

Reduction of *Ins-1* gene expression and tissue insulin levels in n5-STZ rats

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ABSTRACT

Objective: The high global incidence of type 2 diabetes has challenged researchers to establish animal models that resemble the chronic stage observed in type 2 diabetes patients. One such model is induced by neonatal streptozotocin (n-STZ) administration to rat pups at 0, 2, or 5 days after birth. In this study, we assessed *Ins-1* gene expression and tissue insulin levels as well as serum concentration of glucose and insulin, insulin resistance, and histological changes of the islets of Langerhans in n5-STZ rats after 20-weeks post-induction.

Methods: Wistar rat pups were randomly distributed into a control group and a streptozotocin-induced group. Experimental induction involved a single intraperitoneal injection of streptozotocin (150 mg/kg) into neonates at five days after birth.

Results: At 20 weeks post-induction, streptozotocin-induced rats exhibited increased serum glucose levels, reduced serum insulin levels, impaired glucose metabolism and insulin resistance compared to control rats. Histologically, streptozotocin-induced rats exhibited atrophic islets, vacuolization, and significantly fewer insulin-positive cells. *Ins-1* gene expression was significantly decreased in n5-STZ rats in comparison to the control group.

Conclusion: Our findings support that the n5-STZ model 20 weeks post-induction represents an appropriate experimental tool to study T2D and to evaluate novel therapeutic agents and targets that involve insulin gene expression and secretion, as well as complications caused by chronic diabetes.

Key words: chronic hyperglycemia, *in vivo* diabetes models, *Ins-1* gene, type 2 diabetes.

INTRODUCTION

Diabetes is a disease characterized by chronic hyperglycemia; it is mainly classified as type 1 or type 2 diabetes (T1D and T2D, respectively). T1D is distinguished by the autoimmune destruction of pancreatic beta cells, while T2D presents insulin resistance in target tissues coupled with the failure of beta cells to secrete insulin (ADA, 2013; Arulmozhi *et al.*, 2004; Kasuga, 2006). T2D constitutes approximately 90-95% of all reported cases of diabetes. In the early stages of T2D, the beta cells try to compensate for hyperglycemia by increasing insulin secretion; however, chronic hyperglycemia and insulin resistance gradually exhaust their ability to secrete insulin (Weir and Bonner, 2004; Fonseca, 2009). The persistence of hyperglycemia generates micro- and macrovascular complications observed in T2D patients (e.g., diabetic neuropathy, nephropathy, retinopathy, and premature atherosclerosis) (Breyer *et al.*, 2005). Although many studies have generated an understanding of this disease, especially regarding the evaluation of new therapeutic agents and targets, further research with well-characterized T2D experimental models in early and chronic stages is necessary to augment our understanding of the disease. A promising animal model can be exploited to develop biological characteristics and/or complications that resemble the pathophysiology of T2D in diabetic patients. T2D models involve different animal species, including cats, primates,

pigs, dogs and rodents. However, rodent models are preferred for their easy availability and the relative cost efficiency of their maintenance compared to other species (Cefalu, 2006; Srinivasan and Ramarao, 2007). Generally, these models have been obtained spontaneously (genetically diabetic animals) or through surgical manipulation or chemical or dietary induction. Chemical induction is the most frequent procedure used to develop T2D models; intraperitoneal or intravenous administration of either alloxan (ALX) or streptozotocin (STZ) is commonly employed (Cefalu, 2006). ALX is a derivative of uric acid that produces a toxic effect on pancreatic beta cells, inducing diabetes and generating complications including neuropathy, cardiomyopathy, and retinopathy. However, the proportion of animals that acquire diabetes as a result of ALX administration is heterogeneous and is not proportionally related to increasing doses of ALX, owing to the chemical instability of this molecule (Srinivasan and Ramarao, 2007).

Furthermore, ALX-induced animals present a high incidence of ketosis, which results in a high mortality rate. In contrast, STZ (also known as 2-deoxy-2-[3-methyl-5-nitrosourido]-D-glucopyranose) isolated from *Streptomyces achromogenes* is preferred over ALX for the induction of experimental T2D (Arulmozhi *et al.*, 2004; Cefalu, 2006). This molecule accesses pancreatic beta cells through glucose transporter 2 (GLUT-2) and produces DNA fragmentation by alkylation, causing cellular necrosis of beta cells (Szkudelski, 2001; Cefalu, 2006; Takada *et al.*, 2007). One important aspect to consider regarding

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the use of STZ for induction is the dose, which determines the experimental development of either T1D or T2D. High doses of STZ in adult rats generate T1D with ketosis and high mortality. However, STZ dose adjustments partially destroy the beta cells, resulting in lower incidences of ketosis and mortality (Junod *et al.*, 1969; Arulmozhi *et al.*, 2004; Szkudelski, 2001). In contrast to adult rat models, STZ can be used to induce T2D in neonatal stages (nSTZ), which results in the development of T2D in adulthood (Portha *et al.*, 1979; Bonner-Weir *et al.*, 1981; Arulmozhi, 2004; Cefalu, 2006; Takada *et al.*, 2007). Depending on the day of the STZ injection, neonatal STZ models are denominated as n0-STZ, n2-STZ and n5-STZ (STZ administered to neonates on the day of birth, at 2 days old or at 5 days old, respectively) (Bonner-Weir *et al.*, 1981; Arulmozhi, 2004). The characteristics of n5-STZ rats at 12 weeks post-induction are similar to those observed in humans with early-stage diabetes, such as hyperglycemia, polydipsia, polyuria, elevated glycosylated hemoglobin, impaired glucose tolerance and insulin resistance (Takada, 2007; Datusalia *et al.*, 2012).

However, little information is available about the biochemical, histological and molecular changes in n5-STZ rats after 20-weeks post-induction (chronic stage). Therefore, employing this model, we analyzed serum glucose and insulin concentration, glucose metabolism, insulin resistance (homeostasis model assessment, or HOMA index), insulin tissue levels and *Ins-1* gene expression.

METHODS

Animals

Wistar rats were obtained from the Biotherium of the University of Guadalajara. The experimental animals were maintained in standard laboratory conditions (acclimatization of 24 ± 2 °C, $55.0 \pm 5\%$ humidity, and a 12-h light-dark cycle) and were fed *ad libitum* with standard rodent diet (Purina LabDiet® 5001) and free access to water. This protocol was approved by the institutional ethics committee, and all animal procedures were conducted in accordance with the production, care, and use of laboratory animals established in Mexican Official Standard (NOM-062-ZOO-1999).

Experimental groups

Five-day-old male Wistar rat pups (9-11 g body weight) were separated from their mothers and fasted for 8 h. Animals were randomized into two groups. One group (n=5) of animals (diabetic, n5-STZ) was injected intraperitoneally with STZ (150 mg/kg; Sigma, St. Louis, MO, USA, catalog S0130) freshly diluted in citrate buffer (10 mmol/L sodium citrate, pH 4.5) as previously described (Takada *et al.*, 2007). The control group (n=5) received only citrate buffer (vehicle solution) in an equal volume. Mortality (48%) occurred in the STZ-treated group during the first days post-injection. After weaning (day 21), all animals were kept in groups of five in normal cages.

At 20 weeks post-induction, glucose levels were measured to verify the diabetic stage of n5-STZ rats (glycemic values > 200 mg/dL). Glucose levels among the control group remained within the normal range after 20 weeks.

During post-induction week 20, rats were fasted overnight and subjected to a glucose tolerance test. After that, the animals were anesthetized to obtain blood and tissue samples and finally sacrificed.

Glucose tolerance test

Three days before animals were sacrificed, an oral glucose tolerance test (oral administration of 2 g/kg glucose by gastric gavage) was performed in fasted animals of both groups. Using a glucometer (One Touch® Ultra®, Johnson & Johnson), serum glucose levels were quantified from samples collected from tail snips at 0, 30, 60, and 90 min. In addition, area under the serum glucose concentration curve (AUC_{glucose}) values were calculated by subtracting the average of the 0 min serum glucose concentration from each of the 0-90 min OGTT values, employing the trapezoidal rule.

Serum glucose and insulin in n5-STZ and control animals

The day of the sacrifice, animals were anesthetized and blood was collected from the retro-orbital plexus. Serum was separated by centrifugation at 6,000 g for 15 min at 4 °C and stored at -70 °C until biochemical determinations. Serum glucose concentration was determined using the enzymatic method: glucose oxidase-peroxidase (BioSystems, Spain, catalog 11503) in a semi-quantitative spectrophotometer (BTS-330, BioSystems, Spain). Serum insulin concentration was quantified through enzyme-linked immunosorbent assay (ELISA) with the DRG® Rat insulin Elisa kit (catalog EIA-2048, Marburg, Germany) following the manufacturer's instructions.

Insulin resistance index

Insulin resistance in the experimental groups was determined by the homeostasis model assessment of insulin resistance (HOMA-IR). The HOMA-IR index was calculated using the following formula: $HOMA = \text{fasting serum insulin (in } \mu\text{U/mL)} \times \text{fasting serum glucose (in mg/dL)} / 405$ (Matthews *et al.*, 1985). To convert from $\mu\text{g/L}$ to pmol/L , a factor 174* was employed (*provided in the user's manual of the kit DRG® Rat Insulin ELISA, Cat. Number EIA-2048, Germany). Additionally, the relationship $1\mu\text{U/ml} = 6.00\text{ pmol/L}$ was used to convert from pmol/L to $\mu\text{U/ml}$ (Manley *et al.*, 2007; Sapin, 2007). High HOMA scores indicate low insulin sensitivity or high insulin resistance (Bonora *et al.*, 2000). Serum glucose and insulin concentrations were measured as described above.

Pancreatic histological analysis

After laparotomy of anaesthetized animals, a fragment of pancreatic tissue was resected and washed with normal saline solution (0.90% w/v NaCl) and fixed immediately with 4% paraformaldehyde in 1x phosphate buffered saline (PBS). Tissue was gradually dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin. Four-micron sections were cut and stained with hematoxylin and eosin (HE). Finally, the sections were analyzed using light microscopy.

Immunohistochemical insulin expression

To determine the insulin protein expression in pancreatic beta cells, paraffin-embedded sections were cut (4 μm) and incubated with a rabbit monoclonal antibody against rat insulin (Insulin (C27C9), Cell Signaling Technology®, catalog 3014) overnight at 4 °C. The Mouse/Rabbit ImmunoDetector

HRP/DAB Detection System (BIO SB, U.S.A., catalog BSB 0003) was used to detect the primary antibody. The pancreatic tissue was counterstained with HE. For negative controls, the primary antibodies were replaced with 1× PBS. The percentage of beta cell positivity was determined using LeicaQwin (Leica, France) and Motic Images Plus 2.0 (Motic China Group Co. Ltd., China) software.

RNA extraction, retro-transcription, and quantification of *Ins-1* gene expression

To quantify *Ins-1* gene expression, first RNA was isolated from pancreatic tissue using the RNeasy® Protect Mini Kit (QIAGEN, USA, catalog 74124). RNA (2 µg) was converted into cDNA using the transcriptor first strand cDNA synthesis kit (Roche, Germany, catalog 04 897 030 001) according the manufacturer's instructions. *Ins-1* gene expression was determined by real-time PCR using the LightCycler®FastStart DNA Master^{Plus} SYBR Green I Kit (Roche, Germany, catalog 03515885001). The *Mrps 18a* gene was used as a housekeeping gene. Triplicate amplification reactions were performed in a 2.0 LightCycler® (Roche, Germany), using the following cycling conditions: *Ins-1* gene: 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 65 °C for 10 s, and 72 °C for 8 s; *Mrps 18a* gene: 95 °C for 10 min and 45 cycles of 95 °C for 10 s, 61 °C for 10 s, and 72 °C for 6 s. Primer sequences were as follows: *Ins-1* Forward 5'-CCATCAGCAAGCAGGTCAT-3', reverse 5' TGTGTAGAAGAAACCACGTTCC-3'; and *Mrps 18a* Forward 5'-CATGTGGTGTGAGGAAAGCAG-3' and reverse 5'-GGGATCTTGTATTGTCGTGGGT-3'. cDNA was replaced by sterile water in the negative controls. Crossing of threshold (Ct) values obtained for the target gene were normalized against *Mrps 18a* Ct values. Relative quantification of PCR products was determined using the method of $2^{-\Delta\Delta Ct}$. Melting curve analysis was also performed to confirm the amplification of a single amplicon for each gene analyzed (Livak and Schmittgen, 2001; Pfaffl, 2001).

Statistical analysis

The serum glucose and insulin concentration values, as well as the relative expression units of the *Ins-1* gene, were expressed as mean ± standard error of the mean (S.E.M.). Beta cell positivity was expressed as a percentage. Differences between groups were assessed using the Mann-Whitney test. AUC_{glucose} differences were determined by using repeated-measures Analysis of Variance. Data analysis was performed using PASW statistical software version18 (Chicago, IL, USA).

RESULTS

n5-STZ rats exhibited reduced insulin levels and increased glucose levels compared to non-diabetic rats

Mean serum glucose increased 271% in *n5*-STZ rats in comparison to control rats ($P < 0.05$, Fig. 1A). On the other hand, serum insulin concentration decreased in the diabetic group by 47% ($P < 0.05$) with respect to the control group (Fig. 1B). Additionally, the mean HOMA index tended to be slightly higher in diabetic rats than in control animals (Fig.1C), which suggested impaired insulin activity in the *n5*-STZ group.

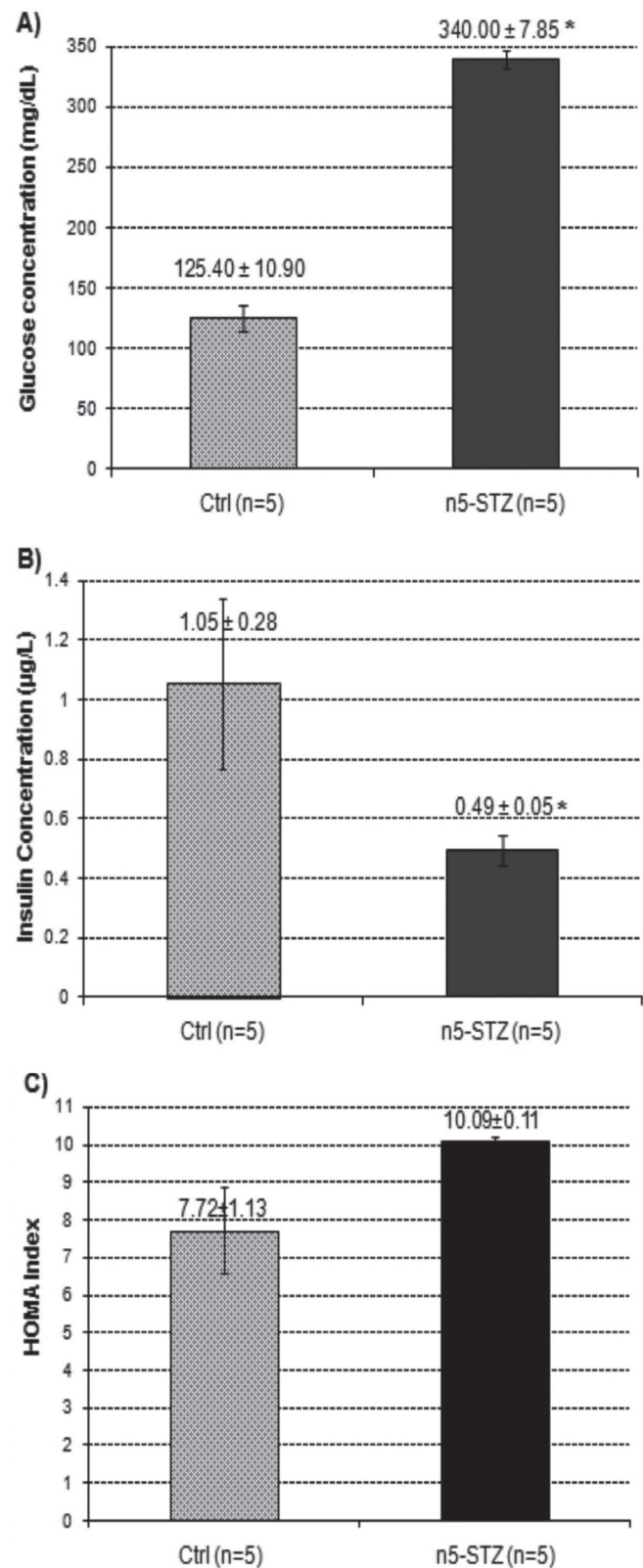


Fig. 1A. Serum glucose (mg/dL) increased significantly in *n5*-STZ rats compared to control rats, * $P < 0.05$. **1B.** Serum insulin (µg/L) was reduced in the diabetic group compared to the control group, * $P < 0.05$. **1C.** Control rats exhibited insulin sensitivity compared to *n5*-STZ rats. The values represent the mean ± S.E.M.

Impaired glucose tolerance in diabetic rats

At the 20th week post-induction the glucose tolerance was evaluated in both groups. In n5-STZ rats, the mean baseline glucose level was 255.00 ± 29.00 mg/dL. Serum glucose increased gradually at 30 and 60 min, and reached 317.00 ± 31.90 mg/dL at 90 min, indicating impaired glucose tolerance. In contrast, the mean baseline serum glucose in the control group was 67.00 ± 2.90 mg/dL. After 30 min of an exogenous oral glucose overload, serum glucose concentration increased and then decreased (at 60 and 90 min as expected, Fig. 2) until it reached the baseline values. In addition, we quantified the OGTT results by calculating the area under the serum glucose concentration curve (AUC_{glucose}) of OGTT values, employing the trapezoidal rule.

Table I shows the mean AUC_{glucose} values in n5-STZ group, which exhibited a persistent upward trend (values of 8455 ± 839 , 9155 ± 1076 and 9290 ± 1120 mg/dL•min). By contrast, in the control group the corresponding values increased between 0 and 30 minutes (3260 ± 213) and then returned significantly towards the baseline values by minute 90 (2745 ± 177).

Histological changes of islets of Langerhans in diabetic rats

Analysis of pancreatic tissue from experimental diabetic rats (n5-STZ) revealed degenerative and necrotic changes demonstrated by shrinking of the islets of Langerhans and reduction of their number. The n5-STZ group showed a 57.97% decrease in its number of islets of Langerhans,

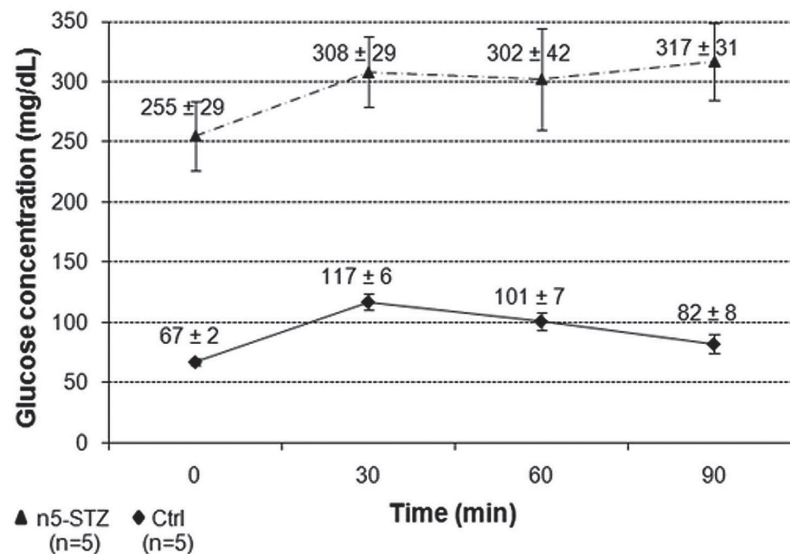


Fig. 2. Oral glucose tolerance test. Serum glucose was assayed at 0, 30, 60, and 90 min. Control rats (rhombus continuous line) exhibited normal glucose metabolism. In contrast, n5-STZ rats (triangle dash dot) exhibited impaired glucose metabolism. Each point of the curve represents the mean \pm S.E.M.

TABLE I

Area under the serum glucose concentration curve from OGTT values in n5-STZ and control rats (AUC_{glucose})

	n5-STZ group		Ctrl group	
	AUC_{glucose} (mg/dL•min)	Average glucose ⁺⁺ 0-90 min (mg/dL)	AUC_{glucose} (mg/dL•min)	Average glucose ⁺⁺ 0-90 min (mg/dL)
1 st interval ⁺ t_0-t_1	8455 \pm 839	94	2760 \pm 57	31
2 nd interval ⁺ t_1-t_2	9155 \pm 1076	102	3260 \pm 213*	36
3 rd interval ⁺ t_2-t_3	9290 \pm 1120	103	2745 \pm 177*	31

⁺1st, 2nd and 3rd intervals of time in the oral glucose tolerance test (OGTT) where t_0 = serum glucose value at 0 min, t_1 = serum glucose value at 30 min, t_2 = serum glucose value at 60 min and t_3 = serum glucose value at 90 min.

⁺⁺Average glucose values (mg/dL) = $AUC_{\text{glucose}}/90$ min

n=5 (for each group)

Repeated-measures Analysis of Variance, *P<0.05.

which is a significant reduction compared to the Ctrl group ($P < 0.05$). In addition, endocrine cells presented damage such as karyopyknosis, cytoplasmic vacuoles and an indistinct border between the endocrine and exocrine regions (Fig. 3B). In contrast, histological evaluation of exocrine pancreatic acinar epithelium, ductal tissue, connective tissue, and islets of Langerhans from control rats demonstrated normal cell architecture (Fig. 3A).

The administration of STZ also revealed severe structural degradation, manifested by empty regions in most pancreatic islets. Tissues from both analyzed groups lacked evidence of inflammatory cell infiltration.

Reduced insulin protein expression at tissue level in n5-STZ rats

The number of insulin-positive beta cells and their reactivity were significantly reduced in n5-STZ rats compared to controls (Fig. 4B). These findings corroborate the described morphological changes of degenerated beta cells in HE-stained

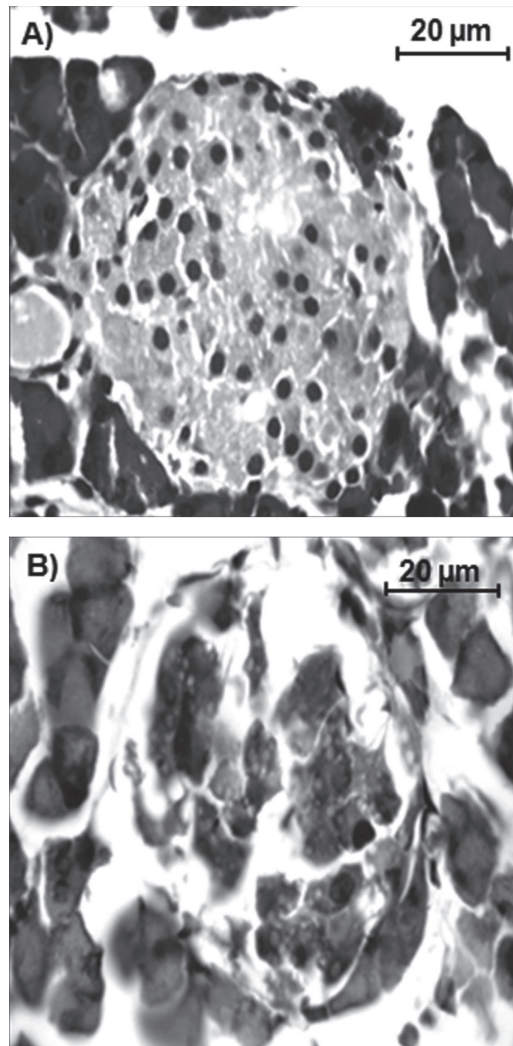


Fig. 3. Representative photomicrographs of islets of Langerhans stained with hematoxylin and eosin, (HE, 400×) **3A.** Islets of Langerhans in control rats exhibit normal histology. **3B.** STZ induces hypochromic nuclei and vacuolar degeneration of endocrine cells, as well as loss of cell architecture in the islets of Langerhans.

sections. Control rats exhibited insulin-immunoreactive beta cells typically distributed in central and peripheral regions of islets of Langerhans (Fig. 4A).

Tissue image analysis indicated that the percentage of insulin-positive cells in n5-STZ rats was one-half that of the control group ($32.74 \pm 13.37\%$ vs. $61.37 \pm 10.38\%$, $P < 0.05$). Figure 4C depicts an immunohistochemically negative control to validate the assay demonstrating the absence of cross-reactivity.

*Decreased *Ins-1* gene expression in n5-STZ rats*

Ins-1 gene expression was measured by relative quantification using real-time PCR. Melting point analysis was performed

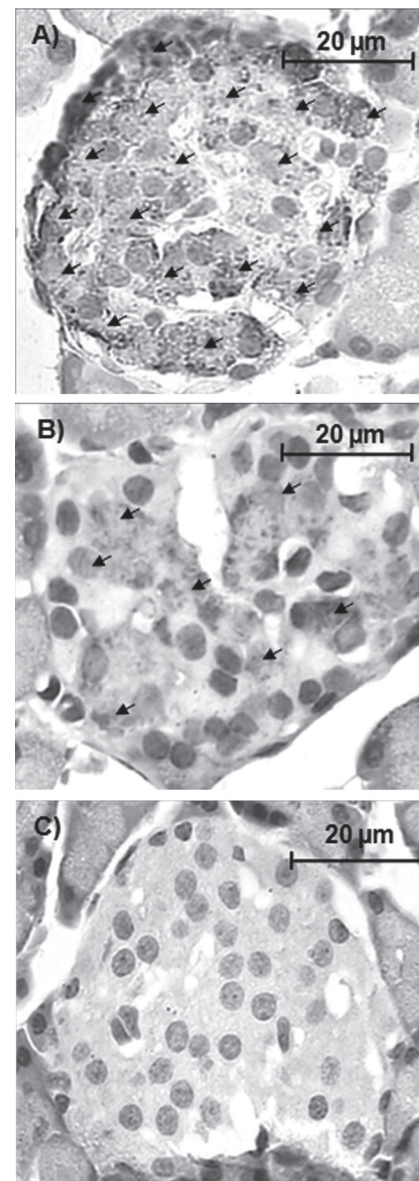


Fig. 4. Representative photomicrographs of insulin expression in the islets of Langerhans (400×). **4A.** Control rats exhibited normal insulin-stained beta cells. **4B.** The number of insulin-positive beta cells is reduced in n5-STZ rats compared to control rats. Arrows indicate insulin-positive cells in islets. **4C.** Absence of cross-reactivity is confirmed by the negative control of the immunochemistry reaction.

to verify the amplification of specific products because the method used employed SYBR Green as dye. *Ins-1* gene expression was significantly lower in n5-STZ rats than in control rats (79% Relative Expression Units, $P < 0.05$) (Fig. 5).

DISCUSSION

The global progression of diabetic patients to advanced stages of the disease has challenged researchers to establish animal models that resemble the chronic phase of diabetes. Spontaneous and chemically induced models are more frequently used for T2D models. In comparison to chemically induced T2D models, the genetic or spontaneous models such as db/db mice, ob/ob mice, Zucker Diabetic Fatty (ZDF) rats and *Psammomys obesus* mice, among others, present several limitations. One of the greatest is that the etiology of T2D in spontaneous models is a result of monogenic mutations, which rarely present themselves in diabetic patients. The difference in the natural course of this pathology in humans, compared to spontaneous models, makes it difficult to equate the findings from spontaneous models to human patients (Cummings *et al.*, 2008). In addition, the spontaneous models of T2D exhibit high mortality rates, a product of ketosis caused by an extensive degradation of pancreatic beta cells, which ultimately requires late-stage insulin treatment in order for the organism to survive (Arulmozhi *et al.*, 2004; Srinivasan and Ramarao, 2007). On the other hand, the rat models with STZ chemical induction, primarily the n5-STZ type, present lesser occurrence of ketosis and reduced mortality. Finally, the care and maintenance of STZ models is more feasible than that of spontaneous models (Srinivasan and Ramarao, 2007). Specifically, the n5-STZ model represents an adequate T2D model. However, as far as we know, biochemical and morphological characteristics of this model have been only described up to 12 weeks after induction (Bonner-Weir *et al.*, 1981; Arulmozhi, 2004; Takada *et al.*, 2007). In this study, we analyzed biochemical and morphological parameters, as well as *Ins-1* gene expression and tissue insulin levels, after 20 weeks of STZ induction in the neonatal n5-STZ model.

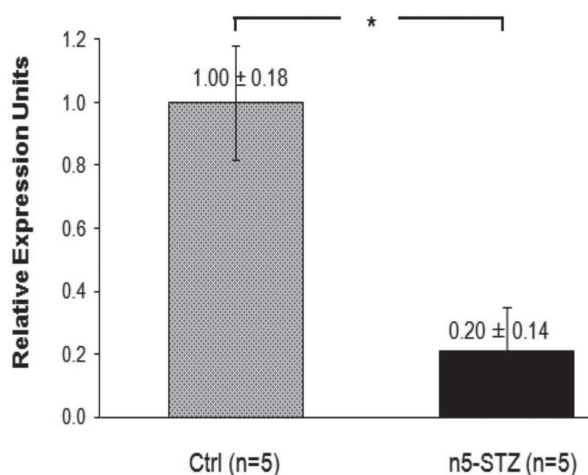


Fig. 5. Relative expression of the *Ins-1* gene is reduced in n5-STZ rats. Data are expressed as relative expression units, using *Mrps 18a* as a housekeeping gene. Bars represent standard errors of the mean (S.E.M.); * $P < 0.05$.

The mean serum glucose in control animals (125.40 ± 10.90 mg/dL) was in agreement with the normal glucose range reported (50-130 mg/dL) (Gouveia *et al.*, 2000; Ghezzi *et al.*, 2012). In contrast (and as expected), mean serum glucose was statistically higher in n5-STZ rats (340.00 ± 7.85 mg/dL) than in control animals. The hyperglycemia observed in n5-STZ rats was higher than the hyperglycemia reported in experiments that lasted for 12 weeks (210 to 240 mg/dL). This difference indicates a worsening of the metabolic condition in 20-week-old rats compared with 12-week-old rats (Takada *et al.*, 2007; Datusalia *et al.*, 2012). In addition, the data obtained in the glucose tolerance test demonstrated the absence of impaired glucose tolerance in control rats. On the contrary, n5-STZ rats exhibited impaired glucose tolerance, observed by their inability to metabolize glucose after 90 min, as reported in other studies (Takada *et al.*, 2007).

Another key feature in n5-STZ rats is their altered insulin levels, which were also observed in our results. Mild hypoinsulinemia was observed at various levels according to both circulating insulin concentration and tissue insulin levels. At 20 weeks post-induction, insulin levels were decreased approximately 50% in n5-STZ rats compared to control rats. A similar reduction has also been observed in n5-STZ rats 10 weeks post-induction (Blondel *et al.*, 1989). However, Takada (2007) reported the pronounced elevation of insulin levels in n5-STZ rats compared to control animals and claimed that this hyperinsulinemia might be transient (Takada *et al.*, 2007). During the early stages of T2D development, insulin concentration increases to compensate for hyperglycemia. Nevertheless, in later stages of T2D progression, insulin reserves are depleted in secretory granules from beta cells, reducing the circulating insulin concentration and maintaining high serum glucose levels (Weir and Bonner, 2004). Our study results confirm the strong reduction of the pancreatic insulin stores during the chronic phase in 20-week-old n5-STZ animals, as indicated by reduced serum insulin concentration and decreased insulin content in beta cells. Finally, this insulin deficiency contributes to marked hyperglycemia as a result of diminished insulin activity.

Furthermore, n5-STZ rats in the present study exhibited insulin resistance, a condition previously described in this model and also observed in T2D patients (Takada *et al.*, 2007; Reaven, 2011). Different indexes that indicate insulin sensitivity/resistance (e.g., QUICKI, HOMA, 1/insulin, Matsuda index) are derived from serum insulin and glucose concentrations under fasting conditions (steady state) (Matthews *et al.*, 1985; Matsuda and DeFronzo, 1999; Muniyappa *et al.*, 2008). Here, the HOMA score was 50% higher in n5-STZ rats than in controls. Insulin resistance was moderate compared to other studies (Takada *et al.*, 2007), possibly because beta cell regeneration reported for the Wistar strain might result in some degree of recovery in the insulin stores. Inter-subject variation of the HOMA index, as indicated by a large coefficient of variation, may explain the variability of this score among many studies; its interpretation should be reviewed carefully, particularly in experimental studies works (Wallace *et al.*, 2004).

One limitation in the use of the current experimental model is the high mortality that occurs during diabetes induction in the first days after STZ injection. Mortality rates of 45-75% have been reported (Takada *et al.*, 2007; Datusalia *et al.*, 2012). In the present study, we obtained a mortality rate of 48%,

which correlates with previously reported mortality rates. In addition, because this model takes quite a long time (at least 12 weeks) to induce diabetes, it may not be particularly suitable for fast, routine pharmacological testing of anti-diabetic drugs.

STZ induction in 12-week-old rats reputedly produces damage in beta cell micro-anatomy, as shown by cytoplasmic vacuoles, atypical delimitation between the endocrine and exocrine regions and changes in the nuclear structure. Our histological findings in 20-week-old n5-STZ rats are in agreement with the pancreatic damage previously described (Nagasao *et al.*, 2005). Additionally, the reduction of insulin-positive beta cells that has been demonstrated in 12-week-old STZ-induced rats was also observed in 20-week-old STZ-induced rats (Adewole and Caxton-Martins, 2006). The described histological findings and the reduction of insulin reactivity exhibited by immunohistochemistry in n5-STZ rats confirmed the previously reported morphological damage to islets of Langerhans, as well as the STZ-selective toxic effect on beta cells (Nagasao *et al.*, 2005; Cefalu, 2006). When all histological and biochemical results are considered together, a feasible explanation for the observed partial damage to the islets of Langerhans by STZ is that it is probably a result of the beta cells' ability to regenerate, particularly in newborn Wistar rats (Wang *et al.*, 1994; Arulmozhi *et al.*, 2004). If this hypothesis is correct, this regeneration might involve the participation of pancreatic endocrine cell differentiation markers such as nestin and insulin-like growth factor 1 (IGF-1), which could explain the increased insulin secretion observed during immediately after STZ treatment, which is likely lost over time (Nagasao *et al.*, 2005). In fact, this study evaluated the chronic phase of experimental T2D in which damage of beta cells is gradual and severe, meaning that beta cells cannot secrete enough insulin to compensate for the increased serum glucose concentration.

A novel finding of this study is the analysis of *Ins-1* gene expression, which revealed a significant reduction of *Ins-1* gene expression in n5-STZ rats compared to control rats that correlates with the observed decrease of insulin levels. One possible explanation of this reduction in *Ins-1* gene expression may be related to the glucose responsive elements in the promoter region of this gene. Accordingly, it might involve the participation of multiple transcriptional factors, possibly including the pancreatic and duodenal homeobox 1 transcription factor (PDX-1) and the transcription factor neurogenic differentiation 1, also known as Beta 2 (NeuroD1/Beta 2). Under normal conditions, PDX-1 and NeuroD1/Beta 2 in pancreatic tissue participate in glucose homeostasis and act synergistically in response to increased glucose to stimulate insulin gene expression (Naya *et al.*, 1997; Andrali *et al.*, 2008). However, chronic hyperglycemia produces a reduction in PDX-1 gene expression and activity which in turn affects the expression of the insulin gene and the secretion of insulin. Additionally, impaired NeuroD1/Beta 2 activity has been implicated in T1D and T2D development (Harmon *et al.*, 1999; Leibowitz *et al.*, 2001; Melloul *et al.*, 2002; Andrali *et al.*, 2008; Sachdeva *et al.*, 2009). Although PDX-1 mRNA and protein levels have been evaluated in some genetic models of diabetes and insulin resistance, such as Goto-Kakizaki (GK) rats, *Psammomys obesus* and non-obese diabetic (NOD) mice, additional studies should be undertaken to evaluate PDX-1 expression in the neonatal STZ-induced model n5-STZ during both early and chronic stages (Fernandes *et al.*, 1997; Harmon *et al.*, 1999; Leibowitz *et al.*, 2001).

Additional studies are also required to evaluate molecules that play an important role in glucose metabolic pathways, such as insulin receptor substrate (IRS), protein kinase B (PKB/Akt), and glucose transporter 4 (GLUT-4), to complement the molecular characterization of the n5-STZ model.

Thus the findings in the present study provide biochemical, morphological, and molecular information about the chronic diabetic stage in n5-STZ rats after 20-weeks post-induction and complement the existing characterization of the neonatal diabetic models induced by STZ.

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