ANÁLISE IN VIVO DA RELAÇÃO ENTRE HIPÓXIA E ESTRESSE OXIDATIVO SOBRE O DESENVOLVIMENTO EMBRIOFETAL DO PÂNCREAS DE DESCENDENTES DE RATAS DIABÉTICAS

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Tese apresentada à Faculdade de Medicina de Botucatu – Unesp, Programa de Pós-Graduação em Ginecologia, Obstetrícia e Mastologia. Área de concentração: Tocoginecologia, para obtenção do título de Doutor.

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Palavras-chave: Estresse oxidativo; Hiperglicemia; Hipóxia; Pâncreas.

"Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos."

(Isaac Newton)

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Artigo

INFLUENCE OF MATERNAL GLYCEMIA, OXIDATIVE STRESS AND HYPOXIA ON FETAL RAT ENDOCRINE PANCREAS

Running title: Maternal glycemia, oxidative stress and hypoxia on fetal rat pancreas

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RESUMO

Durante a gravidez diabética, a hiperglicemia materna pode prejudicar o desenvolvimento embrionário por uma associação de hipóxia e estresse oxidativo. Deste modo, nossa hipótese é de que a combinação desses mecanismos esteja envolvida no desenvolvimento pancreático alterado. Portanto, o objetivo do presente estudo foi avaliar os efeitos do estresse oxidativo e hipóxia no organismo materno sobre o desenvolvimento pancreático fetal em condições hiperglicêmicas. Foram utilizados ratos da linhagem *Wistar* que foram aleatoriamente distribuídos em: Controle (C); Diabete moderado (DM) e Diabete grave (DG). O diabete foi induzido em ratos pela administração de *streptozotocin*. As ratas foram acasaladas e, no 18º e 21º dias de prenhez, foram avaliados parâmetros como hiperglicemia e marcadores de hipóxia e de estresse oxidativo maternos. Nos mesmos momentos, os fetos foram coletados para análise das ilhotas pancreáticas. Foram encontradas alterações na tríade hormonal (insulina, glucagon e somatostatina) e marcadores de proliferação celular (PDX-1 e ki67) e morte celular (caspase-3). Essas alterações foram mais evidentes nos fetos advindos do grupo diabete grave. Além disso, a morfologia das ilhotas pancreáticas fetais e localização correta das células endócrinas foram claramente alteradas. Também houve correlação positiva entre glicemia, estresse oxidativo e hipóxia no organismo materno dos grupos diabéticos. Estes mecanismos também foram positivamente correlacionados com a redução no número de ilhotas e de células por ilhota nos descendentes. Portanto, a presença de estresse oxidativo e hipóxia, induzidos por alterações glicêmicas maternas, causou prejuízo no desenvolvimento pancreático fetal. Este fato demonstra que é necessário um rígido controle glicêmico materno para prevenir complicações embriofetais e perinatais.

Palavras chave: diabete, pâncreas, rato

ABSTRACT

In diabetic pregnancy, hyperglycemia may impair embryonic development by a combination of hypoxia and oxidative stress. Therefore, we hypothesized that a combination of these factors was involved in the impaired pancreatic development in the offspring. Thus, the objective of the present study was to evaluate maternal oxidative stress and hypoxia status on fetal pancreatic development in hyperglycemic conditions. Wistar rats were randomly assigned into three groups: control (C); mild diabetes (MD) and severe diabetes (SD). Diabetes was induced by the beta-cytotoxic drug (streptozotocin) in rats. The female rats were mated and at days 18 (early period of maximum fetal development) and 21 (at term) of pregnancy the maternal hyperglycemia, hypoxia and oxidative stress markers were evaluated. In the fetus, the pancreatic islets were analyzed. The results showed alterations in pancreatic hormone triad (insulin, glucagon and somatostatin), beta cell marker (PDX-1), proliferation (ki67) and apoptosis (caspase-3), which were more pronounced in the SD group. Furthermore, the morphology of the fetal pancreatic islets was clearly changed. There was a positive correlation between blood glucose, oxidative stress and hypoxia of the mothers and the reduction in the number of islets and number of cells per islet in the fetuses from the diabetic groups. Therefore, oxidative stress and hypoxia induced by maternal hyperglycemia led to impairment of fetal pancreatic development. These observations indicate that a rigid glycemic control in diabetic pregnancy is required to prevent the embryofetal and perinatal complications.

Keywords: diabetes, pancreas, rats

INTRODUCTION

Pancreatic development represents an interesting process, which begins with thickening of the endoderm, proliferation of pancreatic progenitors, and evagination of a dorsal and ventral pancreatic bud later become a single gland (Zaret & Grompe, 2008; Jorgensen et al., 2007). The pancreas consists of two functionally and morphologically distinct cell populations derived from one primitive epithelium (Avolio et al., 2013; Pan & Wright, 2011; Gittes, 2009). These two tissue types make up the exocrine and endocrine compartment, respectively. The exocrine portion (98% of the whole organ mass) mostly contains enzyme-secreting acinar cells organized into clusters surrounding a network system of ducts and they contribute to food processing (Slack, 1995). The endocrine compartment (approximately 2% of the whole pancreatic tissue) is organized into highly vascularized and innervated cell clusters called pancreatic islets comprising three major hormone- producing cell subtypes: alpha (α), beta (β) and (δ) cells secreting glucagon, insulin and somatostatin, respectively. Insulin is released in response to an increase in blood glucose levels and acts by reducing glycemia by promoting the storage and/or metabolism of glucose. Glucagon has the opposite function by promoting glucose release and/or neogenesis from glycogen, in the case of hypoglycemia. Somatostatin has been implicated in the regulation of other hormones and exocrine enzyme secretion (Csaba & Dournaud, 2001; Roncoroni et al., 1983; Adrian et al., 1978). The close interaction between the endocrine pancreatic cells and vascular cells regulates hormone release, establishing a fine adjustment of the glucose homeostasis in the organism (Avolio et al., 2013; Konstantinova et al., 2007; Prado et al., 2004; Slack, 1995).

The increasing prevalence of human pancreatic diseases, in particular, *Diabetes mellitus* has encouraged further understanding of pancreas organogenesis. However, human studies are limited not only for ethical reasons but also by the many uncontrollable variables that modify the intrauterine environment. Therefore, it is necessary to develop adequate experimental models (Lopez-Soldado & Herrera 2003). The induction of experimental diabetes using a beta (β)-cytotoxic drug such as streptozotocin (STZ) is well-characterized (Ward et al., 2001). This cytotoxin is derived from *Streptomyces achromogenes* and results in degranulation and necrosis of β-cells

causing insulin deficiency. Depending on the animal strain, dose, route of drug administration, and the period of life in which STZ is administered in rats, different blood glucose levels are found, leading to different diabetes severity from mild diabetes (blood glucose level between 120 and 200/300 mg/dL) (Damasceno et al., 2002; Gelardi et al., 1990; Merzouk et al., 2000; Portha et al., 1974; Sinzato et al., 2011; Tsuji et al., 1988; Volpato et al., 2014) to severe diabetes (blood glucose level above 200/300 mg/dL) (de Souza et al., 2010; Volpato et al., 2008; Rudge et al., 2006; Damasceno et al., 2004; Eriksson et al., 2003; Eriksson et al., 2000).

There is evidence that hyperglycemic environment causes an increase in intracellular oxygen consumption in mitochondria resulting in decrease of oxygen tension in tissues, which leads to hypoxia (Ornoy et al., 2010). Low oxygen tension favors embryonic organogenesis and cell proliferation (Burton et al., 2009). Although hypoxia is essential in early development (Dunwoodie, 2009), as the pregnancy evolves, a control of oxygen tension is necessary in order to maintain normal fetal growth (Burton et al., 2009). The partial oxygen tension regulates embryonic development of several organs, including the pancreas (Fraker et al, 2007; Heinis et al., 2010). Thus, the intrauterine environment in which the fetus develops may contribute to physiological and metabolic changes throughout life, (Collman, 2011; Plagemann, 2011).

There is evidence that diabetes- or insulin resistance-induced hyperglycemia causes oxidative stress and that if oxidant induction of homeostatic mechanisms are insufficient to restore physiologic redox status, normal cellular function may be impaired, and tissue integrity could be affected. However, demonstration that hyperglycemia-induced oxidative stress is responsible for the pathogenesis of diabetic complications has been difficult, and may be tissue-specific (King & Loeken, 2004). Embryos in diabetic environment seem to be more sensitive to high levels of reactive oxygen species, especially during organogenesis. Then, in diabetic pregnancy, hyperglycemia may impair embryonic development by a combination of hypoxia and oxidative stress (Ornoy et al. 2010).

Considering that diabetes causes hyperglycemia, oxidative stress, and hypoxia, we hypothesized that a combination of these variables is involved in the pancreatic development impairment. Thus, the objective of the present study was to evaluate the

effect of maternal oxidative stress and hypoxia status on fetal pancreatic development in hyperglycemic conditions.

MATERIALS AND METHOD

Ethical Approval

The local Committee of Ethics in Animal Experimentation approved all the experimental protocols used (Process number: 916/2011).

Experimental animals

Male and female Wistar rats, weighing approximately 220 and 190g, respectively, were obtained from CEMIB (Multidisciplinary Center for Biological Research —Campinas, São Paulo State). All animals were adapted and maintained in Vivarium of Laboratory of Experimental Research on Gynecology and Obstetrics, Unesp. Water and food were given *ad libitum* in a controlled environment (room temperature: 22 ± 3°C, humidity: 50 ± 10% and 12 hr light/dark cycle) during all experiment. Male and female normoglycemic rats were mated to obtain offspring.

Mild diabetes induction

At birth (day 1 of life), a group of female offspring were subcutaneously injected with 100 mg streptozotocin/kg body weight (STZ – Sigma Chemical Company, St. Louis, Millstone, USA) dissolved in citrate buffer (0.1 M, pH 4.5) to obtain rats with mild diabetes (MD) (Sinzato et al., 2011). Other female rats subcutaneously received only citrate buffer (non-diabetic rats, i.e., control group – C). At day 5 of life, the offspring given STZ and presenting blood glucose level higher than 400 mg/dL were included in the MD group; the animals given citrate buffer and presenting glycemia lower than 120 mg/dL were included in the C group.

Severe diabetes induction

In other group of rats, severe diabetes were induced in adult female rats (90 days of age) by a single intravenous injection of STZ at a dose of 40 mg/kg diluted in citrate buffer (0.1 M, pH 4.5) (de Souza et al., 2009). After five days of STZ injection, the diabetic state was confirmed by a blood glucose concentration ≥ 300 mg/dL by a One-Touch Ultra glucometer (Life Scan, Johnson and Johnson®) and the rats were included in the severe diabetes group (SD). The control animals given only citrate buffer at the same route and period of life and the dams that presented glycemia lower than 120 mg/dL were included in the C group.

Mating and pregnancy

At 100 days of life, severe diabetic, mild diabetic and control adult female rats were mated overnight with non-diabetic males. The morning when spermatozoa were found in the vaginal smear was designated as gestational day zero (D0).

Experimental groups

After mating, the animals were randomly distributed in different experimental groups according to their euthanasia and the presence or absence of hyperglycemia. The control animals for mild or severe diabetes groups presented no difference in the analyzed parameters and consequently were clustered:

GROUPS		
Control (C)	**Mild Diabetes (MD)**	**Severe Diabetes (SD)**
Euthanasia on Day 18 of pregnancy	Euthanasia on Day 18 of pregnancy	Euthanasia on Day 18 of pregnancy
Euthanasia on Day 21 of pregnancy	Euthanasia on Day 21 of pregnancy	Euthanasia on Day 21 of pregnancy

Laboratory procedures

Maternal analysis

Maternal blood samples

Blood samples were collected from the tail and glucose levels were assessed by a One-Touch Ultra glucometer (Johnson & Johnson®) for inclusion criteria. Glucose tolerance test (GTT) was performed at day 17 of pregnancy according to Campos et al. (2007) to assess the development of altered glucose metabolism. The glycemic mean for correlation analyses with hypoxia and oxidative stress markers was calculated by mean of blood glucose levels at timepoints 0, 30, 60 and 120 minutes of GTT. At day 18 (fetal period) and 21 (at term), the female rats (C, MD and SD groups) were lethally anesthetized with 50mg sodium thiopental/kg bw (Thiopentax®, Cristália Produtos Químicos e Farmacêuticos Ltda., São Paulo State, Brazil) to obtain blood samples for measurement oxidative stress markers in washed erythrocytes (superoxide dismutase - SOD, glutathione peroxidase - GSH-Px, thiol groups – SH and malonyl dialdehyde - MDA) and serum samples were used for evaluation of hypoxia status (erythropoietin).

Oxidative stress markers in maternal washed erythrocytes

Blood samples were collected in tubes with anticoagulant was centrifuged at 90 × g for 10 min at 4°C. The supernatant was discarded and erythrocytes were washed with phosphate buffer saline (0.01M, pH 7.4) followed by centrifugation at 263 × g for 1 min at 4°C. This procedure was repeated three times and final cell pellet was used for determination of SOD, GSH-Px, SH and MDA levels as described by de Souza et al. (de Souza et al., 2010).

Fetal pancreatic analysis (Day 18 and 21 of pregnancy)

Pancreas samples

Following maternal procedures, the exploratory laparotomy was performed for fetal pancreas collection. The intestinal loops were moved cranially for uterus exposure. Immediately all viable fetuses were carefully exposed and glycemia was measurement

by a conventional glucosimeter. Pancreas samples were dissected under thiopental sodium anesthesia and placed in 10% paraformaldehyde for 24 hours. Then, the samples were washed and imbedded in 70% alcohol. It was collected 5 pancreas/litter from 5 mothers and the samples were fixed tissues were processed for paraffin embedding.

Morphometric and immunohistochemistry analyses

Serial 5 µm thick sections were prepared and stained. The immunohistochemical staining was performed on paraffin sections. Before staining the sections were subjected to heat-induced epitope retrieval in citrate solution at pH 6.0 for anti-insulin, anti-glucagon, anti-Ki67 and anti-caspase-3. For somatostatin and PDX-1 antibodies, epitope retrieval was not necessary. The tissue sections were washed in phosphate-buffered saline (0.1 M) and incubated with 8% H2O2 (diluted in 92% methanol) for 20 min to inhibit endogenous peroxidase activity. Paraffin sections were incubated with the following primary antibody glucagon (1:500 - Anti-Glucagon antibody, ab8055, abcam®) and capase-3 (1:200 – Anti-Caspase-3, 9661S, Asp175, Cell Signalling Technology) for 1 hour; insulin (1:10.000 - Anti-Insulin + Proinsulin Antibody [D6C4], ab8304, abcam®) and pdx-1 (1:1000 – Rabbit Anti-PDX-1, ab47267, abcam®) for 2 hours; somatostatin (1:2000 – Polyclonal Rabbit Anti-Somatostatin, Dako®) and ki67 (1:100 - Monoclonal mouse Anti-Human, Clone MIB-1, Dako®), overnight. Then, tissue sections were incubated for 30 min with Protein Block (Dako®) and 30 min with Histofine (Simple Stain Max Po. Universal Immuno-peroxidase Polymer/Anti-Mouse and Rabbit) except, for anti-pdx-1 and anti-somatostatin, in which Envision + Dual Link System-HRP (Dako®) was used as secondary antibody. Subsequently, the slides were developed with DAB (3,3-diaminobenzidine - Dako®). Sections were finally stained with hematoxylin & eosin (Mayer's hematoxylin) to assess pancreatic islet histology and morphology in the experimental animals. Then, the slides were thoroughly rinsed in water for 5 min. The antibodies were validated by immunohistochemistry against normal and diseased tissues from rats. The antibodies were further optimized in our laboratory by serial dilution and the specificity of the secondary antibody was tested by omission of the primary antibody.

All morphometric and immunohistochemistry analyzes were performed in an imaging computer system (KS-300 software, version 3.0, Zeiss), which receives image

from a digital camera (CCD-IRIS / RGB, Sony®) coupled to a microscope (DMR, Leica® type). Software Image J was used to determine total area of fetal islets, stained area of insulin, glucagon and somatostatin antibodies and count of number of stained cells for PDX-1, Ki67 and caspase-3. The ratios of stained area/total area for hormonal triad and number of stained cells/total area for proliferation and cellular death markers were performed.

Statistical analyses

Data were presented as mean ± standard deviation (SD). For data presenting a normal distribution, Tukey´s Multiple Comparison test was used. For correlation analysis was applied Pearson´s correlation test. $P < 0.05$ was considered as limit of statistical significance.

RESULTS

Table 1 represents mean number of islets, cells per islets and stained cells of fetuses from all groups (C, MD and SD groups) at each time point (days 18 and 21 of pregnancy) analyzed. At day 18 of pregnancy, there was decreased number of islets and reduced number of insulin, glucagon and caspase-3-stained cells in SD group compared to those of C group. Moreover, in the same group there was also increase of somatostatin-stained cells relative to the MD and C groups. The MD group presented increased number of insulin-stained cells and decreased number of Ki67-stained cells compared to the C group. At term, the islets of fetuses from the SD group remained to have reduced number and fewer insulin-stained cells compared to those of the MD and C groups. In MD and SD rats, there was observed a decrease in mean number of cells per islet and reduced mean number of glucagon, somatostatin, and Ki67-stained cells in relation to those of the C group. In the MD and SD groups, the mean number of capase-3-stained cells was increased compared to those of C group.

The ratio of stained area and total area of islet was determined for insulin, glucagon and somatostatin and the ratio of number of stained cells and total area of islet was also performed for PDX-1, Ki67 and capase-3 positive cells in fetuses from all

groups in each time point analyzed (Table 2). At day 18 of pregnancy, the fetuses in the SD group presented decrease in insulin and Ki67 positive cells and increase in somatostatin positive cells compared to the MD and C groups. At term, the insulin positive cells remained decreased, and PDX-1 and caspase-3 positive cells were also decreased in the SD group relative to the MD and C groups. In the MD group, there was reduced ratios of somatostatin, ki67 and caspase-3 compared to the C group (Table 2).

Figure 1 shows the insulin, glucagon and somatostatin morphological analyses in fetuses from all groups and time points. There was observed disorganized cell distribution at day 18 of pregnancy in all groups (A, B, C, D, E, F, G, H and I) and this finding was more pronounced in the SD group compared to others groups (C, F and I). In the SD group at the same time point, a presence of alpha and delta cells in the center of islets was also observed (F and I). At term the disorganized cell distribution of alpha and delta cells was preserved in the SD group (O and R) in relative to the MD (N and Q) and C groups (M and P).

The PDX-1, Ki67 and capase-3 morphological analysis are shown in figure 2. It was observed that there were cells stained for caspase-3 in the center of islets in the SD group at different time points (I and R). In the MD (H and Q) and C (G and P) groups, the capase-3-stained cells were mostly on the periphery of the islets.

The islet size distribution of fetuses from the SD, MD and C groups is shown in figure 3. At day 21 of pregnancy, the SD and MD fetuses presented decreased number of large islets compared to those of the C group.

Figure 3 displays the maternal oxidative stress markers of different groups and time points. The diabetic groups presented higher MDA concentrations compared to those of the C group at days 18 and 21 of pregnancy. At term , there was observed increased activities of SOD in the SD mothers and of glutathione peroxidase (GSH-Px) in the MD group compared to those of the C group.

The erythropoietin levels were determined and there was an increase in mothers in the SD group (0.004±0.002 IU/mL) compared to those of the MD (0.004±0.002 IU/mL) and C (0.002±0.000 IU/mL) groups at day 18 of pregnancy. At day 21 of

pregnancy, the diabetic groups (MD = 1.53±0.91 IU/mL and SD = 11.22±7.49 IU/mL) presented increased erythropoietin levels in relation to the C group (0.41±0.39 IU/mL).

The fetal glycemic level in fetuses from the SD group (413.85±42.20mg/dL) was increased relative to other groups (MD = 130.02±63.83mg/dL and C = 122.30±34.71mg/dL) at day 21 of pregnancy (at term).

The correlation analysis between hypoxia (erythropoietin level) and glycemic means, and between hypoxia and each marker of oxidative stress of mothers of all groups (SD, MD and C groups - together) in each time point analyzed (at days 18 and 21 of pregnancy) are shown in Table 3. The maternal erythropoietin level was positively correlated ($p<0.05$) with the maternal glycemic mean and malonyl dialdehyde (MDA) levels at days 18 and 21 of pregnancy. At term, the erythropoietin level was also positively correlated ($p<0.05$) with superoxide dismutase (SOD) activity and thiol groups (SH) level.

Table 4 shows the correlation analysis among number of islets and number of cells per islets in fetuses with maternal glycemic mean, oxidative stress markers and hypoxia (erythropoietin level) of mothers (SD, MD and C groups - together) in each time point analyzed (at days 18 and 21 of pregnancy). At day 18 of pregnancy, there was a negative correlation ($p<0.05$) between number of fetal islets and maternal glycemic mean. At term pregnancy, there was negative correlation ($p<0.05$) between the number of fetal islets and all parameter analyzed. Besides, the number of cells per fetal islet was positively correlated ($p<0.05$) with maternal glycemic mean and negatively correlated ($p<0.05$) with MDA level and SOD activity.

DISCUSSION

The developmental origin of adult disease hypothesis postulates that environmental damages during intrauterine life may alter central regulatory mechanisms of the developing fetuses, mainly in organogenesis period (Adamo et al., 2012). In the present study, the maternal inadequate conditions impaired the fetal pancreatic

development by reduction of number of islets and alterations in the number and organization of hormone-containing cells (insulin, glucagon and somatostatin). These hormones were analyzed by number of stained cells and by ratio of stained area and total area of islets. It was observed that fetuses from severely diabetic rats presented a reduced number of beta cells leading to decreased insulin synthesis. The glucagon displayed no changes but the number of alpha cells was reduced, showing that these cells are increasing. These alterations were most pronounced in the end of pregnancy. Interestingly, the fetuses generated in a severe diabetic environment presented increase of somatostatin-stained cells and their formation in fast period of early growth may be responsible by decreased number of somatostatin cells at term. At first, this may be due to the compensatory mechanism due to reduction of insulin synthesis. However, in late pregnancy, the number of somatostatin-stained cells was lower showing that the hyperglycemic intrauterine environment also impaired their function. Even the fetuses from glucose intolerant mothers (mild diabetes) showed the lower number of somatostatin-stained cells and reduced hormonal synthesis. It is known that somatostatin is indirectly involved in glycemia regulation by allowing fine adjustments in the control of insulin and glucagon secretion (Bousquet et al., 2004). These results suggest that this hormone was more susceptible to changes in the intrauterine environment. Iessi et al. (2015 – paper submitted) also observed hormonal alterations and decreased somatostatin levels that persisted in perinatal life in diabetic environment of rats. In addition to the hormonal alterations, the fetuses from diabetic dams showed a decreased number of islets and cells per islets. These data may be due to changes caused by hyperglycemic insults in cell proliferation and death leading to alteration in studied markers. In normal conditions, there must be a balance between cellular death and proliferation in order to have an adequate pancreatic development (Scaglia et al., 1997). In the present study, this balance was not verified in fetal pancreas from diabetic rats. In the end of pregnancy, the proliferation (Ki67 marker) was 50% lower and there was a two fold increase on the number of stained cells with caspase-3 (cell death marker) in islets cells of fetuses from diabetic groups. PDX-1 is a transcription factor expressed in embryonic duodenum and pancreas and later in beta cells and some delta cells, and is necessary for pancreas development (Haber et al., 2006). In this study, it was noted that

there was reduction of this marker in islets of fetuses from diabetic groups. This finding may be related to the decrease in the number of pancreatic cells in diabetic groups.

In humans, the alpha, beta and delta cells are scattered in whole islets. However, in rodents these cells present a very characteristic organization, the beta cells are localized in the center of islets and the alpha are limited in the periphery of islets along with delta cells (Abraham et al., 2009; Kordowich et al., 2010). In this study, there was a delay in islet development in fetuses from SD group at day 18 of pregnancy. At the same time point, aberrant localization of alpha and delta cells in the center of islets was observed. At term the disorganization of alpha and delta cells was preserved in these fetuses. Our findings corroborate with Matsushima et al. (1978) who observed that the arrangement of endocrine cells in the islets of diabetic rats became completely different from the control rats. In the present study cell proliferation and death markers were detected by pancreatic morphological analyses. The most interesting finding was that fetuses from severe diabetic dams presented a different distribution of caspase-3-stained cells relative to the controls. The caspase-3-stained cells were mainly in periphery of islets (i.e. non-beta cells) in normal conditions whereas the fetuses from severe hyperglycemic environment had increased number of caspase-3-stained cells in the center of islets, showing an altered cytoarchitecture. It was noted in other studies (Gallego, 2014, Takeda et al., 2012; Simsek et al., 2012; Reddy et al., 2003) that the distribution of caspase-3 positive cells in islets of control rats is similar to our findings, suggesting that the caspase-3 positive cells are non-beta cells due to normal localization of these cells in the fetal pancreases. However, more studies must be conducted in order to identify the cell types involved.

In this study, a reduced number of large islets in MD fetuses and increased number of these islets in SD group were demonstrated at day 21 of pregnancy. However, we agree that this finding is uncertain. The standardization of number of islets for analyses of fetal pancreatic islet size distribution for all studied groups and time points is important for statistical analyses, but it does not consider the difference related to the number of islets and number of cell per islets existing in each diabetic group, especially in the severe diabetic group.

Besides the maternal hyperglycemic insults, the diabetic mothers presented oxidative stress, confirmed by lipoperoxidation (high malonyl dialdehyde concentrations) in maternal blood. The mild diabetic (MD) group showed twice the MDA concentration and the severe diabetic (SD) group four times more than control animals. In this study we also observed an increased antioxidant system, as shown by increased activities of superoxide dismutase (SOD) in the SD group and glutathione peroxidase (GSH-Px) in the MD group. This fact might be an attempt of the maternal organism to control the glycemic alteration-induced oxidative stress. However, this increase in the antioxidant system may represent an insufficient response in these animals. In addition, it was observed there is a relationship between hypoxia (erythropoietin concentrations) and glycemic alterations (at days 18 and 21 of pregnancy). These data indicate that hyperglycemia leads to hypoxia and both might contribute to the oxidative stress status. According to Ornoy et al. (2010) pregnant diabetic women may impair the embryofetal development due to hypoxia and oxidative stress. The relationship between number of islets and cells per islets in fetuses with maternal hyperglycemia, oxidative stress markers and hypoxia of mothers summarizes the physiological mechanisms involved in the reduction in the number of fetal islets and consequently the number of cells per islet. The relationship between glucose intolerance or decompensated hyperglycemia, oxidative stress and hypoxia is most pronounced at term. Therefore, the oxidative stress and hypoxia induced by maternal glycemic alteration may lead to fetal pancreatic development impairment indicating that rigid glycemic control in diabetic pregnancy is required to prevent the embryofetal and perinatal complications.

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ADDITIONAL INFORMATION

Competing interests

The authors confirm that there are no conflicts of interest.

Author contributions

I.L.I, Y.K.S. and D.C.D conceived and designed the experiments. I.L.I., Y.K.S., F.Q.G., B.D., collected the experimental data. I.L.I., Y.K.S., F.Q.G., B.D., I.C., M.R., J.H.N. and D.C.D analysed and interpreted data. I.L., Y.K.S, J.H.N. and D.C.D. drafted the article and revised it critically for important intellectual content. All authors have read and approved the manuscript.

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Table 1. Number mean of islets, cells per islet and stained cells of fetuses from mild (MD), severe (SD) and control (C) groups at days 18 and 21 of pregnancy.

	Groups		
Day 18 of pregnancy	**C (n=5)**	**MD (n=5)**	**SD (n=5)**
Number of islets	70.21±31.44	51.21±17.94	36.41±11.73*
Number of stained cells			
Insulin	29.12±12.18	38.26±25.06*	21.13±11.37*#
Glucagon	22.40±13.54	19.28±8.63	16.60±8.76*
Somatostatin	0.55±0.17	0.50±0.17	1.45±0.22*#
PDX-1	33.20±18.03	40.70±20.76	29.54±17.99
Ki67	8.27±9.54	4.35±2.75*	9.54±5.53#
Caspase-3	19.48±12.49	18.81±8.29	13.00±7.19*#
Day 21 of pregnancy (at term)	**C (n=5)**	**MD (n=5)**	**SD (n=5)**
Mean number of islets	150.40±35.45	120±30.84	57.40±11.28*#
Mean number of cells per islet	145.36±71.00	90.16±30.73*	90.26±41*
Number of stained cells			
Insulin	51.87±23.27	48.34±21.83	32.14±17.93*#
Glucagon	27.98±11.66	14.14±5.74*	14.64±7.63*
Somatostatin	6.74±2.88	4.44±1.99*	4.45±1.82*
PDX-1	40.80±17.43	29.34±13.66*	31.85±16.01
Ki67	62.38±34.94	36.86±15.69*	36.00±16.93*
Caspase-3	7.90±2.34	12.82±6.12*	13.38±7.12*

Values expressed as mean ± standard deviation.

*$p<0.05$ – statistically significant difference in relation to control group (Tukey's Multiple Comparison test).

# $p<0.05$ – statistically significant difference in relation to MD group (Tukey's Multiple Comparison test).

Table 2. Ratio of stained area/total area of islets for pancreatic hormones, and ratio of number of stained cells /total area of islet for PDX-1, ki67 and caspase-3 of fetuses from control (C), mild (MD) and severe (SD) groups at days 18 and 21 of pregnancy.

	Groups		
Day 18 of pregnancy	**C (n=5)**	**MD (n=5)**	**SD (n=5)**
Insulin	0.10±0.05	0.09±0.05	0.06±0.03*#
Glucagon	0.05±0.03	0.04±0.02	0.04±0.02
Somatostatin	0.002±0.001	0.003±0.000	0.009±0.003*#
PDX-1	2.42±0.65	2.71±1.03	2.48±0.88
Ki67	3.28±1.60	3.63±2.10	1.80±0.07*#
Caspase-3	2.46±0.92	2.12±0.90	2.22±0.94
Day 21 of pregnancy (at term)	**C (n=5)**	**MD (n=5)**	**SD (n=5)**
Insulin	0.21±0.07	0.19±0.07	0.09±0.05*#
Glucagon	0.04±0.01	0.04±0.01	0.04±0.02
Somatostatin	0.011±0.005	0.005±0.004*	0.009±0.005#
PDX-1	4.47±1.03	3.97±1.23	2.70±1.16*#
Ki67	4.30±1.40	3.75±1.13*	3.78±1.07#
Caspase-3	5.76±1.88	2.41±1.66*	3.28±1.70*#

Values expressed as mean ± standard deviation.

*$p<0.05$ – statistically significant difference in relation to control group (Tukey's Multiple Comparison test).

# $p<0.05$ – statistically significant difference in relation to MD group (Tukey's Multiple Comparison test).

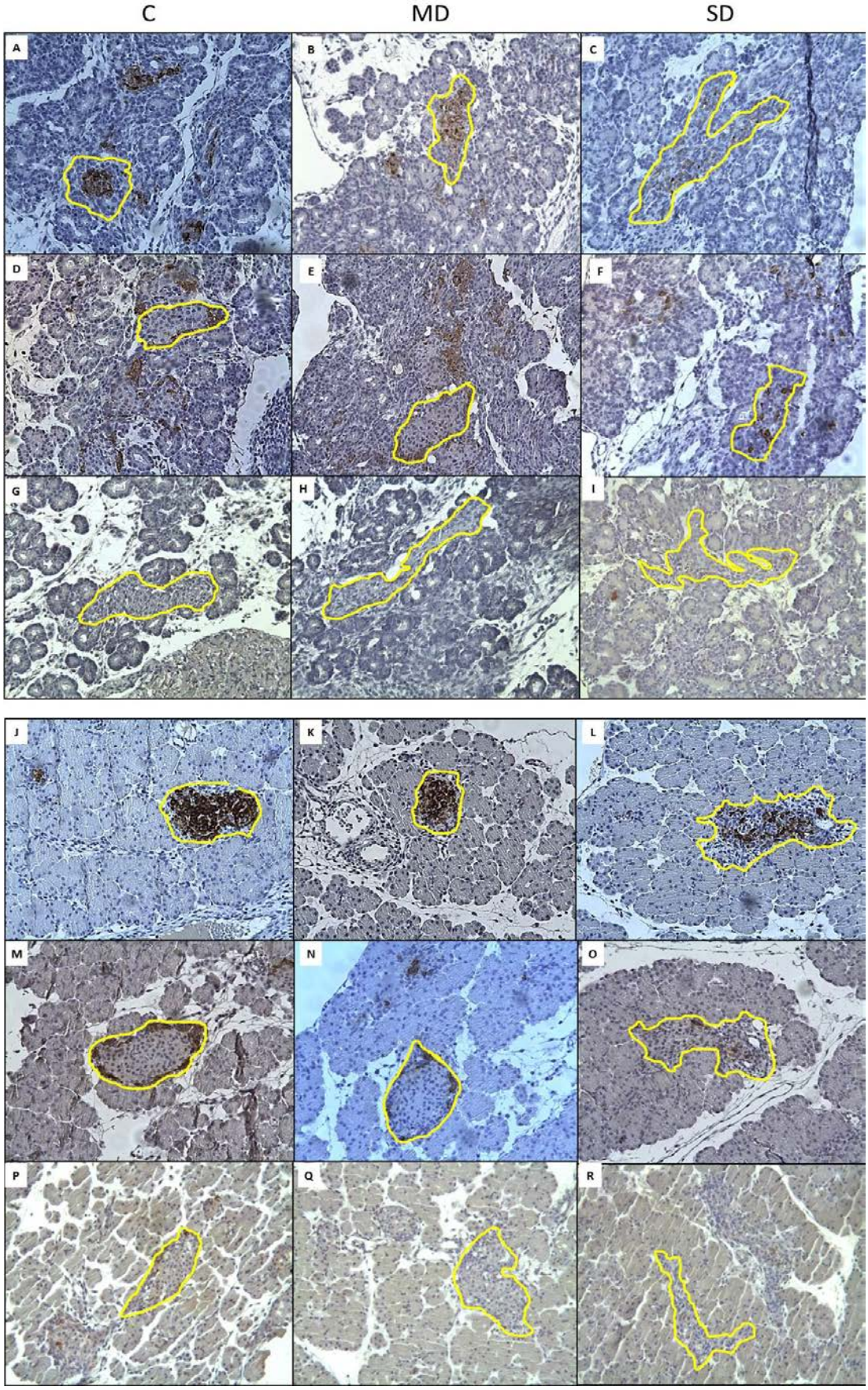
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Figure 1. A: Photomicrography of fetal pancreatic islets from control group immunolabelled for insulin at day 18 of pregnancy.

B: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for insulin at day 18 of pregnancy.

C: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for insulin at day 18 of pregnancy.

D: Photomicrography of fetal pancreatic islets from control group immunolabelled for glucagon at day 18 of pregnancy.

E: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for glucagon at day 18 of pregnancy.

F: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for glucagon at day 18 of pregnancy.

G: Photomicrography of fetal pancreatic islets from control group immunolabelled for somatostatin at day 18 of pregnancy.

H: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for somatostatin at day 18 of pregnancy.

I: Photomicrography of fetal pancreatic islets from severe dibetic group immunolabelled for somatostatin at day 18 of pregnancy.

J: Photomicrography of fetal pancreatic islets from control group immunolabelled for insulin at day 21 of pregnancy.

K: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for insulin at day 21 of pregnancy.

L: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for insulin at day 21 of pregnancy.

M: Photomicrography of fetal pancreatic islets from control group immunolabelled for glucagon at day 21 of pregnancy.

N: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for glucagon at day 21 of pregnancy.

O: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for glucagon at day 21 of pregnancy.

P: Photomicrography of fetal pancreatic islets from control group immunolabelled for somatostatin at day 21 of pregnancy.

Q: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for somatostatin at day 21 of pregnancy.

R: Photomicrography of fetal pancreatic islets from severe dibetic group immunolabelled for somatostatin at day 21 of pregnancy.

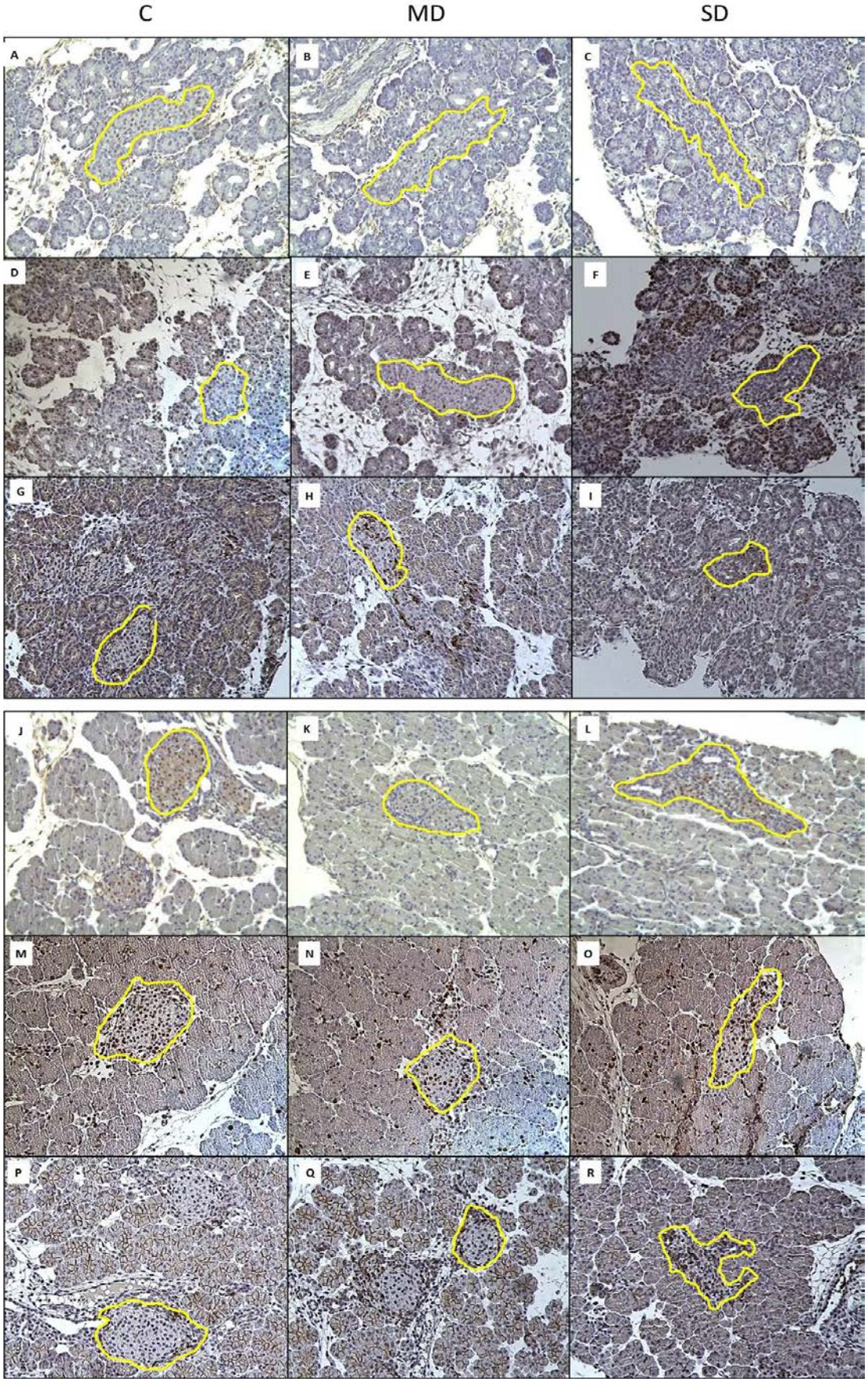
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Figure 2. A: Photomicrography of fetal pancreatic islets from control group immunolabelled for PDX-1 at day 18 of pregnancy.

B: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for PDX-1 at day 18 of pregnancy.

C: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for PDX-1 at day 18 of pregnancy.

D: Photomicrography of fetal pancreatic islets from control group immunolabelled for ki67 at day 18 of pregnancy.

E: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for ki67 at day 18 of pregnancy.

F: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for ki67 at day 18 of pregnancy.

G: Photomicrography of fetal pancreatic islets from control group immunolabelled for caspase-3 at day 18 of pregnancy.

H: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for caspase-3 at day 18 of pregnancy.

I: Photomicrography of fetal pancreatic islets from severe dibetic group immunolabelled for caspase-3 at day 18 of pregnancy.

J: Photomicrography of fetal pancreatic islets from control group immunolabelled for PDX-1 at day 21 of pregnancy.

K: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for PDX-1 at day 21 of pregnancy.

L: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for PDX-1 at day 21 of pregnancy.

M: Photomicrography of fetal pancreatic islets from control group immunolabelled for ki67 at day 21 of pregnancy.

N: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for ki67 at day 21 of pregnancy.

O: Photomicrography of fetal pancreatic islets from severe diabetic group immunolabelled for ki67 at day 21 of pregnancy.

P: Photomicrography of fetal pancreatic islets from control group immunolabelled for caspase-3 at day 21 of pregnancy.

Q: Photomicrography of fetal pancreatic islets from mild diabetic group immunolabelled for caspase-3 at day 21 of pregnancy.

R: Photomicrography of fetal pancreatic islets from severe dibetic group immunolabelled for caspase-3 at day 21 of pregnancy.

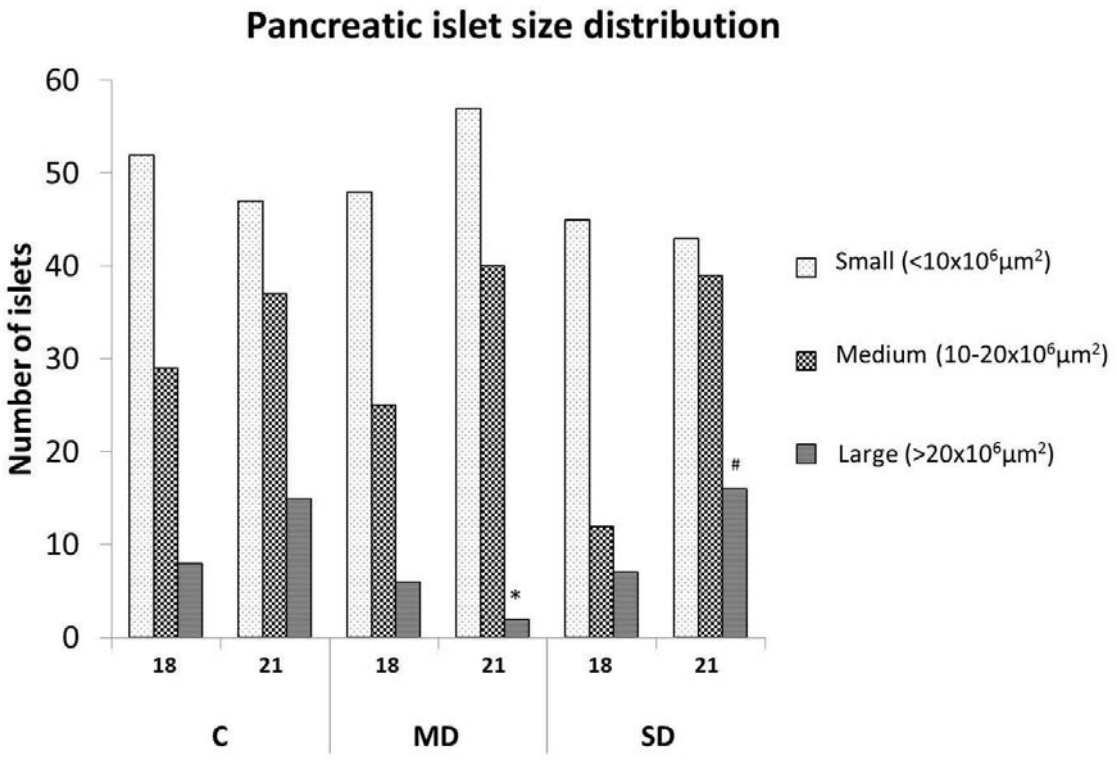

Figure 3. Fetal pancreatic islet size distribution of control (C), mild (MD) and severe (SD) diabetic groups at days 18 and 21 of pregnancy.

Values expressed as mean ± standard deviation.

*$p<0.05$ – statistically significant difference in relation to control group (Tukey's Multiple Comparison test).

$^{\#}$ $p<0.05$ – statistically significant difference in relation to MD group (Tukey's Multiple Comparison test).

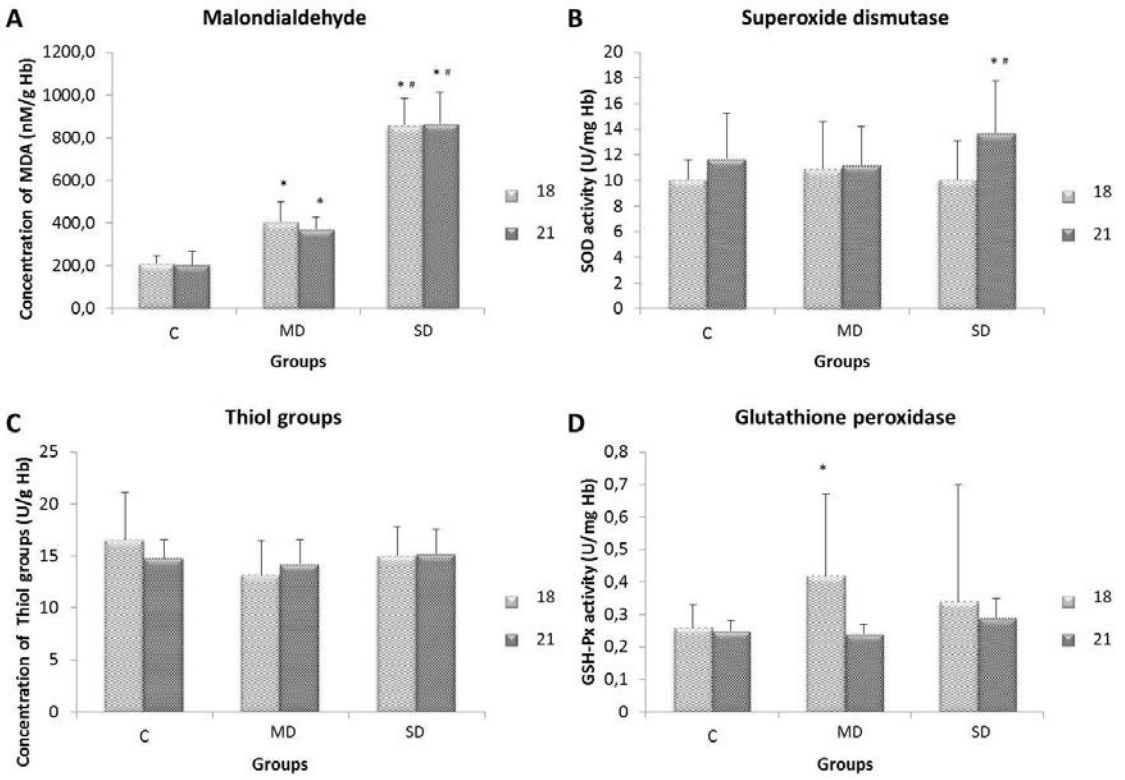

Figure 4. Maternal oxidative stress of control (C), mild (MD) and severe (SD) diabetic groups at days 18 and 21 of pregnancy.

Values expressed as mean ± standard deviation.

*$p<0.05$ – statistically significant difference in relation to control group (Tukey's Multiple Comparison test).

$^{\#}$ $p<0.05$ – statistically significant difference in relation to MD group (Tukey's Multiple Comparison test).

Table 3. Correlation analyses among erythropoietin levels and oxidative stress markers of rat mothers of control (C), mild (MD) and severe (SD) diabetic groups (together) at days 18 and 21 of pregnancy.

Day 18 of pregnancy	**R**	**P**
Erythropoietin x Glycemic mean	0.52	*
Erythropoietin x Malondialdehyde	0.49	*
Erythropoietin x Superoxide dismutase	0.14	>0.05
Erythropoietin x Thiol groups	-0.20	>0.05
Erythropoietin x Glutathione peroxidase	0.08	>0.05
Day 21 of pregnancy (at term)	**R**	**P**
Erythropoietin x Glycemic mean	0.80	*
Erythropoietin x Malondialdehyde	0.62	*
Erythropoietin x Superoxide dismutase	0.58	*
Erythropoietin x Thiol groups	0.54	*
Erythropoietin x Glutathione peroxidase	0.47	>0.05

*$p<0.05$ – statistically significant correlation (Pearson´s correlation test).

Table 4. Correlation analyses among number of islets and cells per islet on fetal organism with maternal glycemic level, oxidative stress markers and erythropoietin of control (C), mild (MD) and severe (SD) diabetic groups (together) at days 18 and 21 of pregnancy.

Day 18 of pregnancy	**R**	**P**
Number of fetal islets x Maternal glycemic mean	-0.52	*
Number of fetal islets x Malondialdehyde	-0.42	>0.05
Number of fetal islets x Superoxide dismutase	-0.05	>0.05
Number of fetal islets x Thiol groups	-0.23	>0.05
Number of fetal islets x Glutathione peroxidase	-0.29	>0.05
Number of fetal islets x Erythropoietin	-0.18	>0.05
Day 21 of pregnancy (at term)	**R**	**P**
Number of fetal islets x Maternal glycemic mean	-0.83	*
Number of fetal islets x Malondialdehyde	-0.79	*
Number of fetal islets x Superoxide dismutase	-0.68	*
Number of fetal islets x Thiol groups	-0.63	*
Number of fetal islets x Glutathione peroxidase	-0.70	*
Number of fetal islets x Erythropoietin	-0.68	*
Number of cells per fetal islet x Maternal glycemic mean	0.90	*
Number of cells per fetal islet x Malondialdehyde	-0.52	*
Number of cells per fetal islet x Superoxide dismutase	-0.57	*
Number of cells per fetal islet x Thiol groups	-0.51	>0.05
Number of cells per fetal islet x Glutathione peroxidase	-0.27	>0.05
Number of cells per fetal islet x Erythropoietin	-0.27	>0.05

*$p<0.05$ – statistically significant correlation (Pearson´s correlation test).