EFFECTS OF INSULIN ON PANCREATIC β-CELLS OF STREPTOZOTOCIN-TREATED DIABETIC RATS: EXPERIMENTAL OBSERVATIONS ON IMMUNOHISTOLOGICAL AND MORPHOLOGICAL CHANGES

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Running Title

Insulin-induced immunohistological and morphological changes in pancreatic β-cells

Summary

This study was undertaken to investigate the effects of insulin administration on immunohistological and histological changes in pancreatic β-cells, plasma insulin, and blood glucose concentrations in streptozotocin (STZ)-induced diabetic rats. Fifty male Wistar rats (200-250 g) were randomly divided into three experimental groups (control, STZ-treated, and insulin + STZ-treated groups). Diabetes was induced in the diabetic animal groups by single intraperitoneal injections of STZ (75 mg/kg body weight), while the 'control' group animals received equal volume of citrate buffer (pH 6.3) solution intraperitoneally. The diabetic rats in the insulin-treated group were given daily subcutaneous injections of lentel insulin (0.5 U/kg) as from day 11 of the study period, and stopped on the 30th day of the study period. The rats were sacrificed at different time intervals (10th, 20th, 30th and up to 40th day following treatment with STZ). The pancreases of the rats were resected and randomly processed for immunohistological staining, and pancreatic insulin content. In the diabetic state, pancreatic β-cells showed a weak immunostaining for insulin on day 10. Thereafter, insulin administration caused a significant decrease ($p<0.05$) in the elevated blood glucose, and a significant increase ($p<0.05$) in the plasma insulin concentrations. The surviving β-cells, with insulin administration, regenerated and almost regained their normal immunostaining and functional status for insulin. The pancreatic insulin contents showed approximately 45-fold increases when compared with immunoreactivity on the 10th day. Forty days post STZ treatment, the pancreatic staining intensity decreased further, signifying decrease in the β-cell mass. Furthermore, the blood glucose concentration increased, while pancreatic insulin contents and plasma insulin secretion were markedly impaired and showed approximately 70%-fold decrease when compared with the control rats. In conclusion, the present study illustrates the sequence of morphological changes that occur in the islets of Langerhans, and confirms that a moderate single dose of streptozotocin in Wistar rats produces specific necrosis of β-cells, typical of type-1, insulin-dependent diabetes mellitus (IDDM). This experimental model appears to be suitable for studying factors that can improve the plasticity and functions of the pancreas in IDDM.

Key Words

Insulin; Streptozotocin; Immunohistological and Morphological Changes; Pancreatic β-Cells.

Introduction

Diabetes mellitus is a group of hormonal and metabolic diseases characterized by hyperglycemia, glucosuria, polyuria, and so on; resulting from defects in insulin secretion, insulin action, or both [1]. The chronic hyperglycemia of diabetes mellitus is associated with long-term damage, dysfunction, and failure of various organs, especially the kidneys, eyes, nerves, heart, pancreas itself, and blood vessels [2].

Type 1 diabetes mellitus usually appears during childhood age, and results from autoimmune destruction of insulin-producing β-cells in the pancreas [3]. However, the face of type 1 diabetes is now changing, and it is no longer considered a disease confined to childhood. The incidence of type 1 diabetes in Western countries is clearly rising, affecting younger children, adolescents and adults [4]. Chronic hyperglycemia due to failure to maintain proper glycemic control, often leads to the development of diabetes-specific microvascular pathology in the retina, renal glomerulus, neurological and macrovascular complications [5]. Prompt intensive insulin therapy can delay the onset, and slow the progression of micro- and macro-vascular complications [6].

Several pathogenic processes are involved in the development of type 1 diabetes. These processes range from autoimmune destruction of pancreatic β-cells with consequent insulin deficiency, to abnormalities that result in resistance to insulin action [7]. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of the hormone's action. One of the greatest medical events and scientific breakthroughs of the 20th century was the discovery of insulin in the 1920s [8]. The importance of insulin is juxtaposed with that of glucose, the body's basic unit of fuel, and both insulin and glucose are required for life. Insulin was the first hormone to be synthesized completely in the laboratory, and it is the pillar of treatment for type 1 diabetes mellitus [9].

In attempts to produce new, long-acting insulin analogues, pancreas and islet transplantation have also been used as alternatives for restoring endogenous insulin secretion [10]. However, the limited availability of pancreatic tissue donors, and the potential autoimmune reactions, constitute severe restrictions. Thus, studies based on the use of surrogate cells to deliver insulin are seriously emerging. Approaches centered on developing and transplanting cell lines derived from β-cells or neuroendocrine cells [11]; [12], insulin-secreting cells derived from embryonic stem cells [13], islets generated *in vitro* from pancreatic stem cells [14], and also non-β-cells, such as hepatocytes [15], muscle [16], and fibroblast cells [17], are being used in pursuit of this goal. In addition, *in vivo* approaches have been developed to induce the liver to express regulated insulin [18]; [19].

For a long time, it was believed that the endocrine pancreas belongs to a category of tissues that were finally differentiated and irreplaceable in the adult. This was mainly supported by the low replication rate of endocrine cells in adulthood [20]. In the light of the recently available biomedical data, however, this point of view has been drastically changed, and nobody disputes today that the endocrine pancreas is a plastic organ, especially because of the high ability of the β-cell mass for change according to the insulin demand [21]. This plastic property has been demonstrated in physiological, as well as in pathophysiological conditions, such as pregnancy [22] and obesity [23] respectively.

In several mammalian species including humans [24], glucose appears to play a key regulatory role in pancreatic plasticity because it is a potent stimulus of pancreatic β-cell growth, both *in vivo* [25]; [26], and *in vitro* [27]. The effect of insulin on β-cell growth *in vivo* is more controversial. β-cell proliferation is stimulated by insulin treatment in fetal islets transplanted to diabetic rats [28]. Moreover, insulin therapy improves β-cell regeneration in adult mice, with streptozotocin-induced experimental diabetes [29]; [30]. Furthermore, the study of Koiter *et al*. [31] has stressed the interplay between glucose and insulin for the control of islet cell proliferation *in vivo*. Elucidating the precise role of insulin is of particular importance because a series of recent studies have shown the tight dependence of β-cell mass homeostasis and function on insulin receptor and insulin receptor substrates-1 and -2. [32].

The core aim and objectives of the present study were to (i) examine the effect of early treatment of type 1 diabetes mellitus with insulin administration, (ii) investigate the *in vivo* role of insulin on short-term β-cell mass changes, and (iii) probe some of the mechanisms associated with these histomorphological changes.

Material and Methods

Animals

Fifty male Wistar rats (*Rattus norvegicus*), three months old and weighing 200-250 g, were randomly assigned to three (A, B and C) groups of 'test' and 'control' rats. They were kept and maintained under laboratory conditions of temperature, humidity and light; and were allowed free access to standard laboratory diet pellets (Ladoke Feeds, Ibadan, Nigeria) and water *ad libitum*. All the animals were fasted for 16 hours, but still allowed free access to water, before the commencement of our experiments. The control group of animals (C) consisted of ten rats, while the treatment groups consisted of forty rats. Group A (20) rats received STZ (75 mg/kg i.p.) only, while Group B (20) rats received STZ (75 mg/kg i.p.) and insulin (0.5 U/kg s.c.).

Induction of experimental diabetes

Diabetes mellitus was induced (in groups A and B diabetic 'test' rats) by single intraperitoneal injections of STZ (75 mg/kg, freshly dissolved in 0.1mol/l citrate buffer, pH 6.3). Control rats were injected with only citrate buffer solution intraperitoneally. The 'test' animals in both groups A and B became diabetic within 48 hours after STZ administration. Diabetic state was confirmed by measuring basal blood glucose concentration 48 hours after STZ injection. At the end of the 16 hour fasting period – taken as zero time (i.e., 0 hour), blood glucose levels (initial glycaemia – G_o) of the fasted normal (normoglycemic) and STZ-treated, diabetic (hyperglycemic) rats were determined and recorded. Group B diabetic rats were injected daily from the 10^{th} to 30^{th} day with 0.5 U/kg body weight of lentel insulin subcutaneously.

Determination of blood glucose concentrations

Blood samples (20 µl each) were obtained from the caudal vein of each animal for determination of blood glucose levels. The samples were obtained 1 day before STZ-treatment, and on various days after induction of diabetes mellitus. Diabetes was allowed to develop and stabilize in the STZ-treated rats over a period of 1 day to 40 days. Blood glucose concentrations were determined by means of Bayer Elite® Glucometer and compatible blood glucose test strips [33]. The mean fasting blood glucose levels of the normal, non-diabetic rats were found to vary between 4.01±0.04 and 4.20±0.13 mmol/L. Fasted STZ–treated rats with blood glucose concentrations ≥ 18 mmol/L were considered to be diabetic, and used in this study. Differences in blood glucose concentrations between groups were analyzed statistically by one-way analysis of variance (ANOVA; 95% confidence interval) followed by Student's t-test. Probability levels lower than 0.05 were considered to be statistically significant. The rats received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by National Academy of Sciences, and published by the National Institutes of Health, USA.

Immunohistological staining for insulin

The animals were sacrificed by cervical dislocation, some at the end of the 16-hour fasting period, and others on various days following STZ administration. Pancreatic tissues were excised and weighed after the fat and lymphnodes had been removed. The splenic parts of the pancreas of each rat were fixed in aqueous Bouin's solution, and embedded in paraffin. Each pancreatic block was serially sectioned (6 µm) throughout its length to avoid any bias due to changes in islet distribution or cell composition, and thereafter mounted on slides. For each pancreas, 10 sections were randomly chosen at a fixed interval through the block (every 30^{th} section), a procedure that has been shown to ensure that the selected sections are representative of the whole pancreas [29]. Sections were immunostained for insulin, using a peroxidase indirect labeling technique. The sections were incubated for 1 h with guinea-pig anti-insulin serum (final dilution 1:1,000, Ref. 64-104-1; Aurora, OH). Thereafter, sections were incubated for 45 min with peroxidase-conjugated rabbit anti-guinea pig IgG (final dilution 1:20; Dako, Carpinteria, CA). The activity of the antibody-peroxidase complex was revealed with 3,3'-diaminobenzidine-tetrahydrochloride, using a peroxidase substrate kit (DAB; Biosys-Vector, Compiegne, France). A standard concentration of Hematoxylin was added as a counterstain.

Pancreatic insulin content determination

The splenic regions of the pancreatic tissues from euthanized rats were weighed and homogenized on various experimental days, in acid-ethanol solution (75% ethanol, 23.5% distilled water, 1.5% concentrated HCl). After overnight incubation at 4^0C, the suspensions were centrifuged, and the supernatants were collected and assayed for insulin

content, using a competitive ELIZA kit [34]. Plates were coated with rabbit anti-guinea-pig Ig secondary Ab (Organon Teknika, Durhan, NC), followed by incubation with a guinea-pig anti-human insulin Ab (Cortex Biochem, San Leandro, CA). Following two washing steps, various extract dilutions or insulin standards (Linco Research, St. Louis, MO) were mixed with constant concentration of HRP-conjugated rat insulin (Organon Teknika) for 4 h at room temperature, or at 4^0C overnight, before competitive capturing was allowed for 3 h. After washing five times, Sigma FAST OPD tablets (Sigma, St. Louis, MO) were used as substrate. Results were analyzed using ceres 900 C ELIZA-reader.

Plasma insulin

Blood samples were collected sequentially before and on each experimental day. The plasma was separated by centrifugation, using polyethylene glycol, and plasma insulin concentrations were determined by Radioimmunoassay (RIA), with an anti-porcine insulin anti-body (INC; Biomedicals, Orsay, France) and pure rat insulin (Novo, Copenhagen, Denmark) as the reference standards [35]; [36].

Statistical analysis

Experimental data obtained from 'test' rats treated with STZ, as well as those obtained from citrate buffer-treated 'control' rats, were pooled and expressed as means (±SEM). The differences between the 'test' and 'control' means were analyzed statistically with one-way analysis of variance (ANOVA; 95% confidence interval) followed by Student's t-test. Values of $p<0.05$ were taken to imply statistical significance.

Results

Characteristics of diabetic state

Forty-eight hours after STZ administration, all animals that had been treated with STZ displayed glucosuria, ketonurea, hyperglycemia, hypoinsulinemia and a moderate loss of body weight. The pancreatic weight, blood glucose, plasma insulin, and pancreatic insulin contents of both STZ-treated diabetic, and normal, non-diabetic rats were either significantly increased or decreased ($p<0.05$) in value respectively (Table 1).

Blood glucose and plasma insulin concentrations

In our control set of experiments, pretreatment of the rats with citrate buffer alone did not significantly modify ($p>0.05$) the plasma insulin and blood glucose concentrations of either the fasted normal or the fasted diabetic rats. As shown in Fig. 1, there was a gradual rise in the blood glucose concentrations of the STZ-treated rats as from day 1 following injection of STZ, and the blood glucose values were significantly higher ($p<0.05$) than those of control animals. Furthermore, high levels of blood glucose concentrations of the STZ-treated rats were persistently observed till the 10^{th} day (20.2±0.1 mmol/l) post STZ administration in both groups A and B. The blood glucose concentrations significantly reduced in value ($p<0.05$) in group B rats after the commencement of parenteral insulin administration (0.5 U/kg body weight), and throughout the period of insulin treatment. With the first dose of insulin, a marked decrease (≈ 40%) in blood glucose concentration was noted in the STZ-treated rats, and the blood glucose value decreased until 30^{th} day. Thereafter, the blood glucose level rose again when insulin administration was discontinued (21.0±0.52 mmol/l) (Table 2).

Histopatological findings

Histologically, pancreatic islet necrosis was diffused, but exocrine pancreatic acinar epithelium, ductal and connective tissues appeared normal. Identifiable pancreatic islets on day 10 were of low cellularity. There was no evidence of inflammatory cell infiltration or fibrosis.

Immunohistological staining of the pancreatic tissues before STZ treatment showed the presence of a strong islet insulin immunoreactivity at a level of 0.59 islet/mm^2 of total pancreatic tissue. This was limited to cytoplasmic staining of individual β-cells (Fig. 2B). In diabetic rats without insulin treatment, there was absolute negativity for insulin-immunoreactive β-cell in the islet of Langerhans (Fig. 2B). In diabetic rats treated with insulin, a few surviving β-cells in the islets showed insulin-immunopostivity in every small granule as from day 20 (Fig. 2C). On the 30^{th} day of the study, both the number of insulin-immunoreactive β-cells and their granules increased markedly, and displayed intense immunostaining when compared to that of day 10 of the study (Fig. 2D).

One of the most interesting findings was the rise in pancreatic insulin content of the insulin-treated rats as from day 15 (4.9±1.0 μU/ml). In contrast with the previous day 10, insulin levels were ≈35-fold higher than that of day 10 (Table 2 and Fig. 1). This increase in pancreatic insulin values was also observed to reflect in the immunohistological staining (Fig. 2C and 2D) of the 20^{th} to 30^{th} day. However, the observed increase in immunohistological staining intensity was probably an evidence of increase in β-cell mass, regaining its normal immunostaining for insulin and functional status up to the day of normoglycemia.

Treatment groups	Body weights (g)	Pancreas weights (g)	Plasma glucose levels (mmol/L)	Plasma insulin contents (μU/ml)	Pancreatic insulin contents (μU/mg)
Diabetic rats	176.3±0.3*	0.74±0.08*	20.13±0.25*	5.25±1.12*	4.82±1.25*
Control rats	192.6±0.5	1.08±0.06	4.20±0.6	9.65±2.10	7.55±2.52

*p < 0.05 vs control.

Table 1.

Various parameters recorded in diabetic (40) and normal (10) rats just before and after removal of pancreas. Values presented represent the means (±SEM) of 10 observations.

Treatment groups	Fasting values (0 day)	day 5	day 10	day 15	day 20	day 25	day 30	day 35	day 40
Plasma glucose (mmol/L)	4.2±0.6	16.5±0.4	20.2±0.1*	10.2±0.3	8.9±0.3*	6.5±0.1	5.2±0.5	11.6±0.2	21.1±0.2*
Plasma insulin (μU/ml)	7.8±2.1	5.3±1.4	4.2±2.5*	5.7±3.1*	6.4±2.4*	6.8±4.2*	7.4±2.0	5.6±1.3	4.1±1.5
Pancreatic insulin (μU/mg)	8.5±1.6	4.5±2.3	3.8±1.4	4.9±2.0	5.8±1.2	6.6±3.1	6.8±2.1	4.5±1.2	3.4±1.3

Values given represent the means (±SEM) of 10 rats. *p < 0.05 vs control

Table 2.

Changes in blood glucose concentrations, plasma and pancreatic insulin contents in diabetic rats treated with insulin as from the 10^{th} to 30^{th} day.

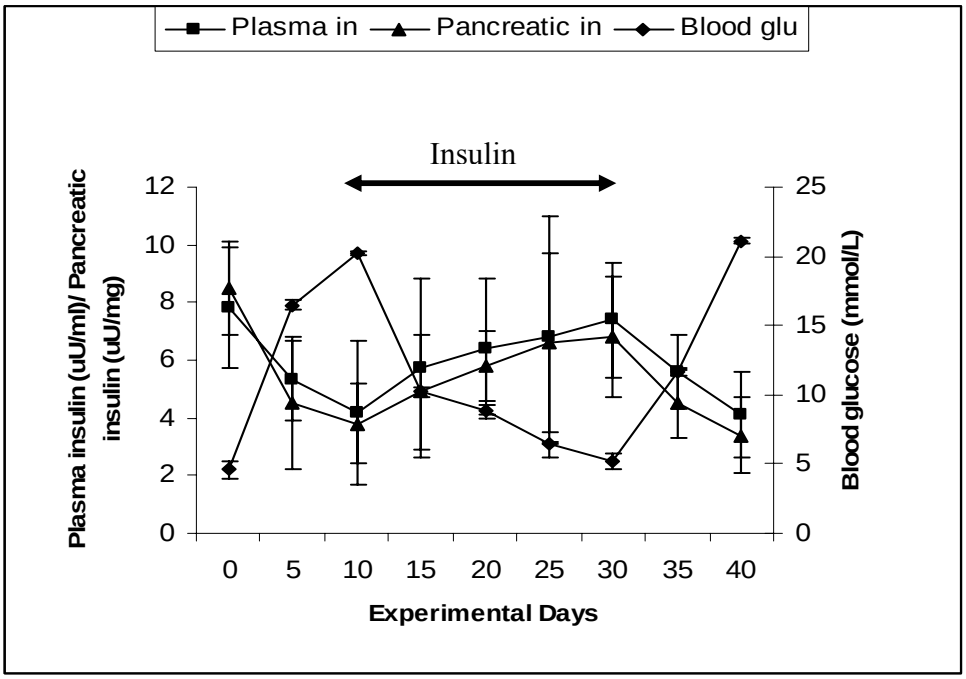

Figure 1.

Characteristics of the animals during development of diabetes following STZ injection, and correction of hyperglycemia as from day 10 of the study period by insulin administration (subcutaneous insulin injections of 0.5 U/kg daily up to 30th day). During the period of insulin treatment, all the animals normalized their blood glucose level to <6.5 mmol/l, and this was sustained till 30th day. During the insulin treatment period, there was a remarkable increase in the pancreatic insulin contents, as well as in the plasma insulin concentration. At this stage, the blood glucose level was significantly low ($p<0.05$) compared with blood glucose concentration of untreated, STZ-induced diabetic rats. Plasma and pancreatic insulin levels were also significantly high ($P<0.05$) compared with untreated STZ-induced diabetic rats. When insulin treatment was stopped on the 30th day, hyperglycemia and hypoinsulinemia ensued by the 40th day.

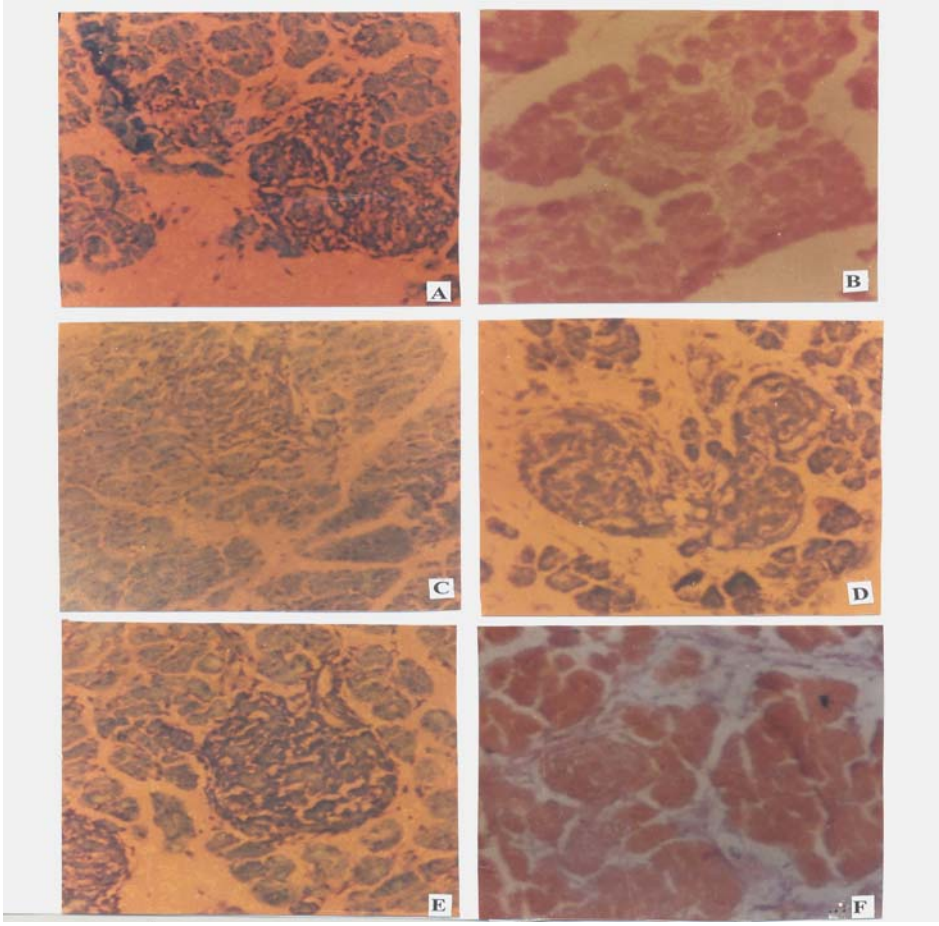

Figure 2.

Immunohistological staining of the pancreatic tissues, represented by dark granules. (A) Control group: Showing normal cells in the islets of Langerhans and showing β-cells in the islets of Langerhans that are strong staining with the anti-insulin antibody. Immunoperoxidase, haematoxylin counterstain. (B) Diabetic untreated group: Shrunken islets of Langerhans displaying degenerative and necrotic changes with weak to absolute negativity for immunoreactive β-cells in the islet of Langerhans. Immunoperoxidase, haematoxylin counterstain. (C) A few β-cells in some islets of Langerhans displaying insulin-immunopositivity in very small granules 10^{th} day post STZ administration. Immunoperoxidase haematoxylin counterstain. (D) Majority of the β-cells in the islets of Langerhans showing moderate staining with the anti-insulin antibody 10^{th} day after the commencement of insulin treatment. Immunoperoxidase haematoxylin counterstain. (E) Remarkable increase with distinct anti-insulin antibody staining of the β-cells in the islets of Langerhans on the 20^{th} day of insulin treatment. Immunoperoxidase haematoxylin counterstain. (F) Weak insulin immunoreactivity in a few β-cells in the islets of Langerhans, 10^{th} day after insulin treatment was discontinued. Immunoperoxidase haematoxylin counterstain. Final magnification X400.

Discussion

It has been estimated that 16 million people in United States suffer from diabetes mellitus, and about 800,000 new cases are diagnosed each year [37]. Probably as many as 1 million diabetics in USA have type 1 diabetes, with some additional 50,000 newly diagnosed patients appearing each year [38]. In both human and economic terms, diabetes is one of the most costly diseases of the United States. At present, there is no proven method to prevent or cure diabetes, and the number of people afflicted by the disease continues to increase at an alarming rate [39]. Available insulin replacement treatments for type 1 diabetes only have limited success in controlling its devastating consequences [40]. The United States Diabetic Preventive Services Task Force (USPSTF) recommends that clinicians should screen all high risk individual patients for obesity and IDDM, and offer effective interventions, combine nutrition, education, diet and exercise counseling with behavioral strategies, to help patients acquire the skills and support needed for normal, active live [41].

The growing evidence of the important role of insulin and insulin receptor signaling in β-cell growth and homeostasis [42]; [43] stimulated us to examine the question of the role of insulin in β-cell mass expansion. Several studies have shown that insulin treatment accelerates β-cell regeneration in diabetic rats and mice [44]; [30]. Whether insulin exerts a direct effect on β-cell growth, or acts indirectly through an alteration in plasma glucose concentration, remains questionable. Our finding that insulin administration leads to a substantial increase in β-cell mass in the presence of normoglycemia provides a clearer evidence of the ability of insulin to directly promote β-cell expansion *in vivo,* independent of its modulating effect on plasma glucose concentration. The recovery of pancreatic β-cell mass after its initial decrease could result from either increase in β-cell proliferation, or increase in neogenic activity. Thus, unlike a recent study [45] that claims β-cell neogenesis to be minimal in normal adult animals, our observation from the diabetic animal model studied suggests an increase in both β-cell proliferation and neogenesis. In the present study, the improvement that followed lowering of blood glucose to the extent of replenishing pancreatic insulin stores to 40-50% of control levels was sufficient to control the effects of diabetes, and restore almost normal islet morphology in human diabetic patients. Currently, the goal towards the management of type 1 diabetes mellitus (generally believed to be immune mediated diabetes) is early detection of high risk individuals [46]. In the present study, prompt institution of insulin management regimen towards controlling blood glucose level gave much relief to β-cells, their cellular integrity and insulin homeostasis.

The first step of this study was to constitute a well-defined rat model of moderate diabetes, typical of insulin-dependent diabetes mellitus (IDDM), through moderate loss of pancreatic β-cells. By a single injection of a moderate dose of STZ (75 mg/kg), we have been able to obtain a rat model characterized by moderate basal hyperglycemia and hypoinsulinemia. This experimental model appears to be suitable for studying factors that can improve the plasticity and functions of the pancreas in IDDM. The finding of this laboratory animal study would, therefore, seem to support the advocate of screening high risk individuals on at least two occasions; before age 5 and before puberty, since early diagnosis and treatment of type 1 diabetes with tight glycemic control preserves β-cell

function, prolonging honeymoon period, and preventing the development of diabetic ketoacidosis, which is very common at this early stage of life.

In conclusion, this study has demonstrated an improvement in the cellular integrity of β-cells of the islets of Langerhans following prompt initiation of insulin therapy in the STZ-treated diabetic rats.

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