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Research Article

Effects of in Utero Severe Hyperglycemia on the Development and Function of the Endocrine Pancreas: Repercussions on Postnatal Life of Rats

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Abstract

Gestational diabetes and hyperglycemia during pregnancy are risk factors for developing chronic diseases in adulthood and are considered significant contributors to the diabetes epidemic. The intrauterine environment is determinant in fetal programming and responsible for the structural, physiological, and metabolic changes in the offspring. This study analyzed the developmental alterations induced by severe in utero hyperglycemia in the streptozotocin (STZ) induced diabetic rats on E17.0, E21.0, and on days 20, 28, and 90 of postnatal life. A reduction in size and weight of fetuses and offspring, a delay in pancreas and islet morphogenesis, a decrease of pancreas weight, reduction in the islet's diameter, area, and β cell mass that are associated with the β cell dysfunction and the development of diabetes, were demonstrated. Alterations in the expression of Pdx1, a critical transcription factor for the development and function of the pancreas and the β cells, and a reduction in the expression of GLUT2 and insulin, key factors for glucose homeostasis were, also demonstrated. At 90 days of postnatal life, the glucose tolerance test confirmed the diabetic profile of the offspring of STZ rats. The multiple and permanent changes induced during development that altered the differentiation, reorganization, maturation, and function of the β cells exerted the deleterious effect on glucose homeostasis in the postnatal life of the STZ offspring.

Keywords: In utero severe hyperglycemia; STZ-induced diabetes; β cell mass; glucose homeostasis; epigenetic origin of diabetes

Introduction

Gestational diabetes and hyperglycemia in pregnancy are important health problems and are partially responsible for the increasing prevalence of diabetes and other metabolic disorders. According to the IDF report, in 2021, 16.7% (21.1 million) of live births were affected by hyperglycemia during pregnancy [1]. Considering these numbers, 21.1 million infants born from mothers with diabetes or hyperglycemia in pregnancy have a high risk to develop T2DM, obesity, insulin resistance, and other metabolic disorders in adulthood due to the altered intrauterine milieu where the fetus developed, evidencing the role of fetal programming of future chronic diseases [2-6]. Glucose homeostasis is a complex process that depends mainly on the synthesis and secretion of insulin by the β cells of the pancreatic islets. Modifications of the in utero and early postnatal life environmental factors can induce

alterations in the differentiation, development, and function of the pancreas and β cells, and in consequence, in glucose homeostasis [7,8].

Pancreas development is initiated from a pool of precursor cells that differentiate into endocrine or exocrine cells under the control of a network of transcriptional factors, expressed in a precise spatial and temporal sequence, which are capable of initiating and maintaining the differentiation of the pancreas cell types and, its function. Among the earliest and essential transcription factors required for the pancreas development, the β cell differentiation, function, maintenance, and Survival, Pdx1 (pancreatic duodenal homeobox 1) is a key factor and is responsible for the regulation of genes related with the β cell function, as the insulin gene and other genes involved in glucose sensing and metabolism, like GLUT2 and glucokinase [9-13].

Animal models are of great value to analyze several aspects of hyperglycemic pregnancies. To have a homogenous genetic background, control of , and and external stressors allows a better identification of the alterations induced by high glucose levels during gestation. In relation to the alterations induced in diabetic gestations in animal models, reports are controversial. Only some studies have demonstrated a reduction of the β cell mass and an altered β cell function in the offspring [14-17]. Still, the precise mechanisms involved in the dysfunction of the fetal pancreas that will contribute to developing diabetes have not been totally clarified. With the aim to identify some of the alterations induced by the in utero severe hyperglycemia, we analyzed the size and body weight, the pancreas morphogenesis, weight and structure, the islets differentiation and function, the β cell mass, and the expression of Pdx1 and its targets, insulin, and GLUT2 in fetuses and offspring of rats with STZ induced severe hyperglycemia.

Materials and Methods

Hyperglycemia Induction

Thirty-five female Sprague-Dawley rats weighing 250-300g were included in the study and maintained under controlled conditions (22-25°C, light/dark 12/12 h, and ad libitum access to food and water). After two weeks of observation and acclimatization to the new conditions, rats were mated with proven breeder males for one night, the next morning the presence of spermatozoa in the vaginal smear determined this day as the first day of gestation. Pregnant rats were allotted in 2 groups (Experimental and Control), on the 5th day of gestation; before implantation [18], the experimental group (STZ) (n=25), received an intraperitoneal injection (i.p.) of a single dose of 50 mg/kg BW of streptozotocin (STZ) (Sigma No. 242-646-8) in acetate buffer 0.1M pH 4.3 (vehicle). The control group (Control) (n=10) received an i.p. injection of the vehicle (1 ml/kg of acetate buffer). Blood samples were obtained from overnight fasted rats from a cut of the tip of the tail, and glucose was determined with the Accouches glucometer (Roche®).

Rats with glucose levels >250 mg/dl after 48 hrs. of the injection were considered hyperglycemic (diabetic) and included in the experimental group (STZ), and those with glucose levels <100 mg/dl were included in the control group (Control). Pregnant rats were transferred to individual cages and maintained under controlled conditions (22-25°C, light/dark 12/12 h and, ad libitum access to food and water). Weight and glucose levels, after an overnight fast, from all the pregnant rats were registered every 2 days. Weight, size, and blood glucose levels, after an overnight fast, of all offspring were registered every week from birth to sacrifice. After an overnight fast, five diabetic pregnant rats (STZ) were sacrificed by decapitation on day 17 of gestation, and five were sacrificed on day 21. Two control rats (Control) were sacrificed on the same days. Fetuses were obtained, and weight and size were registered. Fetuses of 17 days and pancreas of fetuses of 21 days of gestation were washed in saline solution (0.9%) and fixed in buffered formaldehyde (10%).

The number of lactating pups per dam was adjusted to 7, 48 hrs. after delivery. The offspring of five STZ rats and two control rats were sacrificed by decapitation on days 20, 28, and 90 by decapitation. Blood was obtained and centrifugated at 3,000 rpm for 15 min. in a Beckman GS-15R centrifuge, and plasma was maintained at -70°C for the later determination of insulin. On the day of sacrifice, the body and the pancreatic weight were registered. The pancreas was fixed in buffered formaldehyde (10%). All samples were processed according to conventional histological techniques. All procedures followed the National Institutes of Health guide for the care and use. Of Laboratory Animals (NIH Publications No. 8023, revised 1978) and the Mexican regulations for the use and care of laboratory animals (NOM-062-ZOO-1999). The study was approved by the National Research Committee and the Ethics Committee of the Health Research Coordination of the Mexican Institute of Social Security and registered (R-2013-3604-9).

Glucose Determination

Blood glucose levels were determined in a drop, taken from the tail vein (according to Guideline 9 (3/10/99) IACUC, 1999) with the Accouches glucometer (Roche ®).

Insulin Determination

Insulin was determined in plasma samples of all the offspring on the established days, with the Rat Insulin RIA kit Merck-Millipore, according to the manufacturer's protocol.

Histological Analysis

The fixed embryos (E17) and pancreas (E21, 20, 28, and 90 days) were processed with conventional histological techniques and embedded in Para last. Consecutive tissue sections of 4μ were obtained with a semiautomatic microtome (Kedee KD 3358) and stained with hematoxylin-eosin. The histological analysis was performed with the Nikon Eclipse E400 ® microscope. Digital images were obtained with the Olympus® digital camera.

Morphometric Analysis

The morphometric analysis was performed at 20x and 40x magnifications. The digital images were obtained with the Olympus DP71 camera and analyzed with the Image Proplus (v. 6.15 for Windows) Media Cybernetics®. The diameter of the islets was determined with the $D=2\sqrt{A*B}$ formula, considering that the islets are elliptical. A is the equatorial radio and B is the polar radio of each islet. The total area of each islet was obtained by selecting the endocrine structures in all the images with the Image Proplus® software. Hundred islets from each pancreas were analyzed in the HE-stained sections for each of the studied times.

β Cell Mass Determination

The β cell mass was obtained by multiplying the average of β cell area (positive to insulin by immunohistochemistry) by the weight of the corresponding pancreas [19].

Immunohistochemistry

The 4μ consecutive sections were mounted on Poly-L-lysine charged slides. The immunodetection was performed with the double antibody technique with the Envision kit (K1392, DAKO). Antigenic recuperation was performed with the Rodent Decloaked 1X (BioCare Medical®). The activity of the endogen peroxidase was eliminated with Peroxidized 1 (BioCare Medical®) for 20 minutes. The unspecific union from the primary antibody was blocked by a 20-minute incubation with the reductor component of the Envision (DAKO®) system. Primary antibodies were anti-Pdx.1 (AB47267, Abcam®), anti-insulin (Neo markers® MS1379-P), and anti-GLUT2 (AB1342, Chemi-Con®), with dilutions in PBS 1:750 for anti-PDX1, 1:300 for anti-insulin and anti-Glut2. As a negative control, PBS was used. After an hour of incubation, at room temperature, with the primary antibody, slides were washed and incubated for an hour with the multi-specie polymer coupled to peroxidase (DAKO®) and developed with diaminobenzidine (DAB). Sections were counterstained with Harris´ hematoxylin, and the analysis was performed with the Nikon Eclipse E400 ® microscope, and digital images were obtained with the DP71 camera.

IOD (Integrated Optical Density) Analysis

A semi-quantitative determination of the expression of Pdx1, insulin, and Glut2 was obtained with the Image Proplus® software for IOD that reports the average intensity/density of each DAB (3,3′-Diaminobenzidine) stained region. The analysis was performed in all the islets from twenty sections of each pancreas.

Glucose Tolerance Test

After an overnight fast of 16 hr, 90 days offspring of control and STZ rats received an i.p. injection of 2g/kg body weight of glucose. Glucose samples were obtained from the tip of the tail at 0, 15, 30, 60, 90 and 120 minutes. Blood glucose levels were determined with the Accouches glucometer (Roche ®).

Statistical Analysis

The values of all parameters are represented as media ± standard error (SE). The ANOVA one way test was performed using the Minitab® (version 15 for Windows®) and a post hoc Turkey test for the specific comparison of the different groups. Differences were considered significant $p \leq 0.05$. For the data obtained with IOD analysis, the nonparametric Mann-Whitney test was applied. Differences were considered significant $p \leq 0$.

Results

Weight and Glucose Levels of Pregnant Rats

The initial body weight of control and STZ rats was similar 260 + 6 g for control rats vs. 265 + 5.1 for STZ rats. Weight gain throughout gestation was reduced in the STZ pregnant rats; on day 21 of gestation, the weight was 311.2 + 6.3 g for control rats vs. 275.9 + 3.5 g for STZ rats ($p<0.05$). Glucose levels on day 1 of gestation were also very similar, 98 + 2.1 mg/dl for control rats vs. 97 + 2.9 mg/dl for STZ rats. A significant increase in glucose levels was observed throughout gestation in STZ rats. On day 21 of gestation, the glucose levels of the control rats were 98 + 3.1 mg/dl vs. 302 + 2.5 mg/dl for the STZ rats. ($p< 0.0001$) Data not shown.

Weight and Size of Fetuses and Offspring

The average weight of E17 fetuses of control rats was 0.7 ± 0.04 g vs. 0.4 ± 0.01 g of STZ rats ($p<0.0001$). This significant reduction was also observed on E21, with values of 3,8 ± 0.08 g for control rats vs 2.19 ± 0.07 g for fetuses of STZ rats ($p<0.0001$) (Figure 1A). The expected age-related increase of size was not the same in fetuses of control and STZ rats; on E17, the size of fetuses of control rats was 24.8 ± 0.6 mm vs. 20.6 ± 0.3 mm for STZ rats ($p<0.05$). The size of the E21 fetuses of control rats was 48.9 ± 0.3 mm vs. 39.8 ± 0.7 mm for STZ rats ($p<0.0001$) (Figure 1B). These changes were maintained and on the day of birth, the average weight of the offspring of control rats was 8.25 + 0.1 g vs. 5.65 + 0.01 g for the STZ offspring ($p<0.05$). The significant reduction in the STZ rats was maintained from birth to 14 days ($p<0.05$) on day 28 (<0.0001) and from 70-90 days ($p<0.05$) (Figure 1C). On the day of birth, the average size of the offspring of control rats was 72.8 ± 0.8 mm vs. 67.6 ± 1.19 mm for STZ rats ($p<0.05$). The reduced size of the STZ offspring could be observed during the 90 days, although it was not significant on days 49 to 70. From day 77 to 90, the reduction was significant ($p<0.05$) (Figure 1D).

Glucose and Insulin Levels (Offspring)

On the day of birth, offspring of STZ rats had higher glucose levels (100 ± 6.43 mg/dl) than offspring of control rats (78 ± 4.1 mg/dl) ($p<0.0001$). A significant reduction of glucose levels was observed in the offspring of STZ rats on day 7 ($p<0.0001$). No significant differences were observed until day 70, when the glucose levels of the offspring of STZ rats increased ($p<0.05$) and were maintained up to 90 days ($p<0.0001$) (Figures 2 & 3).

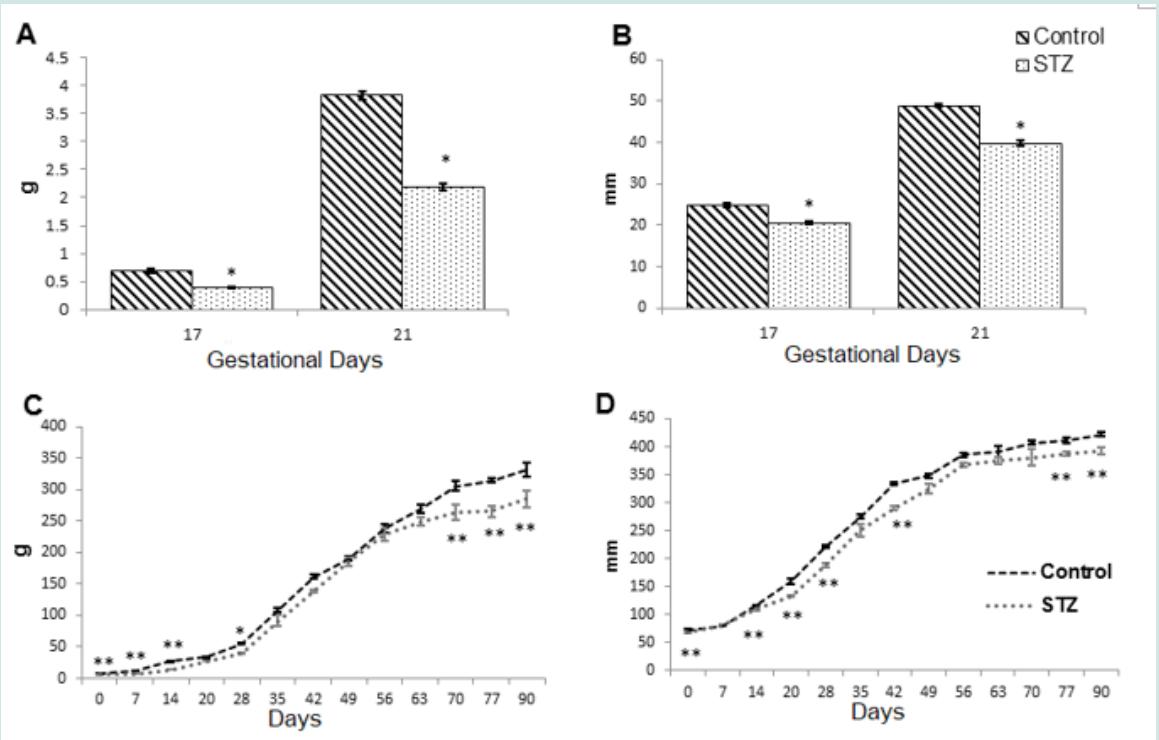
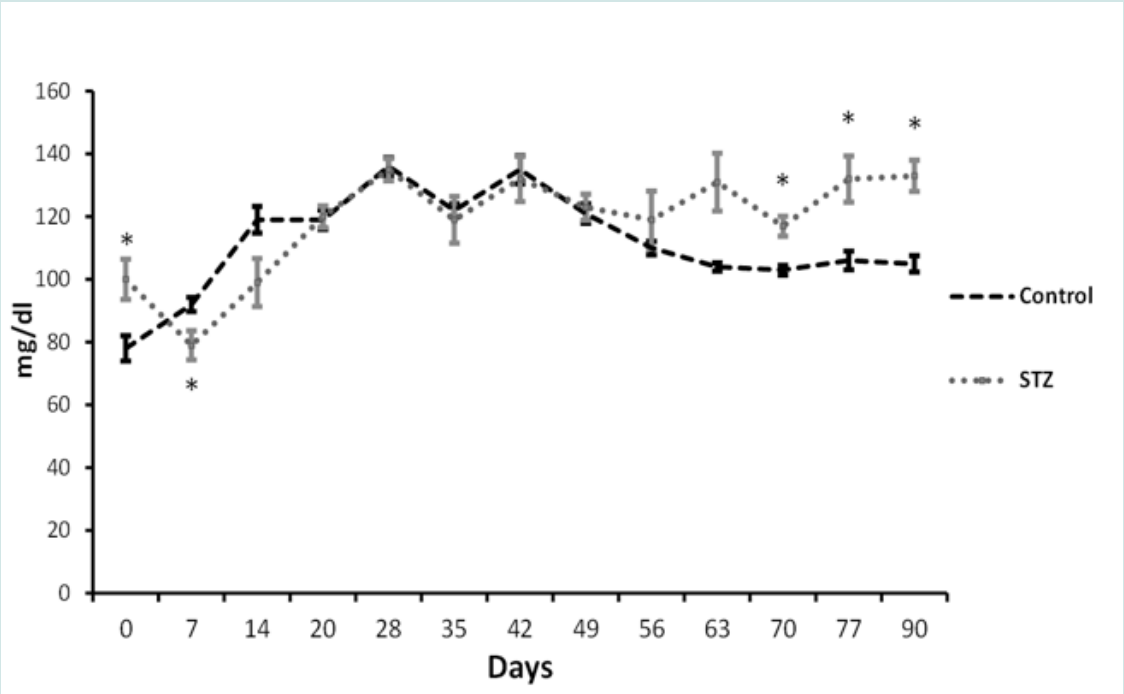
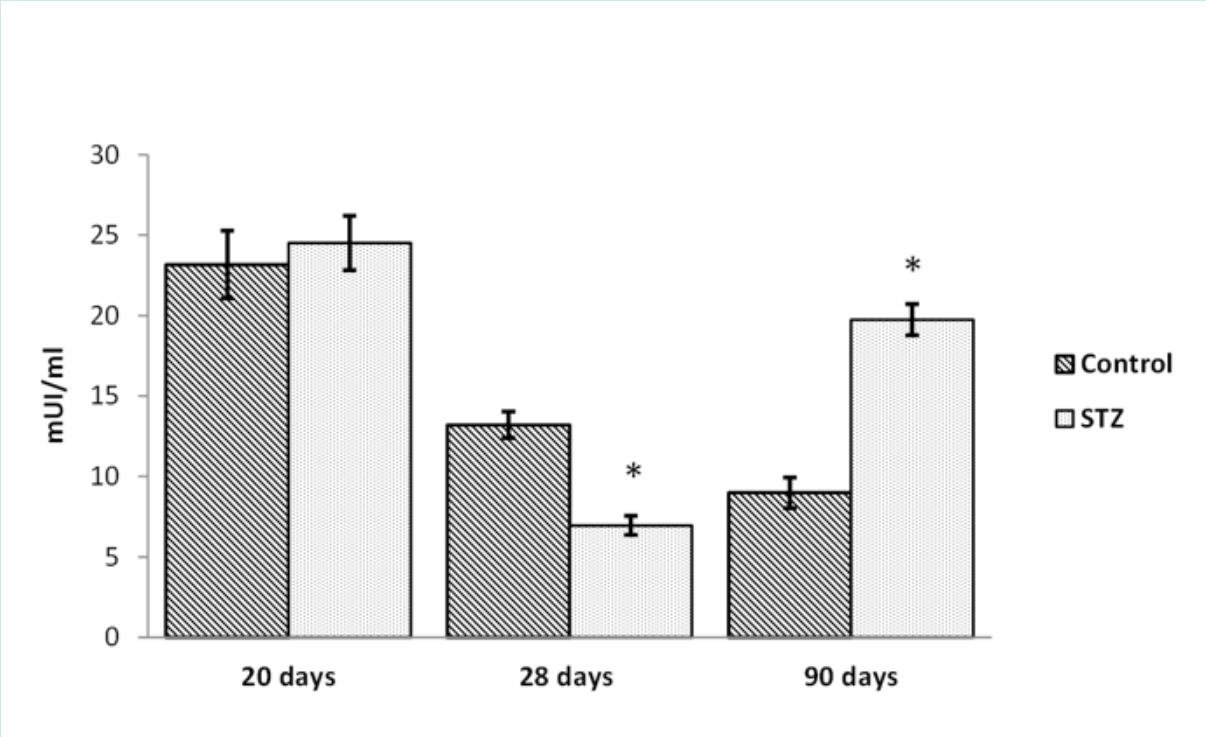


Figure 1: Weight and size of fetuses and offspring of STZ and control rats.
a) Bar graphs represent the weight of fetuses (E17 and E21) of control and STZ rats.
b) Represent the size of fetuses (E17 and E21) of control and rats STZ.
c) Progressive increase of weight in relation to age.
d) Progressive increase of size in relation to age of control and STZ rats (*) p<0.0001 (**) p<0.05.



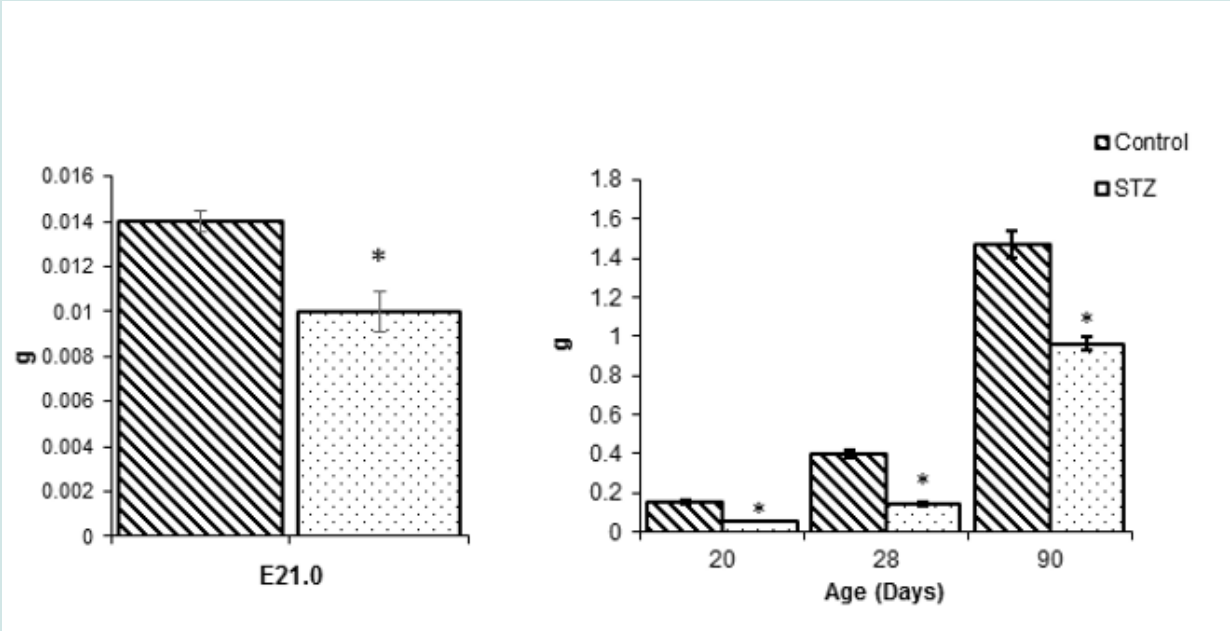
Data are expressed as mean +SE.
Figure 2: Glucose levels from birth to 90 days Blood glucose levels of offspring from STZ and control rats, determined from birth to 90 days of life. (*) p<0.0001.



Data are expressed as mean +SE.

Figure 3: Insulin levels of offspring of STZ and control rats. Bar graphs represent the insulin levels on days 20, 28, and 90. A lower level was detected on day 28 in STZ offspring, followed by an increase on day 90. (STZ n=10, Control n=10 for each studied time) (*) p<0.0001.

Insulin



Data are expressed as mean +SE.

Figure 4: Pancreas weight from fetuses of 21 days and offspring of 20, 28, and 90 days from (STZ) rats and control rats. (A) pancreas weight of E21 fetuses. (B) pancreas weight of 20, 28 and 90 days of postnatal life. (STZ n=10, Control n=10 for each studied time). (*) p<0.0001.

Insulin levels determined on day 20 were higher in offspring of STZ rats but with no significant difference. (24.51 ± 1.68 vs. 23.17 ± 2.11 µUI/ml). On day 28, by weaning, insulin levels were significantly lower in the offspring of STZ rats (6.96 ± 0.59 µUI/ml vs. 13.21 ± 0.815 µUI/ml p<0.0001). A significant increase in the insulin levels could be observed on day 90 in offspring of STZ rats (19,74 ± 2,97 µUI/ml in STZ rats vs. 8,99 ± 0,95 µUI/ml in control rats) p<0.0001) (Figure 4).

Pancreas Weight

Pancreas weight was significantly lower in fetuses and offspring of STZ rats in all the analyzed times. On E21, the pancreas weight of fetuses of control rats was 0.014 ± 0.005 g vs 0.0097 ± 0.01 g for fetuses of STZ rats (p<0.0001). The low pancreas weight was maintained on days 20, 28, and 90 in offspring of STZ rats (0.06 ± 0.004 g, 0.14 ± 0.01 g, and 0.96 ± 0.03 g respectively) (p<0.0001) vs. the weight of offspring from control rats (0.16 ± 0.009 g, 0.37 ± 0.02 g and 1.47 ± 0.07 g respectively) (Figure 3A & 3B).

Morphological Analysis

On day E17, the analysis of the pancreatic morphology of fetuses of STZ rats showed a delay in the organization of the pre-ductal epithelium and in the formation of islet-like associations only few a few islet-like associations were present. On E21 in pancreas of fetuses of STZ rats a delay in the islet-like associations could be observed, evidencing a delay in the islet formation, and the differentiated islets were smaller (p< 0.05). The reduced islet size was maintained on days 20, 28 and 90 of postnatal life in offspring of STZ rats (p<0.0001) (Figure 5).

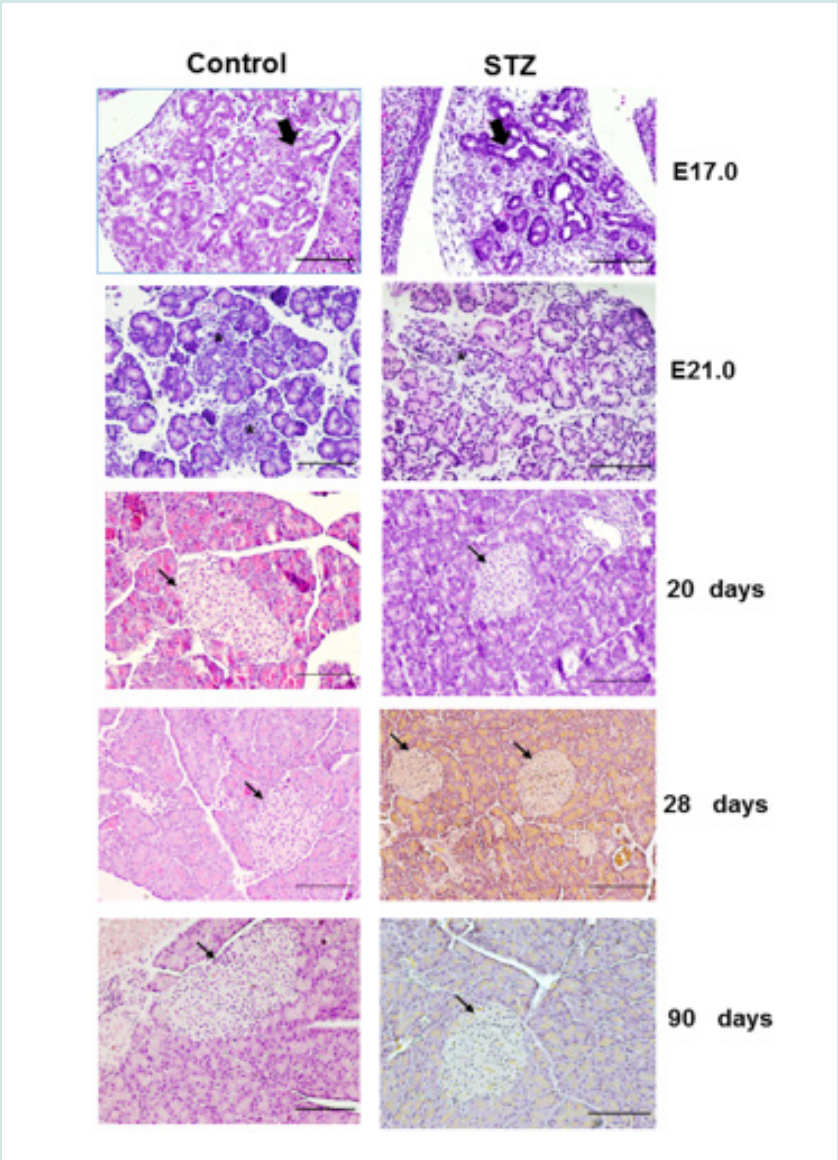


Figure 5: Micrographs of hematoxylin-eosin-stained 4 µ sections of the pancreas of fetuses and offspring of control and STZ rats.

Morphometric Analysis

As expected, islet diameter increased with age in fetuses and offspring of control and STZ rats (E21, 20, 28 and 90 days), but the increase was less in fetuses and offspring of STZ rats in all the studied times. (Figure 6). The total islet area also increased with age in fetuses and offspring of control and STZ rats but was significantly lower in fetuses and offspring of STZ rats (E21,20, 28 days). On day 90, the total area of STZ rats was also smaller, but with no significant difference (Figure 6).

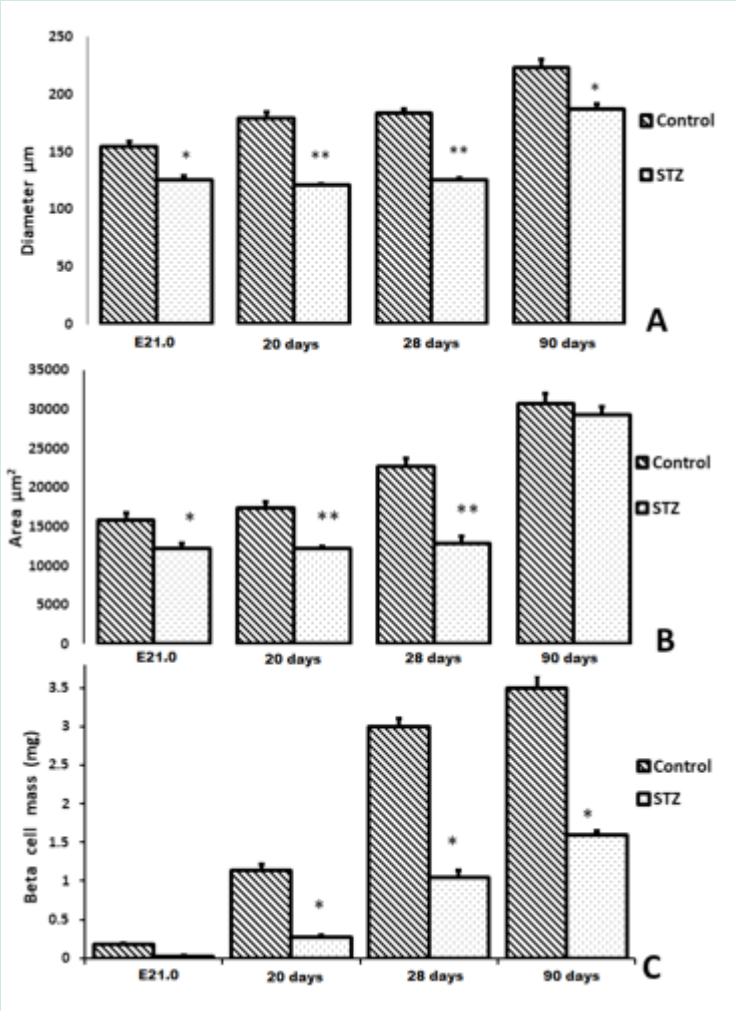


Figure 6: A. Islet diameter, B. Islet area, and C. β cell mass. The islet diameter, area, and β cell mass increased progressively in relation to age, but the increase was significantly less in fetuses (E21.0) and offspring (20, 28 days) of STZ rats (*) p<0.0001.

β Cell Mass

The β cell mass increased progressively in fetuses and offspring of control and STZ rats in relation to age. The increase was significantly less in fetuses (E21) and offspring (20, 28, and 90 days) of STZ rats (p<0.0001) (Figure 6).

Immunohistochemical and IOD Analysis Pdx1 (E17 and E21)

Expression of Pdx1 was observed on E17 in preductal cells of fetuses of control and STZ rats, in the nuclei of the epithelium (Figure 7) with no differences in the expression (Figure 8). On E21, Pdx1 was evident in most of the pancreatic cells, including acinar and epithelial cells and in the nuclei of endocrine cells of islets like associations (Figure 7). The IOD analysis demonstrated a reduction in the expression in the fetuses of STZ rats (p<0.05) (Figure 8).

Insulin (E17 and E21)

Only a few insulin immunopositively cells were observed between the preductal epithelium in the pancreas of E17 fetuses of control and STZ rats, with a higher number in fetuses of STZ rats (Figure 7) but with no differences in the expression (Figure 8). On E21, insulin positive cells were observed mainly between the ductal epithelium and in small islet-like associations and in small islets (Figure 7). The IOD analysis demonstrated a reduction in the expression of insulin in fetuses of STZ rats (p<0.05) (Figure 8).

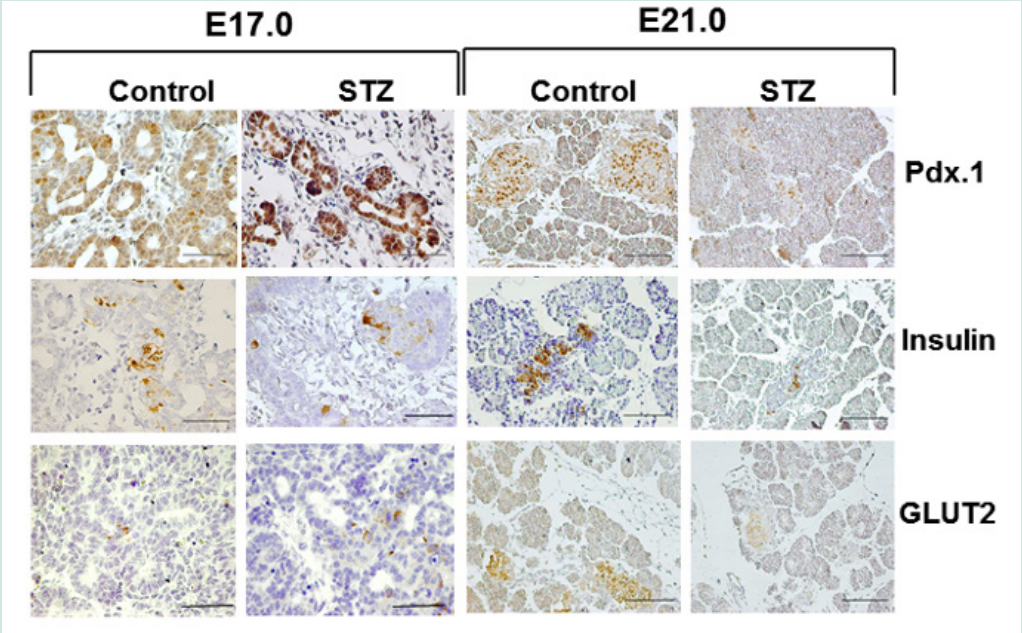


Figure 7: Immunopositivity to Pdx.1, insulin and GLUT2.

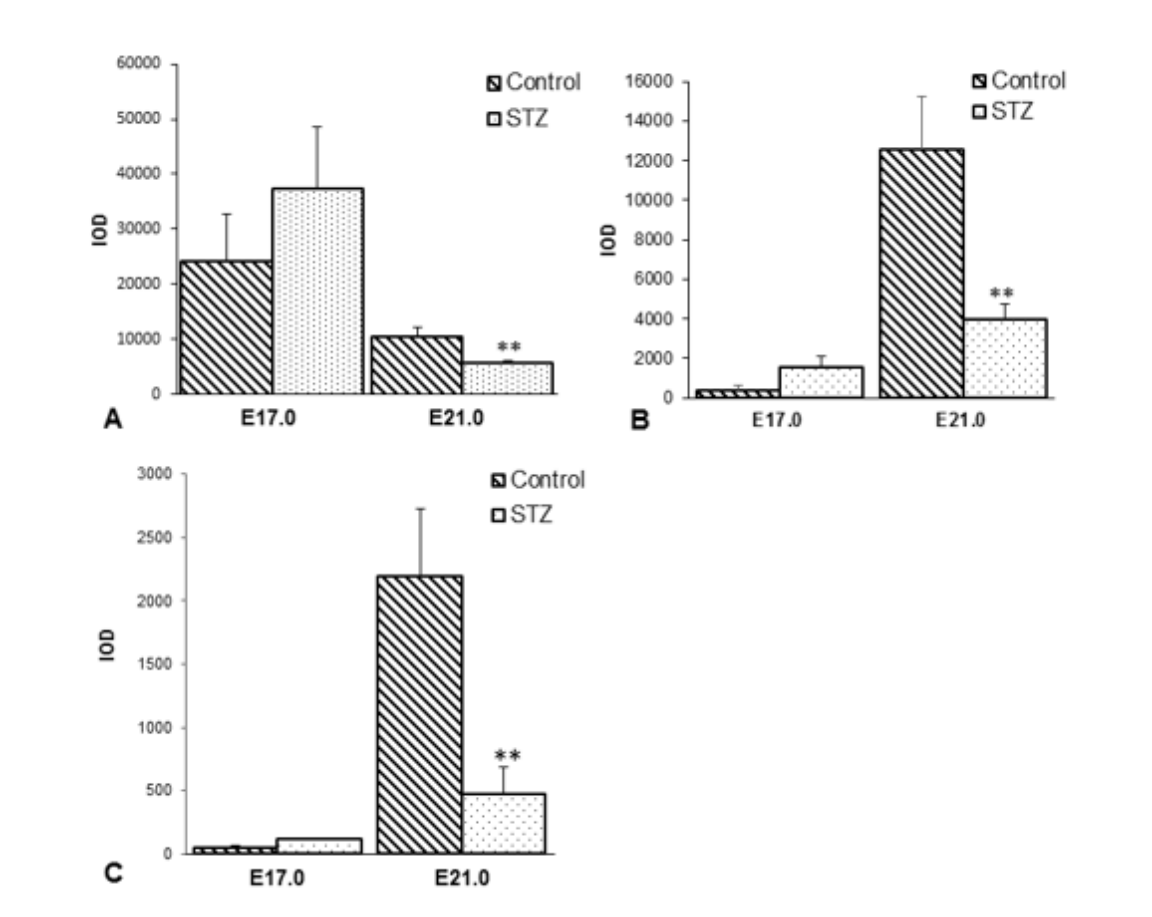


Figure 8: Integrated Optical Density (IOD) analysis. Bar graphs show the semi-quantitative determination of the expression of Pdx1 (A), insulin (B), and Glut2 (C) of fetuses E17 and E21 of control and STZ rats. A reduction in the expression of Pdx1, insulin, and Glut2 in fetuses of 21 days was observed ($p<0.05$).

Glut2 (E17 and E21)

On E17, only a few Glut2 positive cells could be observed in the islet like associations of fetuses of control and STZ rats (Figure 7). No differences in the expression were observed (Figure 7). The expression of Glut2 was detected in E21 fetuses in the central area of islet-like. In fetuses of STZ rats, only few cells were positive to Glut2 (Figure 7); the reduction of the expression was significant was demonstrated by IOD analysis ($p<0.05$) (Figure 7).

Pdx1 (20, 28 and 90 days of postnatal life)

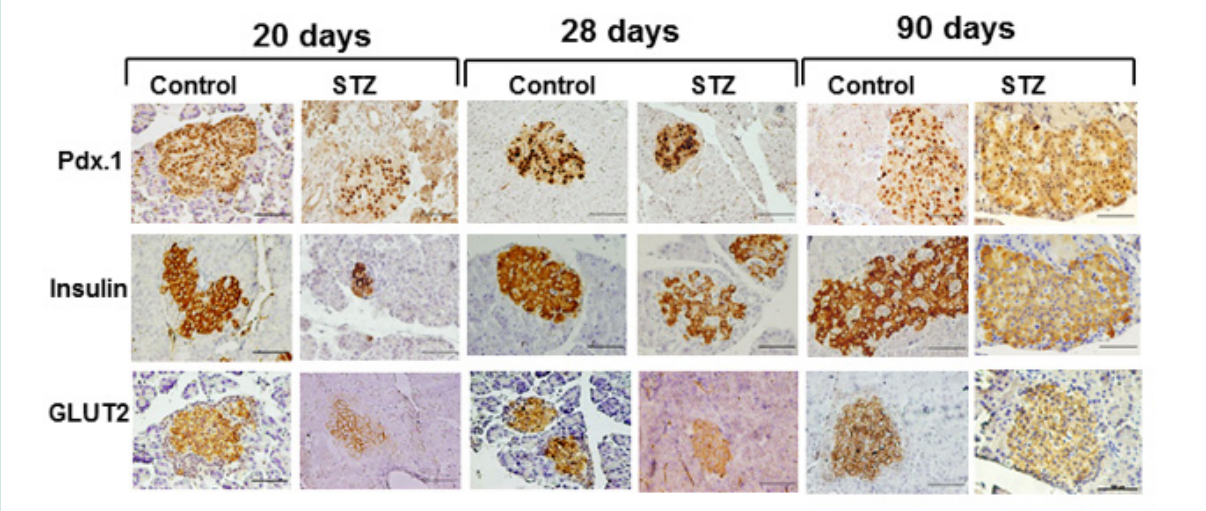
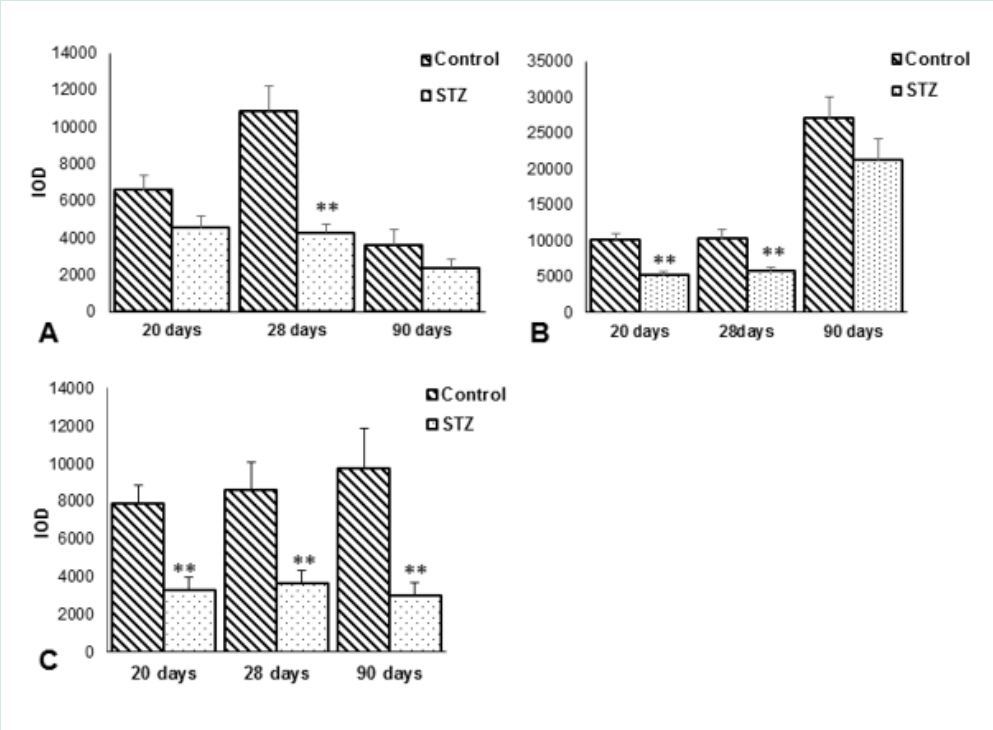


Figure 9: Immunopositivity for Pdx! insulin and GLUT2. Micrographs show Pdx1, insulin, and GLUT2 immunopositivity in 4 μ sections of pancreas from offspring (20, 28, and 90 days) of control and STZ rats.
Scale bar=100 μ m.



Data are expressed as mean +SE.
Figure 10: Integrated Optical Density (IOD) analysis. Bar graphs show the semi-quantitative determination of the expression of Pdx1 (A), insulin (B), and GLUT2 (C) in offspring (20, 28, and 90 days) of control and STZ rats. A reduction in the expression can be observed on day 28 for Pdx1, on days 20 and 28 for insulin and on days 20, 28, and 90 for GLUT2. (**) $p<0.05$.

On days 20 and 28 of postnatal life, expression of Pdx1 was observed in the nuclei and cytoplasm of the endocrine cells (Figure 9). Although the expression was reduced on days 20, 28, and 90 in the offspring of STZ rats, the IOD analysis demonstrated a significantly reduced expression only on day 28 in the offspring of STZ rats ($p<0.05$) (Figure 10). On day 90 the expression of Pdx1 was observed in cells located in the central part of the islets (Figure 9) with no differences in the expression in offspring of control and STZ rats (Figure 10)

Insulin (20, 28 and 90 days of postnatal life)

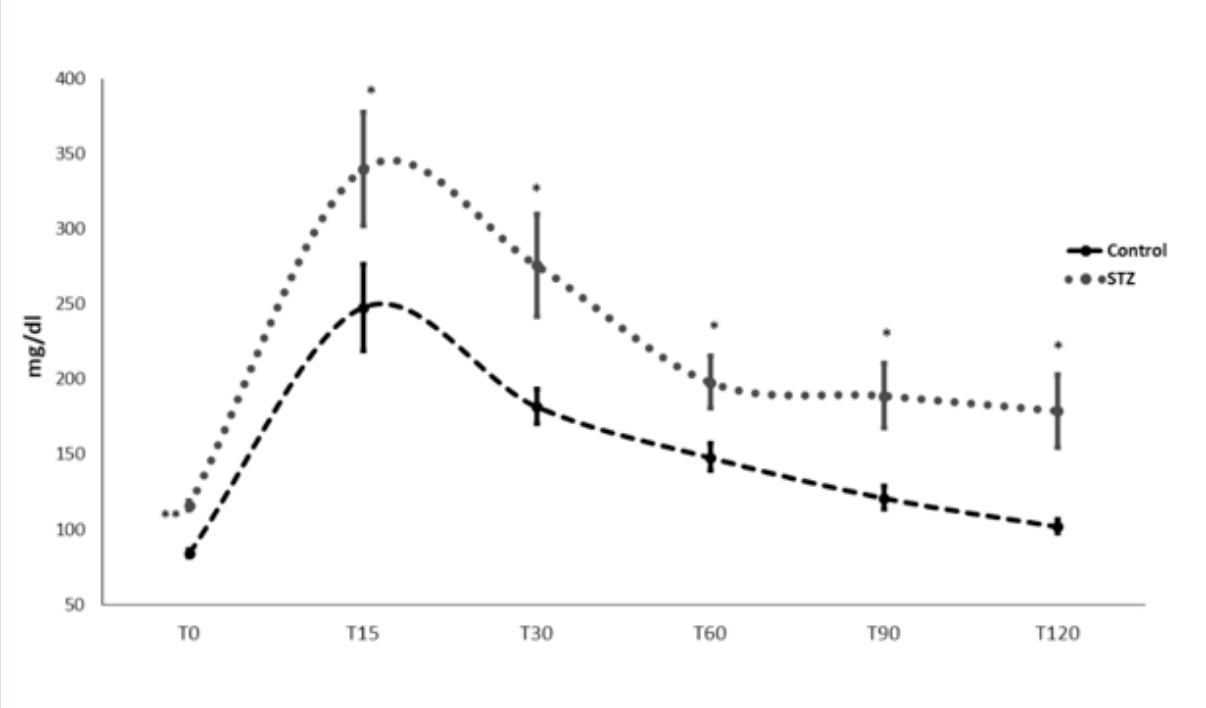
On days 20 and 28, immunopositivity was detected in the β cells of the islets (Figure 9). The IOD analysis demonstrated a decrease in the expression of insulin in the offspring of STZ rats ($p<0.05$). In offspring of 90 days, the IOD analysis demonstrated an important increased expression when compared to the previous times ($p<0.0001$), but no significant could be detected between offspring of control and STZ rats (Figure 10)

Glut2 (20, 28 and 90 days of postnatal life)

In offspring of 20, 28, and 90 days, the expression of Glut2 was detected in cells localized in the islets. The expression in the offspring of control rats was similar in all the analyzed days (Figure 9). The IOD analysis demonstrated a reduction in the expression of Glut2, for all the studied times, in offspring of STZ rats $p<0.05$ (Figure 10).

Glucose Tolerance Test

Basal glucose levels from offspring of control and STZ rats were significantly different ($p<0.05$). After the glucose administration the difference increased in all the studied times ($p<0.001$). In both groups the glucose peak was observed 15 minutes after the administration of the glucose, but a significative difference of the glucose levels was observed from 15 minutes to 120 minutes (Figure 11).



Data are expressed as mean+ SE (*) $p<0.0001$.
Figure 11: Glucose tolerance test: Glucose levels after the i. P administration of 2g/kg BW in offspring of control and STZ rats where an incomplete remission of 120 minutes can be observed in the offspring of STZ rats. (STZ n=10, Control n=10).

Discussion

The high incidence of gestational diabetes and hyperglycemia during gestation is the principal reason for the growing interest to understand the underlying mechanisms of all the alterations induced during fetal life that will have an impact on the adulthood of the offspring. For a long time, it has been established that the intrauterine milieu is responsible for the fetal programming of postnatal diseases and epidemiological studies have demonstrated that offspring of mothers with gestational diabetes have an increased risk of obesity, increased adiposity, insulin resistance, diabetes, hypertension, and cardiovascular diseases, but the mechanisms by which the effect is exerted are not fully identified [20-22]. By working with animal models, we can be able to analyze several aspects of hyperglycemic pregnancies that will allow us to better understand and prevent the development of the well-known adverse maternal and fetal outcomes during pregnancy and long-

term health problems.

STZ has been extensively used in animal models to induce diabetes, the additional benefit is that STZ induced diabetes is that it allows the study of the long-term effects of diabetes independent of any genetic influence. In our study, we injected a single dose of STZ on day 5 of gestation, before implantation when the blastocyst is free in the uterus [18]. STZ has a half-life of 5-15 minutes [23] is excreted (70-80%) mainly in urine and (8-9%) in the feces in a 6 hours period and that STZ has a considerable metabolic transformation and a rapid renal clearance [24,25] so we can be sure that the observed alterations in fetuses and offspring of STZ rats are due to the induction of STZ and not for a direct effect of STZ on the fetuses [26,27]. STZ has the additional benefit of inducing permanent nonreversible hyperglycemia [28].

Gestational diabetes and hyperglycemia during gestation induce developmental alterations that include modifications in the expression pattern of transcription factors involved in the differentiation and development of different organs. It has been demonstrated that gestational diabetes induces a delay in the development of the pancreas and an altered function of the β cells after birth [29-32]. The severity of the induced hyperglycemia affected the general condition of the STZ rats, the weight did not increase in the same proportion as the weight of control rats, but this reduction is also related with to reduction in the number, size and weight of the fetuses. It is important to mention that STZ rats had an increased number of reabsorptions (7+2 data not shown); no reabsorptions were observed in the control rats. No macroscopic malformations were observed in our study. The delay in the fetal growth was evidenced by the reduced size and weight of fetuses ($p<0.0001$) of STZ rats [33].

Alterations in the growth pattern were maintained after birth; offspring of STZ rats were smaller and had lower weight during the first 20 days of life, after weaning and up to adulthood ($p<0.05$). Our results are in accordance with reports that have demonstrated that no matter the abundance of nutrients received by the fetuses of STZ rats, they have a delayed growth [34-38]. The mechanisms responsible for the growth delay have not been clearly demonstrated, but different reports suggest that it is a multifactorial event [34-38]. Specific cellular and molecular mechanisms coordinate the pancreas development and all of them must proceed in a specific order to ensure its function. During the second transition, an undifferentiated epithelium initiates a massive cellular differentiation. On E17 the differentiation is initiated in the primitive pre-ductal epithelium and the precursors of the endocrine cells migrate from epithelial cords or primitive ducts, probably through a mechanism of epithelial-mesenchymal transition, forming groups of endocrine cells in the proximity of the primitive ducts or are even associated to them [39-43].

In our study, fetuses of control rats showed these associations, but in fetuses of STZ rats, these associations were not observed on E17, associations that are crucial for the differentiation of the β cells (positive to insulin and Glut2) [44-46]. On E21, one day before birth, cellular proliferation and growth of the pancreas can be observed, but in fetuses of STZ rats, smaller islet-like associations (<0.0001) and a delayed differentiation from ductal progenitors were evident. Islet hyperplasia in fetuses of STZ rats has been reported and related to the fetuses' hyperglycemic condition [47]. In this study the islet morphometric analysis showed a progressive increase of the islets diameter in relation to age, from E21 to 90 days of postnatal life, but the increase was significantly less in fetuses and offspring of STZ rats ($p<0.05$).These results are in accordance with the studies from other authors who reported that the reduction of fetal weight, islet size, and β cell mass are due to an alteration in the synthesis of insulin during development that has been associated with a delay in the fetal and pancreas growth [48-52].

In fetuses and offspring of control and STZ rats the pancreas weight increased in relation to age but in STZ fetuses and offspring the weight was significantly reduced ($p<0.0001$) in all the studied times, a condition that is in relation to the reduced body weight and can in part explain the reduction in diameter and area of the islets and the reduction of the β cell mass [48-51]. In offspring of control and STZ rats, a remodeling of islets was evidenced by the increase in the area on day 21, although the increase was less in the offspring of STZ rats. On day 28, which corresponds to the expected processes of pancreatic reorganization and maturation induced by weaning, we found that the area was reduced in the offspring of STZ rats when compared to the area of the offspring of the control rats, only on day 90 the increase was almost the same as the observed in the offspring of control rats, but with no significative difference. The reduced increase in the area and diameter of the islets can be related to the reduced expression of key proteins for the differentiation and function of the β cells and to the high levels of glucose [31,49,50].

In addition to the reduced increase in the diameter and area of the STZ offspring we found that although the β cell mass increased progressively in relation to age, the increase was less for fetuses and offspring of STZ rats. This loss of β-cell mass has been linked to epigenetic downregulation of pancreatic homeobox transcription factor (Pdx1), which is essential for normal β-cell differentiation in the embryo [52]. The reduction in the β-cell number may be associated with a functional defect in glucose-stimulated insulin secretion, a dysfunction that favors the development of diabetes [45,46,53]. According to different reports, we found that the pre-ductal epithelium (E17.0) was positive to Pdx1 and was surrounded by mesenchymal, negative to Pdx1. An increase of the IOD for insulin and Glut2 was observed, probably due to the increase of the expression of Pdx1, but the differences were not significant [54-57]. In E21 fetuses of STZ rats the IOD analysis showed a significant reduction of the expression of Pdx.1, insulin, and GLUT2.

On days 20, 28 and 90 of postnatal life the reduction in the expression of Pdx1, insulin and Glut2 persisted in the offspring of the STZ rats. The implicated mechanism is still not known, but it has been proposed that various factors regulate the transcription of Pdx1, and that glucose is one of them [58,59]. Different studies have suggested that other factors, like lipo and glucotoxicity, are related

to alterations in the union of Pdx1 to the insulin promoter [60]. In hyperglycemic conditions, the oxidative stress is also related to the reduced expression of the insulin gene due to the activity of Pdx1 [61-65]. All these reports confirm that the alteration in the expression of Pdx1 is multifactorial. It is well known that an increase in blood glucose levels is responsible for the stimulation of insulin gene transcription and insulin secretion. In this study we observed the progressive increase of glucose from birth to 90 days of postnatal life coinciding with significant high insulin levels in the offspring of STZ rats ($p<0.0001$).

According to our results in adulthood the offspring of STZ with severe hyperglycemia were hyperglycemic and hyperinsulinemia, conditions that will favor the development of overt diabetes and other metabolic and cardiovascular disorders. It is important to mention that insulin levels were not affected by the reduction of GLUT2 and Pdx1 observed from E21 to 90 days of postnatal life, since insulin levels do not depend on the number of transporters but on its capacity of transport. Many transcription factors have been implicated in the regulation of the insulin gene transcription but the more specific are Pdx-1 (pancreatic and duodenal homeobox -1), NeuroD1 (neurogenic differentiation 1) and MafA (V-maf musculoaponeurotic fibrosarcoma oncogene homologue A) that must act in a specific and coordinated form to stimulate insulin gene expression in response to glucose levels. In this study we only analyzed Pdx1 because it is a critical transcriptional regulator, responsible not only for the secretion of insulin but also the responsible of the differentiation and development of the pancreas, β cell differentiation, function, and survival and in the regulation of genes related with the β cell development and function [66].

Only a few studies have analyzed the function of Glut2 and its expression during pancreas development, and it has been demonstrated that its expression and the ionic channels K^+_{ATP} are reduced in the fetal pancreas of rats and humans [67,68]. Glut2 is expressed very early in the development of the pancreas (E11-E18) and is present in two different $Glut2^+$ cell populations related to the development of β cells. During E14-E16, $Glut2^+$ cells are located mainly in the preductal epithelium, and some cell groups scattered in the interstitial tissue are positive to insulin and negative to Glut2. As development progresses, on E17 and E18, the insulin positive cells are also positive to Glut2, and the ductal epithelium is negative to Glut2. The results of this report are consistent with the results of our study, on E17, some preductal cells were positive to Glut2, and as the development progressed, immunopositivity was observed in the β cells that were also positive to insulin [67]. The reduced immunopositivity observed in the β cells of fetuses and offspring of STZ rats evidenced the insensibility of these cells to glucose because they were immature to the glucose response.

The IOD analysis suggests that Glut2 is more sensible to the regulatory action of Pdx1 on the promoter of Glut2 than to insulin. In a study with mice with an inactivated allele for Pdx1, an altered response to glucose was observed at eight weeks; the immunochemical analysis demonstrated that the expression of Glut2 and Pdx1 was reduced when compared with the expression in the wild mice. The glucokinase expression was not altered, and that suggests that the target genes have differential sensitivity to the activity of Pdx1 [64], but the role of other transcription factors cannot be discarded for the expression of Glut2 [68]. There is much information on humans that relates the intrauterine environment with the developmental origins of disease especially with metabolic diseases like insulin resistance, obesity, and diabetes [69]. In a recent review that analyzed the epigenetics and gestational diabetes the authors pointed out that the fetal epigenomic modification is an important risk factor for developing type 2 diabetes and obesity and that determining the epigenetic mechanisms could help to propose intervention targets and reduce the increasing numbers of diabetic patients [70-72].

Conclusions

In this study, we demonstrated that an in utero severe hyperglycemic environment induced multiple alterations in the fetuses and offspring of diabetic rats. The reduction in size and weight of fetuses and offspring persisted up to adulthood. The delay in the pancreas morphogenesis affected its weight, islets diameter, area, and the β cell mass, alterations that are is associated with the β cell dysfunction and the development of diabetes. Severe in utero hyperglycemia also induced a permanent reduction in the expression of Pdx1, a key factor for the development and function of the pancreas the β cells and the expression of insulin and GUT2 key factors for the glucose homeostasis. We also demonstrated an increase in glucose and insulin levels in adulthood, a condition that will favor the development of diabetes and other metabolic alterations. All these permanent changes induced during development that persisted afof developing and up to adulthood, altered the glucose homeostasis in adulthood and will increase the risk to develop different metabolic diseases. Identifying the alterations and understanding the mechanisms through which high glucose levels exert the deleterious effect determinant to prevent, reduce, and treat the effects of in utero high glucose levels in fetuses and offspring of diabetic mothers and contribute to reduce the diabetes epidemic.

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Conflicts of interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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