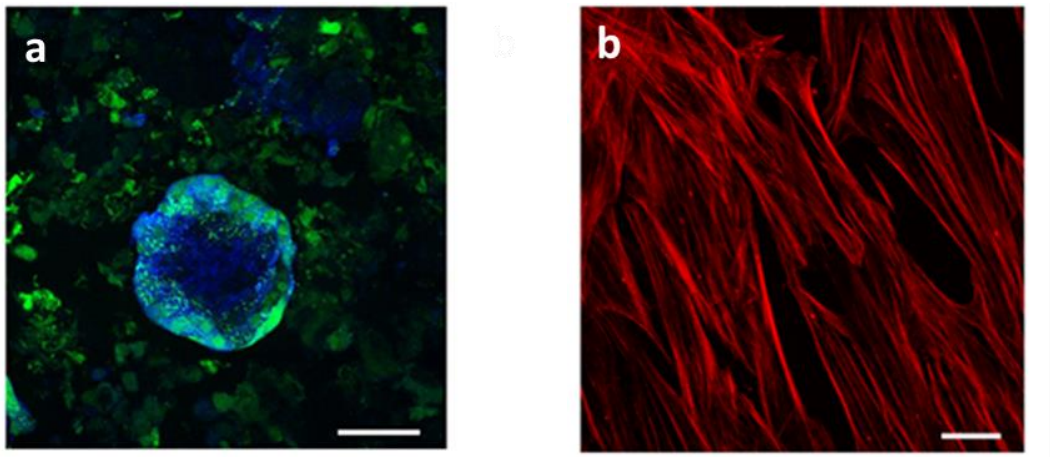


Fig.17 Immunofluorescence of (a) Pancreatic islets cultured alone, stained with calcein (green), insulin in blue;(b)MCSs stained with DiI (red), insulin in blue,. Scale bar 100µn

In direct co-culture, Fig.18 first of all we observed that the red stained resulted very different compared with MSCs cultured alone, so it was possible to affirm that MSCs in direct contact with pancreatic islets lost their peculiar morphology, acquiring a round-shape one. Furthermore we observed a co-localization between insulin (blue) and MSCs (red), indicating that MSCs were able to express insulin, this unexpected result perfectly fit with ELISA assay results, suggesting that when in direct contact MSCs acquired an insulin-releasing phenotype, this finding could explain the increase of insulin release in direct co-culture medium.

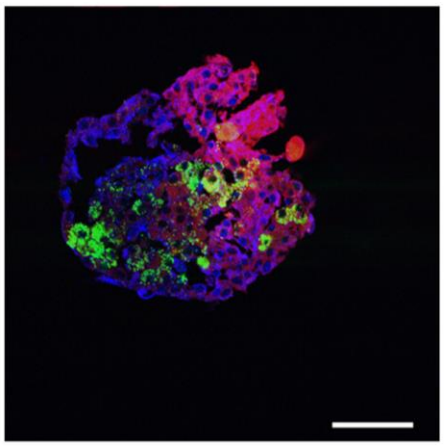


Fig.18 Immuno fluorescence of direct co-culture in green pancreatic islet, in red MSCs and in blue insulin. Scale bar 100µm

Regarding indirect co-culture, islets resulted positive for insulin expression (Fig 19.a), while we did not observed any expression of insulin in MSCs, that appeared only red (Fig 19.b), indicating that the direct contact was essential for the differentiation towards insulin-releasing cells.

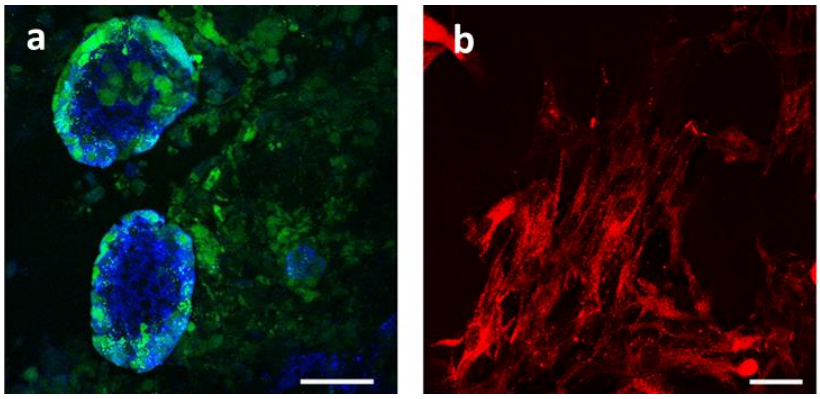


Fig.19 Immunofluorescence of indirect co-culture (a) in green pancreatic islet, in blue insulin; (b) in red MSCs and in blue insulin. Scale bar 100µm

In mixed co-cultures we observe a co-localization of insulin (blue) and MSCs (red) carried in transwell (Fig.20.a), that is MSCs in direct contact with pancreatic islets, indicating as reported for direct contact, that MSCs acquired an insulin-releasing phenotype; while insulin was absent in those seeded on the bottom of the dish (Fig 20.b), meaning that they did not express insulin.

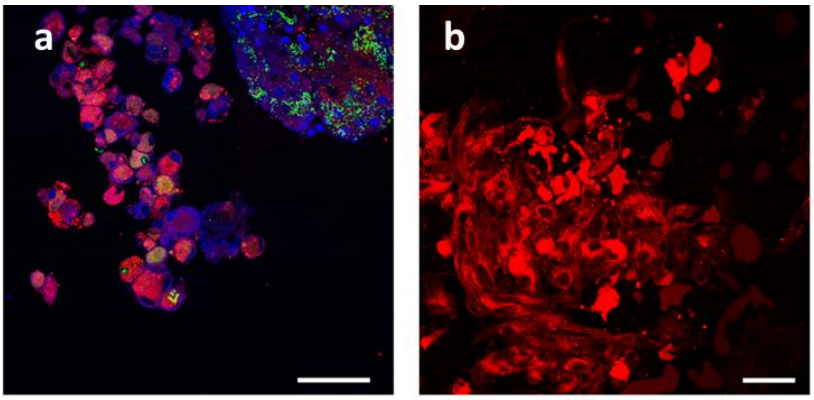


Fig.20 Immunofluorescence of mixed co-culture, (a) pancreatic islets in green, MSCs in red and insulin in blue; (b) MSCs in red, insulin in blue. Scale bar 100µm.

3.2 In vivo experiments

Our experiments were carried out on Lewis rats; diabetes was induced by a single intra-peritoneal injection of Streptozotocin. The animals were randomly assigned to five groups:

Group A - healthy rats (control); Group B - diabetic rats; Group C - diabetic rats transplanted with 3000 islets; Group D - diabetic rats transplanted with 2000 islets and MSCs and Group E - diabetic rats transplanted with MSCs. Transplantations were performed after 8 weeks from diabetes induction, when even long-term complication, as neuropathy, was evident.

3.2.1 Blood Glucose Levels

As reported in the graph below, Fig.22, Group A, healthy rats, for all experiment duration showed normoglyceaemia, on the contrary diabetic rats, Group B always showed hyperglycaemia. Group C and D had significant lower blood glucose levels with respect to diabetic rats, but without statistical differences between them. Basing on these results, we can assert that both transplantations were effective in terms of glycemic control, and that the same results were reached in rats receiving 2000 islets and MSCs with respect those received 3000 islets. Group E presented glucose level comparable to diabetic rats, indicating that MSCs alone were not able to ameliorate glycemic levels. (**$p<0.001$ Groups C,D vs Group E).

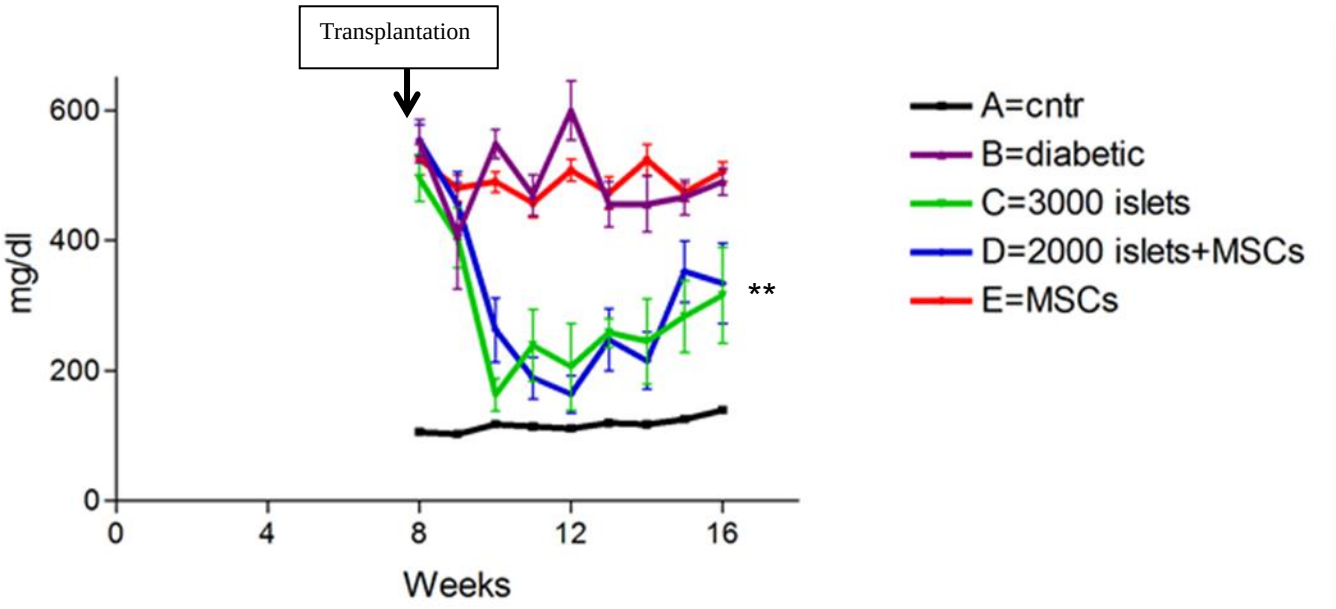


Fig.21 Blood glucose levels was measured weekly, glucose concentrations are reported as mean ±SD. **p<0.001 Groups C,D vs Group E

3.2.2 Body weight

Results obtained from the evaluation of body weight, shown in graph below (Fig.22), run parallel to those obtained by glycemic levels ones, indeed as aspected healthy rats showed a progressive gain of weight, while it was considerably decreased in diabetic rats. Another time transplanted Group C and D trends were comparable and body weights were increased with respect to healthy rats (Group A), while Group E rats showed a body weight similar to diabetc rats (**p<0.001 C and D vs B and E).

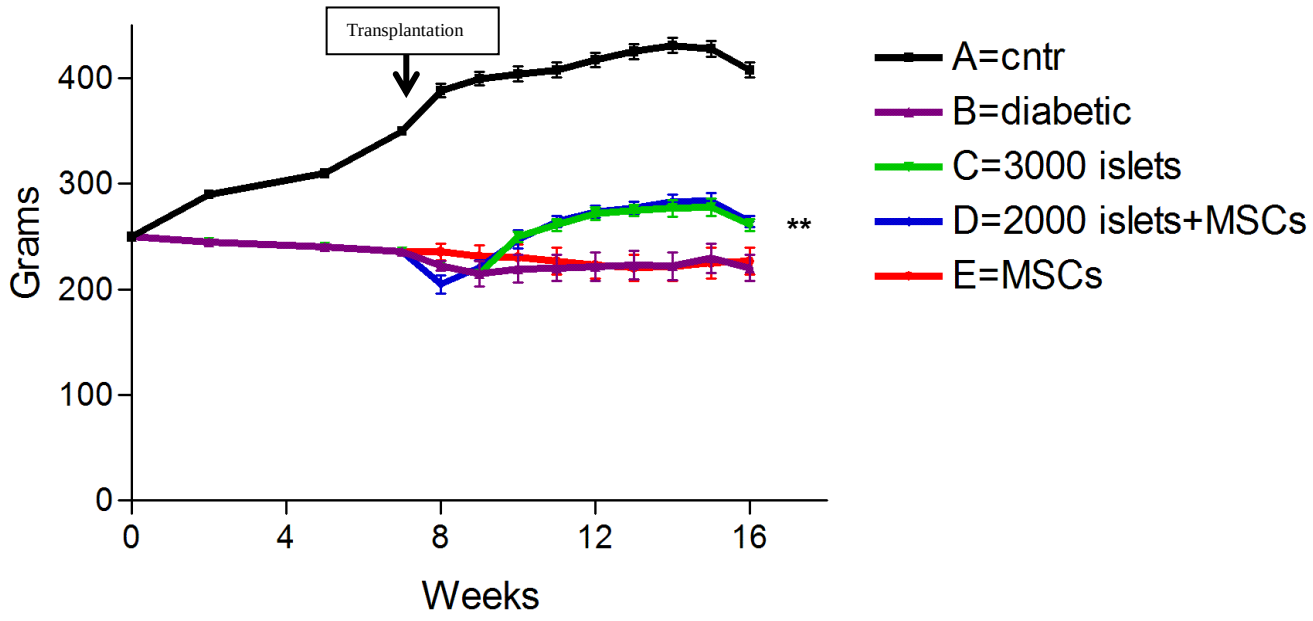


Fig.22 Body weight, body weights were measured weekly,

weights are reported as mean ±SD._**p<0.001 C and D vs B and E

3.2.3 Nerve conduction velocity

In order to analyzed the effect of neuropathy, a long-term complication of diabetes, we evaluated the nerve conduction velocity in caudal nerve.

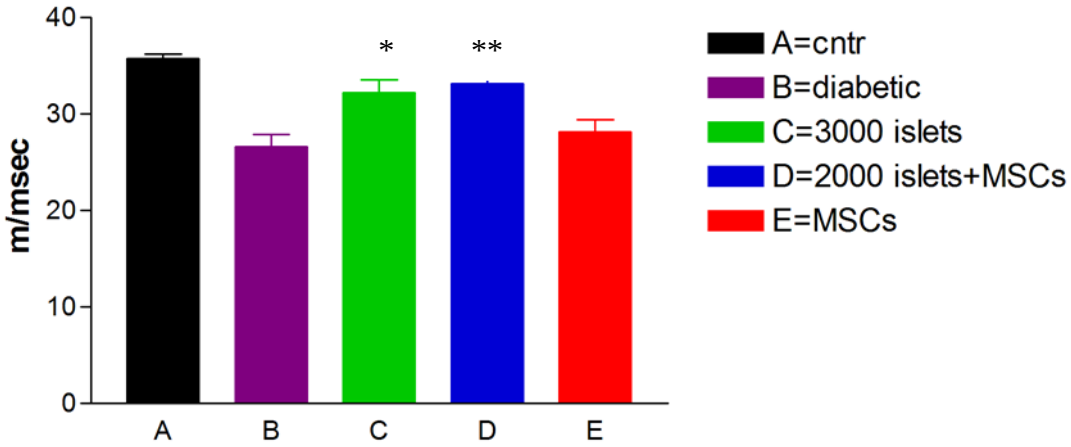


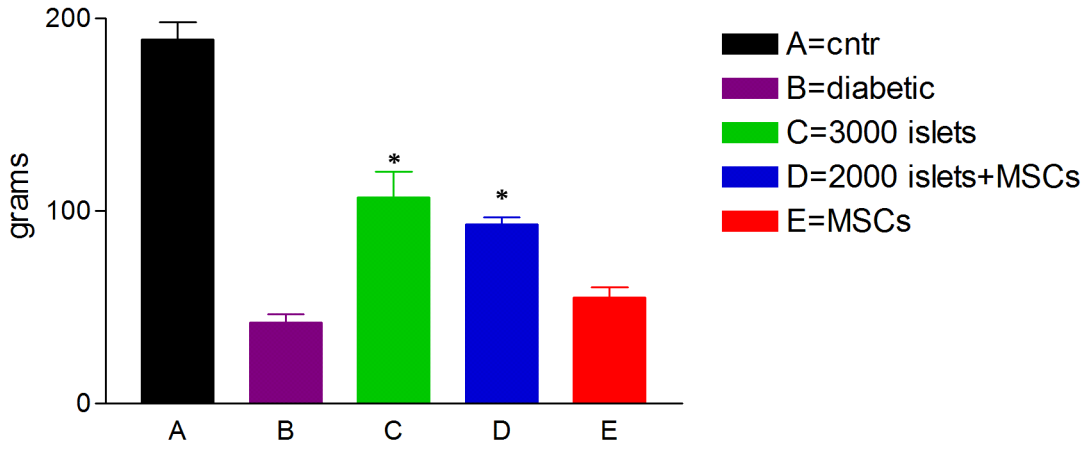
Fig.23 Nerve conduction velocity was measured in caudal nerve, velocities

are presented as mean±SD; *p<0.05 C vs B, **p<0.01 D vs B

As shown in graph, Fig.23, the velocity was reduced in diabetic rats (Group B) with respect to healthy rats (Group A), while it was mainly restored in transplanted groups, C and D. Group E showed nerve conduction velocity similar to diabetic one, indicating that MScs alone were not sufficient to restore neuropathy. The difference between Group B vs Group D, that received 2000 islets and MSCs, were more statistically significant, $p<0.01$ (**), with the respect to the difference between group B and C (*$p< 0.05$), that received 3000 Islets; so the co-transplantation of pancreatic islets and MSCs leaded to a better improvement of diabetic profile.

3.2.4 Randal Selitto Test

We also performed behavioral tests for pain; indeed diabetic neuropathy is characterized by a painful component, which is evaluable by mechanical nociceptive threshold by Randall Selitto test, that indicates the pressure tolerated on the paw.



reported as grams mean±SD. $p<0.01$ C and D vs B

As shown in graph tollerated pressure on the paw was very low in diabetic rats (Group B), while was increased in both transplanted gropus (C and D; $p^*<0.01$), with a similar trend.

Since the difference betweeh diabetic rats, Group A and Group E was not statistically significant, we can affirm that MSCs transplanetd alone were not able to improve diabetic profil.

3.2.5 Hot plate test

Another behavioural test, the Hot Plate test lets to evaluate thermal nociceptive threshold, shown as latency to paw withdrawl from a hot plate.

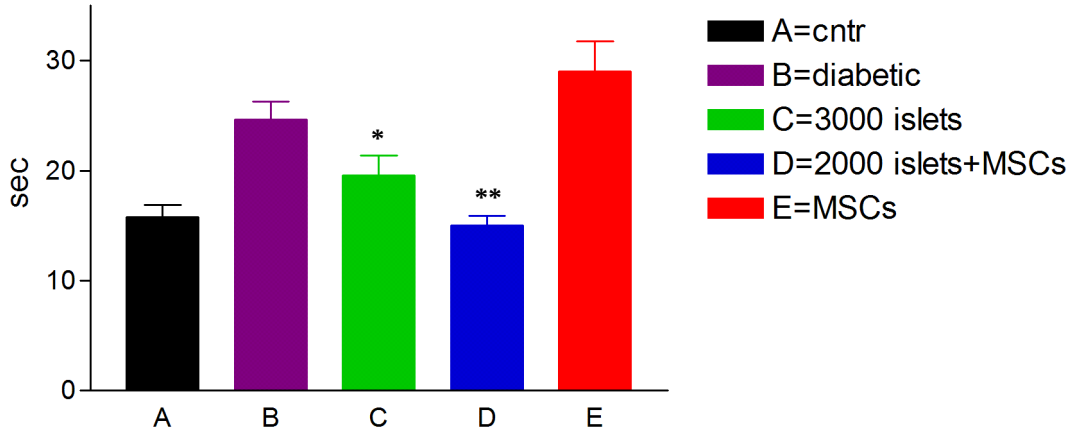


Fig. 25 Hot Plate test, time of latency are reported as mean ±SD.

*p<0.01 C vs B, **p<0.001D vs B

As attended time of latency was increased in diabetic rats with respect to healthy rats (Group A). The time of latency was significantly reduced in group C with respect to diabetic rats (*p<0.01), but was inresult was obtain in Group D, in which treshold levels were comparable to those of healthy group, A. We can affirm that in this case the prsence of MSCs contribute to a better

3.2.6 Intraepidermal Nerve Fiber Density

Small-diameter nerve fibers, which subserve nociception, can be affected early in peripheral neuropathies, although their injury may not be detectable by routine neurophysiologic tests. On the other hand, skin biopsy has proved to be a reliable tool to examine nonmyelinated nerve fibers, as assessed by the quantification of intra-epidermal nerve fiber (IENF) density not only along with the degenerative process but, noteworthy, IENF density could be very helpful in evaluating treatment efficacy.

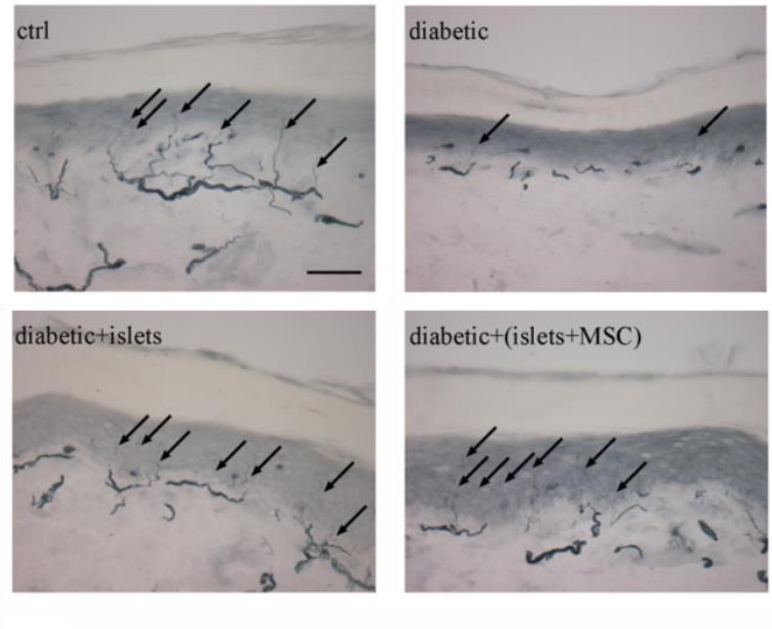


Fig.26 Intraepidermal Nerve Fibers. Scale bar 50µm

It is possible to observe a significant decrease of small fibers in diabetic rats epidemis, that indicates a neuropathy; the number is increased in transplanted groups indicating a rigeneration of C fibers; these data were confirmed by fibers counting.

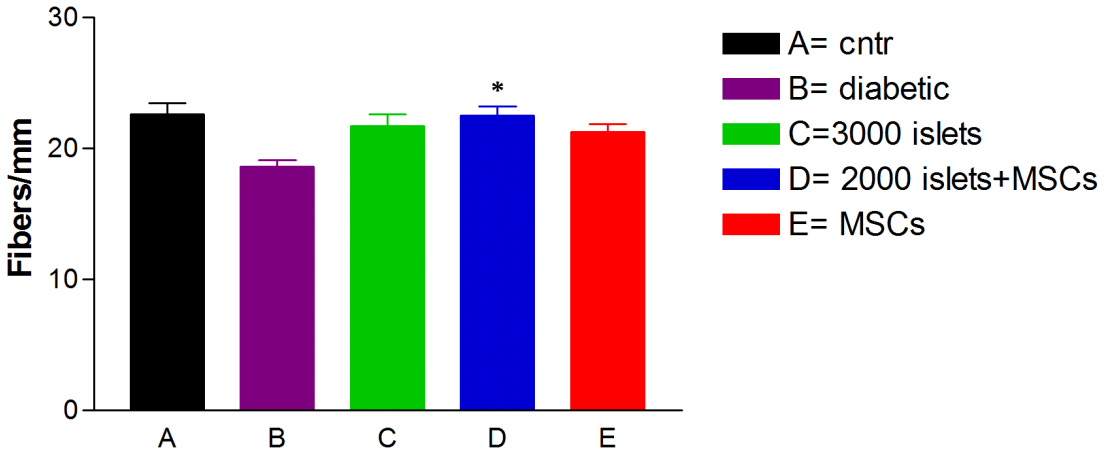


Fig 27. Intraepidermal Nerve Fiber Density Intraepidermal unmyelinated axons were counted for all experimental groups , and the intraepidermal nerve fibers density per millimeter of skin is determined

3.2.7 Plasma and tissue thiobarbituric acid-reactive substances (TBARS)

Since oxidative stress is involved in diabetic neuropathy, we performed the T-BARS (thiobarbituric acid-reacting substances) analysis.

As shown in Fig.28 T-BARs plasma levels were significantly higher in diabetic rats with respect to healthy rats, while both transplanted groups show lower levels; another time is possible to confirm that MSCs alone are not able to improve diabetic profile.

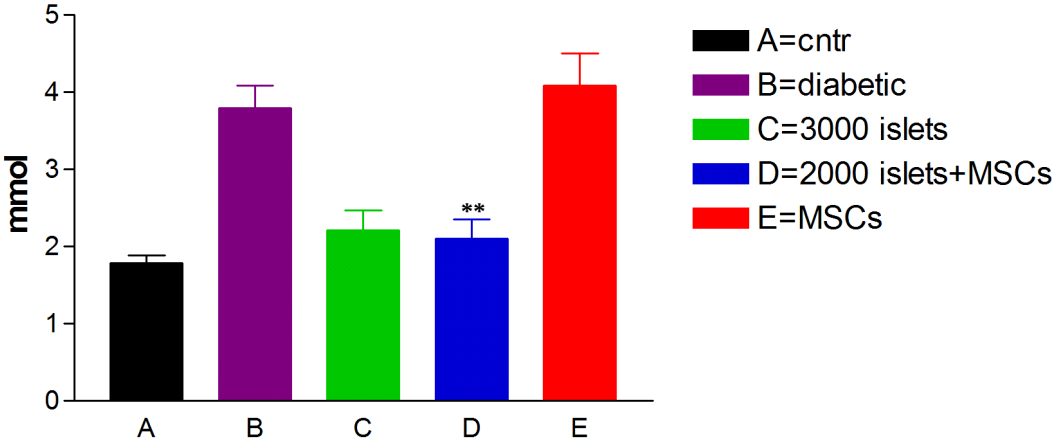


Fig.28 T-BARs plasma level. T-BARS plasma levels were measured for all experimental groups, results are reported as mean ±SD.

The results obtained by plasmatic T-bars levels were also confirmed by those found in tissue.

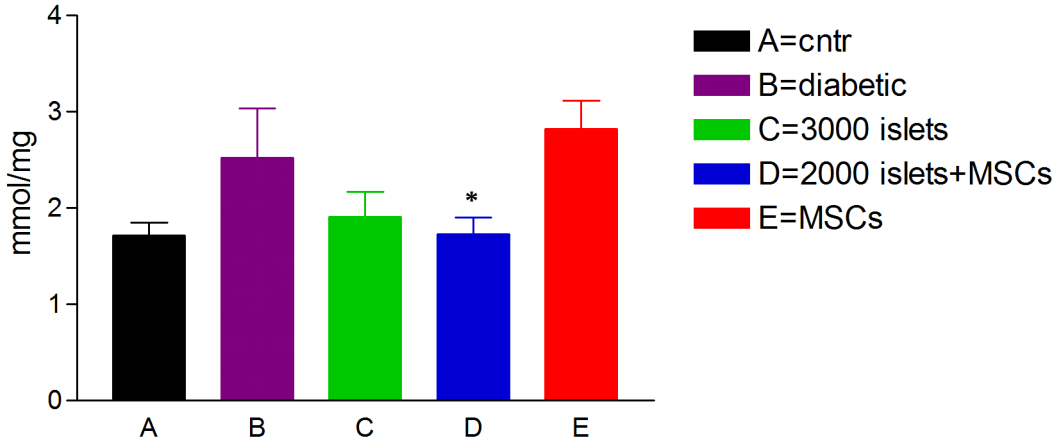


Fig.29 T-BARs tissue level. T-BARS tissue levels were measured for all experimental groups, results are reported as mean ±SD. (*p<0.05 D vs B)

As shown in graph, both transplanted groups show lower levels with respect to diabetic rats, and with those received only MSCs. Only the difference between Group D and B was statistically significant, indicating that the presence of MSCs were able to better improve the stress oxidative aspect of diabetic neuropathy.

3.2.8 Histological kidney analysis

Since one of diabetes long-term complications is nephropathy, we performed PAS staining, which evidence basal lamina, on kidney sections of all experimental groups.

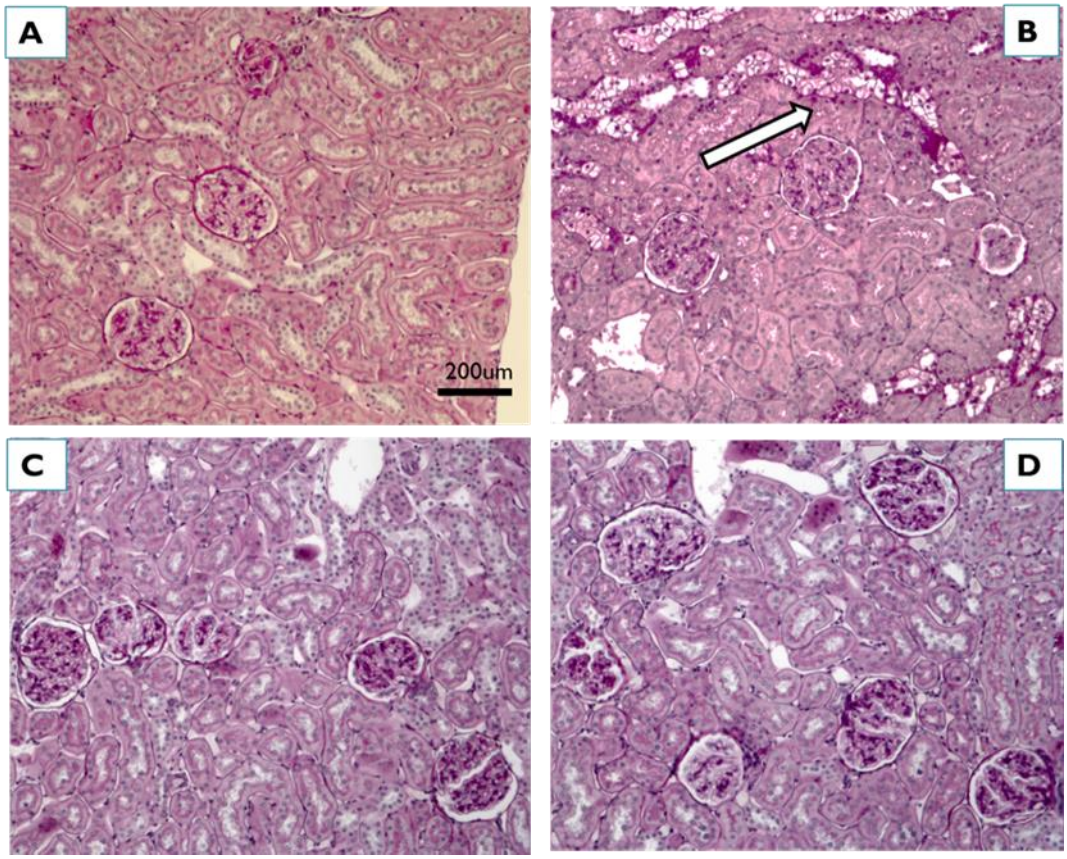


Fig.30 Pas Staining. Pas staining was performed on kidney sections of all experimental groups. The arrow indicates a large area of vacuolization

This staining evidenced basal lamina, that results intense purple. As shown in Fig. 30.A no-evident alterations were visible in the glomerulus and tubules; on the contrary Group B presented evident alterations, localized in the distal tubules, where wide vacuolization areas were well visible. In Groups C and D we observed a regression of vacuolization area, indicated a regression of nephropathy.

3.2.9 Morphometric evaluation of sciatic nerves

We performed a morphometric analysis of sciatic nerves; the differences among groups were assessed morphologically by means of G-ratio calculations.

G-RATIO	Group A	Group B	Group C	Group D
Total number of values	2480	2347	2288	2147
Number of excluded values	0	0	0	0
Number of binned values	2480	2347	2288	2147
Mean	0,719	0,755	0,764	0,742
Std. Deviation	0,064	0,053	0,045	0,055

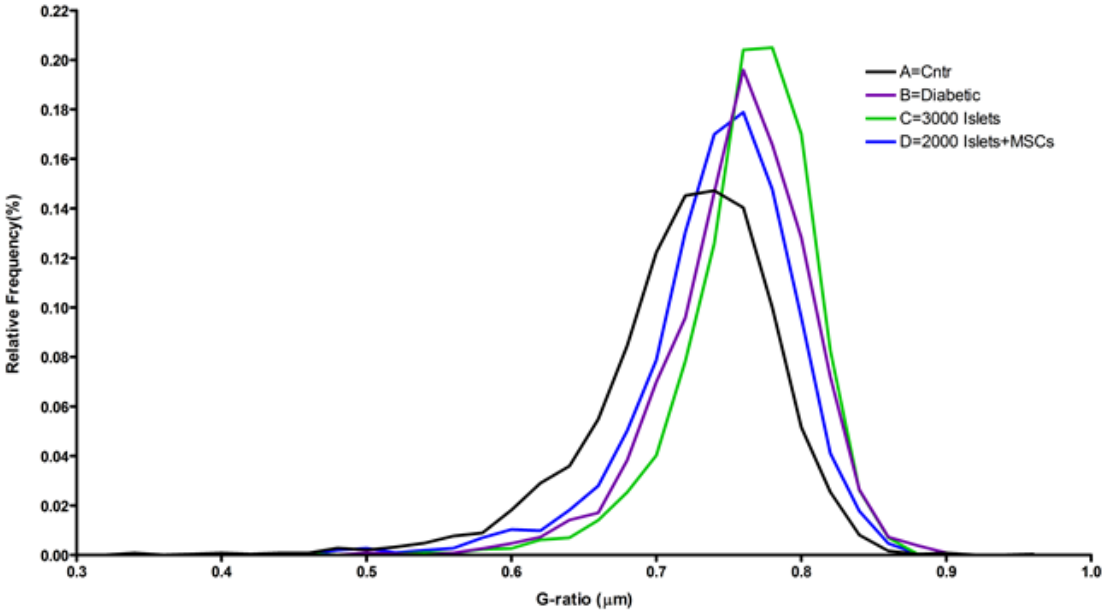


Fig.31 G-ratio. G-ratio were measured for all experimental groups.

Results are reported in tabular form (a), and in graph form (b).

Differences between the groups were statistically significant $p<0.001$

Comparing the G-ratio values of different groups shown in the Fig.31 and the light microscopy observation reported in Fig.32 below, we can affirm that in Group B, diabetic rats, there was an evident morphological alteration, in particular in the largest fibers; the G-ratio was higher with respect to healthy group, suggesting myelinopathy. On the contrary Group A, healthy rats, presented regular myelin sheath structures. Group C,

which received 3000 islets seemed to have altered myelin structure as well as diabetic rats and g-ratio value confirm our observation. Group D, which was transplanted with 2000 islets and MSCs showed fibers with more regular structure and also g-ratio value indicates a restoration of myelinopathy.

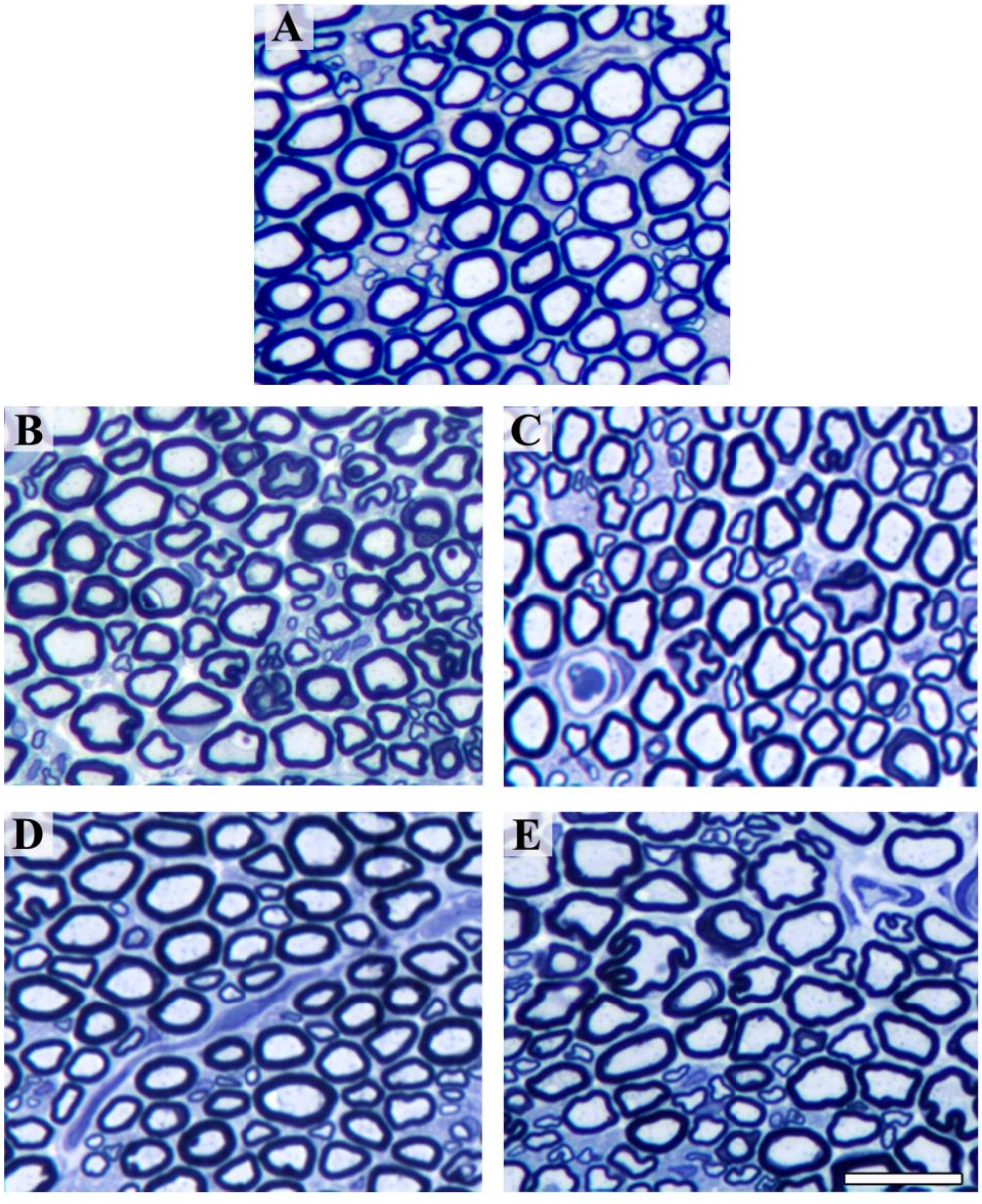


Fig 32. Representative images of sciatic nerve of experimental groups. Scale bar 20 μm

3.2.10 Morphometric evaluation of Dorsal Root Ganglia (DRG)

We performed a morphometric analysis of DRG; the differences among groups were assessed morphologically by means of soma, nucleus and nucleolus calculations. As shown in graph (Fig.32) is possible to notice that the trend for each groups was similar for soma, nucleus and nucleolus; the areas are smaller in diabetic rats (Group B), while the difference between both transplanted Groups C and D and Group B, diabetic rats, was statistically significant (**p< 0.001)

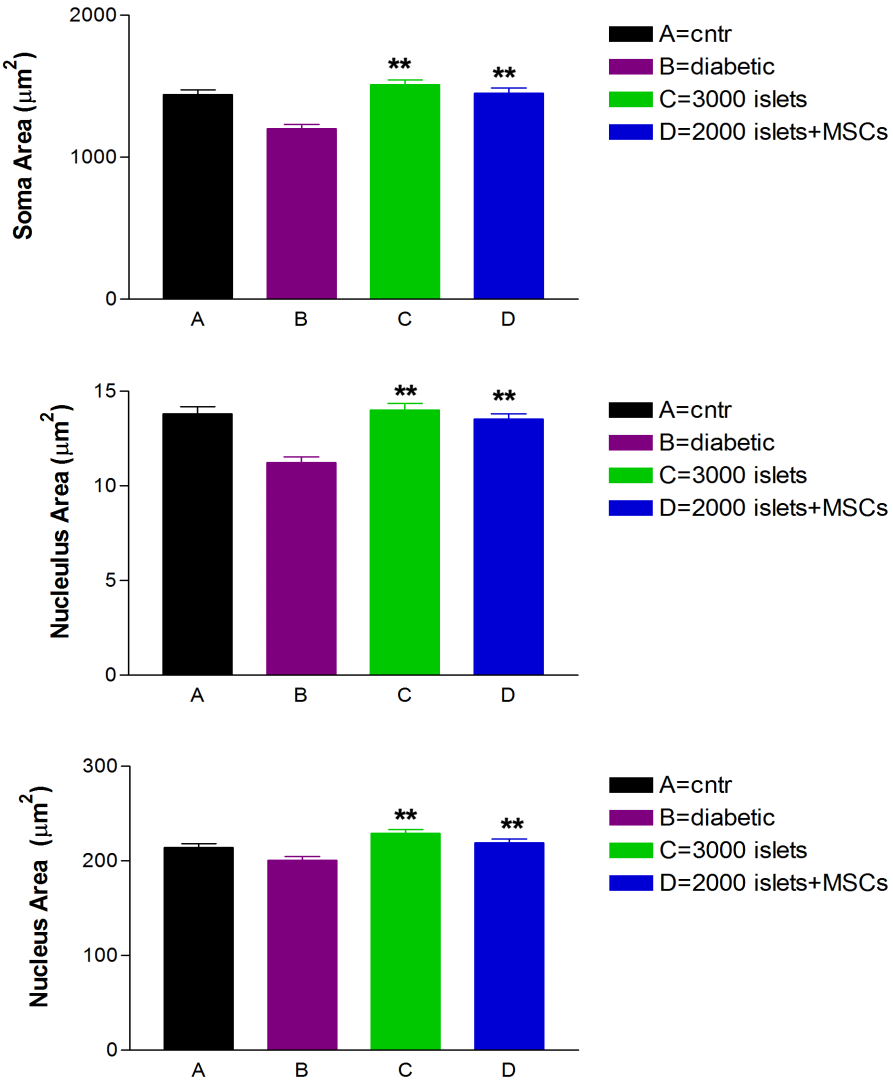


Fig.33 Morphometric evaluation of DRG. Area of soma, nucleus and nucleolus is reported as mean ±SD. **p<0.001 C, D vs B

3.2.11 Pancreas Insulin-staining

We performed Dithizone staining that; as shown in Fig.34 Group A present a well-defined pancreatic islet, with β-cells, resulted as deep pink spots. Groups B and E showed a complete degeneration of pancreatic islets, while islets of transplanted Groups C and D were organized in structures, with some clusters of β-cells, similar to the native islets in pancreas of control group A, indicating that in transplanted groups a mild regeneration of pancreatic β-cells occurs.

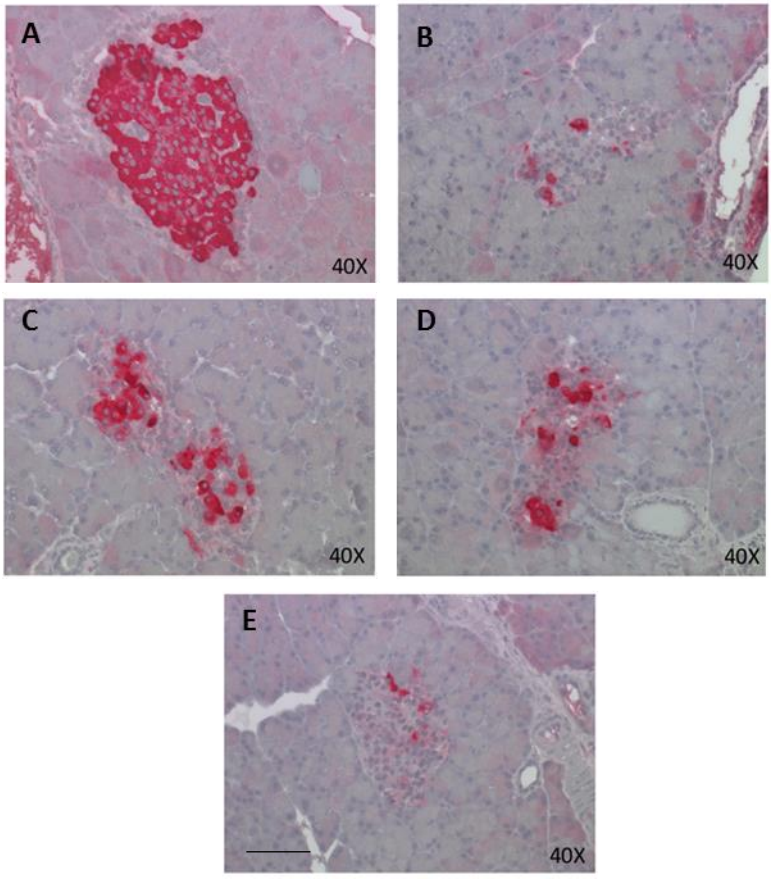


Fig.34. Dithizone staining of pancreas sections. β-cells result as deep pink spots. Scale bar 50μm.

Discussion

Among new strategies for the treatment of T1DM pancreatic, as alternative of whole pancreas transplantation, islets transplantation has been proposed for its minimal related complications and because a milder immunosuppression regimen is needed. The advantage of pancreatic islets is that stricter glycemic control is possible, in respect of traditional exogenous insulin administration. The limitations are representing by the high number of pancreatic islets needed in order to achieve normoglycaemia and that; as reported in literature, no lost-lasting results was reached, in term of exogenous insulin independence. As reported by Johnson, after 1 year whole pancreas transplantation and pancreatic islets transplantation have a very similar follow up, but at 5 years the majority of patients, which received pancreatic islets return to be exogenous insulin dependent (Johnson et al 2012). For these reasons new strategies have been investigated in order to improve this alternative approach. One of these consists in co-transplantation of pancreatic islets and MSCs. MSCs properties have been widely investigated, and due to immune-modulatory, differentiating and survival supporting capability, they are used in many clinical trials for the treatment of multiple sclerosis, heart failure and acute respiratory distress syndrome (Dulamea 2015; Mathiasen et al 2015; Wilson et al 2015). MSCs effect on pancreatic islets in vitro and in vivo models are reported by many authors (Jung et al 2011; Borg et al 2014), but due to the complexity of pathways involved actually not all mechanism(s) at the base of the effect have been clarify.

In order to deeply investigate this aspect we focused our attention both on in vitro and in vivo models. We firstly set up different co-culture conditions: direct ones, in which pancreatic islets were in direct contact with MSCs; indirect co-cultures, in which they shared the medium and mixed ones, in which both previous conditions exist. We have demonstrated that the direct co-culture were feasible, even if pancreatic islets culture condition is floating, while MSCs culture condition is in adhesion. Even if we can assert that direct contact is not sufficient it-self to increase islets survival, surprisingly it seems to be the strictly essential condition for the differentiation of MSCs in insulin releasing phenotype cells, this result not only is confirmed by IF, since we observed insulin expression in MSCs, but even by ELISA test, that shows an increase of insulin concentration in medium of direct co-cultures. We have also observed that MSCs in direct contact lost their peculiar fibroblast-like morphology acquiring a round shape one, indicating a not only a morphology change but even a functional one.

In literature MSCs differentiation towards insulin releasing cells (Chen et al 2004; Moriscot et al 2005) was described, but after factors exposure (Marappagounder et al 2013) or after genetic manipulation (Wang et al 2012; Yuan et al 2012). Even if many promising data were obtained by these authors, our aim was to verify if MSCs by them-selves were able to modify pancreatic islets survival and functionality. In our experimental conditions the simple contact between pancreatic islets and MSCs is sufficient to trigger the intracellular pathways that lead to insulin secretion. On the contrary indirect co-cultures show a significant increase in terms of pancreatic survival, but MSCs do not differentiate; so the increase of insulin release with respect to pancreatic

islets cultured alone is to attribute to the increased islets survival rather than to a differentiation of MSCs. This result finds an explanation in the ability of MSCs to release trophic factors; this aspect is widely described in literature (Ito et al. 2010). In particular VEGF, Von Willebrandt (Sakata et al 2010) and IL-6 are released by MSCs, after co-culturing with pancreatic islets, so we can conclude that probably in this case the immune modulatory and survival supporting properties emerged. As reported by Sakata and collaborators, bone marrow can promote angiogenesis following the co-transplantation with islets. The authors found that the blood glucose was lower and serum insulin was higher in mice administered both islets and MSCs. Moreover, significantly more new peri-islet vessels were detected in these mice with respect to those implanted with pancreatic islets alone. VEGF was expressed at higher levels in mice implanted with MSC than in mice implanted with islets. PDX-1-positive areas, a pivotal gene involved in insulin secretion, were identified in bone marrow cells. However, there were no normoglycemic mice and no insulin positive cells in mice implanted with bone marrow cells alone, suggesting that the co-transplantation of MSCs with pancreatic islets is associated with the islets graft vascularization (Sakata et al 2010).

It is indeed evident that, depending on the co-culture paradigms and through various mechanisms, MSCs directly contribute to insulin production through a differentiation process and also increase the survival of pancreatic islets, so mixed co-culture, in which both direct and indirect contact coexist, should represent the most promising model for increasing islets transplantation's therapeutic potential (Scuteri et al 2014).

We moved to in vivo models, and in order to mimic mixed condition, 2000 encapsulated pancreatic islets and 10^6 MSCs were transplanted in intraperitoneal cavity and contextually 10^6 MSCs were also injected into caudal veins. Our attention was focused between group C, which received 3000 islets alone and group D, which is the group that received 2000 islets plus MSCs. In fact we wanted to prove the positive effect observed in vitro, in order to demonstrate that a lesser number of pancreatic islets is needed if co-transplanted with MSCs. We did not included a diabetic group transplanted with 2000 islets alone, because Figliuzzi and collaborators have already demonstrate, in 2009, that the minimal number to revert diabetic profile in rats are 3000 islets (Figliuzzi et al 2009); and our choice is in accord to guidelines 2010/63/EU for animal welfare in preclinical research ("RRR": reduce, replace, refine). Furthermore a very important aspect is that pancreatic islets with or without MSCs were encapsulated before transplantation; the encapsulation in alginate matrix has been already demonstrate to protect islets (Duvivier-Kali et al 2001; Vos et al 2004), and in addition seems to influence immune response, prolonging islets survival. Our protocol did not include immunosuppressive treatment, but we did not observe any rejection events; this result may be the synergic effect between MSCs immune-modulatory property and the presence of alginate microbeads. Basing on our observation in vivo, group E rats, which received only MSCs, most of the time presented values very similar to those obtained from diabetic rats, indicating that MSCs alone are not them-selves sufficient to improve diabetic profile. Our data are not in contrast to those found in literature, indeed all authors which affirm that MSCs alone are able to revert diabetic profile have used or different diabetic animal model or modified

MSCs. For example Holmes in 2014 have demonstrated that MSCs are able to promote regeneration of endogenous pancreatic islets, but he used a partially pancreatomized rats, so it was not a real diabetic models.

Anyway are data suggest that both pancreatic islets transplantation and in combination with MSC, are able to immediately restore glycemic profile and general conditions, since we observed a body weight gain. Furthermore even one of the long-term T1DM complications is reverted, in fact in transplanted groups, C and D, we observed that NCV was restored to healthy rats' levels, but the best improvement was observed in Group D, which received both MSCs and pancreatic islets. We also evaluate the painful component of diabetic neuropathy, and another time our hypothesis was confirmed by behavioral tests, indeed Group D outcomes were always statistically significant. The morphometric analysis of sciatic nerve has evidenced that neuropathy characteristic of diabetic rats, in terms of g-ratio and histopathological analysis is better reverted in Group D. We also evaluated nephropathy, which is a well-known long-term complication, and through histological staining, that evidence basal lamina, we observed large areas of vacuolization in diabetic rats in particular within distal tubules, that were not still visible in transplanted groups. We also analyzed pancreas though an insulin-staining that evidenced that in transplanted groups were present islets organized in structure similar to the native islets in pancreas of control, indicating surprisingly a mild regeneration of endogenous pancreatic islets. This finding suggest that some trophic factors are released both by transplanted pancreatic islets and MSCs. The question of whether β cell regeneration occurs mainly via the replication of preexisting β cells or via neogenesis

from stem cells in the islets remains a major focus of interest. In recent years, doubts about the existence and importance of β cell neogenesis, especially by stem cells, have been raised. Lineage-tracing studies have only found evidence for β cell replication (Dor et al., 2004 and Nir et al., 2007), and strong evidence to support the presence of a population of stem cells that gives rise to pancreatic endocrine cell types in the adult pancreas remains lacking. Akt and Erk are potent regulators of beta cell proliferation. Some studies have reported that the overexpression of active Akt1 in mouse β cells substantially affects compartment size and function (Tuttle et al., 2001). Another study demonstrated that the anti-proliferative effect of pro-inflammatory cytokines in cultured β cells is associated with an extracellular signal that is regulated by kinase 1/2 pathway inhibition (Blandino-Rosano et al., 2008). Thus, which signal is involved in beta cell proliferation remains unknown. Since MSCs are able to release many pro-inflammatory cytokines and are also able to activate MAP kinase pathway, the combination of pancreatic islets transplantation with MSCs and MSCs systemic administration could be a very promising approach.

The majority of clinical parameter and histological evaluations show that the co-transplantation of MSCs and pancreatic islets is able to better revert diabetic condition with respect to transplantation of islets alone; we can hypothesize that this further improvement may be ascribed to a direct support to both pancreatic islets and other cell populations, such as neurons. This MSCs properties has been already reported in different papers (Scuteriet et al 2011; Crigler et al 2006), as well as the ability of MSCs to

reduce the oxidative stress, which plays a pivotal role into diabetic neuropathy onset (Caliò et al 2014).

In conclusion our data demonstrate that co-transplantation of 2000 pancreatic islets and MSCs and 3000 pancreatic islets alone had very similar effects in terms of improvement of diabetic profile, but co-transplantation of pancreatic islets and MSCs lead to better results, as demonstrate by the majority of clinical parameter and histological evaluations Our findings suggest that the co-transplantation of pancreatic islets and MSCs is able to revert diabetic profile, but not all mechanisms at the base of this positive effect have been clarified. We can assert that MSCs alone, in our experimental conditions, were not able to revert diabetic condition.

Co-transplantation of pancreatic islets and MSCs offers a credible alternative to insulin injections for treating and reverting T1DM, since not only a lesser amount of pancreatic islets is needed in order to reach normoglycaemia, but also because they are able to ameliorate chronic complications, in particular diabetic neuropathy and nephropathy.

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