BRUNA DALLAQUA

ESTUDO DOS MARCADORES DE HIPÓXIA E DE ESTRESSE OXIDATIVO EM DIFERENTES MOMENTOS DA VIDA DE RATAS E EM SEUS DESCENDENTES NO QUADRO DIABÉTICO

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor(a) em Ginecologia, Obstetrícia e Mastologia. Área de conhecimento: Farmácia

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota.”

Madre Tereza de Calcutá

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"Para todas as coisas tenho força em virtude daquele que

me confere poder"

Filipenses 4:13

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"É na educação dos filhos que se revelam as virtudes dos pais"

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"Cultive o hábito da preferência pelas pessoas que são gratas pelo fato dos espinhos conterem rosas, mais do que pelas pessoas que vivem se queixando de que as rosas têm espinhos"

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Artigo

INFLUENCE OF DIABETES ON OXIDATIVE STRESS STATUS IN DIFFERENT LIFE MOMENTS OF RATS AND THEIR OFFSPRING

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Abstract

The purpose of this study was to investigate hypoxia and oxidative stress markers in the blood and liver of diabetic rats and their offspring. Mild diabetes (D) was induced in the newborn rats using streptozotocin-STZ. Non-diabetic newborns (control-C) received the buffer. The newborn were distributed randomly into groups according to their euthanasia (5, 15, 30, 60, or 90 days of life). Female rats (C and D groups) were weighed and lethally anesthetized to obtain blood samples for measurement glucose and insulin levels, and evaluate oxidative stress markers (SOD, catalase, MDA, GSH-Px, and thiol groups) in washed erythrocytes and in the liver. A group of C and D adult rats were mated, and at term pregnancy washed erythrocytes and liver were sampled from the dams and their fetuses to evaluate the same markers before pregnancy. Another group of dams was also assembled to obtain offspring (Days 5 and Day 15 of life) to study same markers. The HIF-1α and PGC-1α gene and protein expression was analyzed in all experimental groups. There was higher glycemia on Days 5, 30, 90 in the life and pregnancy and lower insulin levels on Day 15, 60 and 90 of the diabetic dams. The 5 days old-diabetic rats presented increased SOD, GSH-Px and thiol in washed erythrocytes, and increased MDA concentration in the liver, while at term pregnant animals showed higher MDA concentration, and decreased SOD and catalase activities. The 15 days old offspring from diabetic dams presented higher insulin levels and antioxidant enzymes. These results suggest that glycemic alterations at different moments in the life of rats stimulate an antioxidant defense system in these rats and in their offspring in the first days of life.

Key words: diabetes; oxidative stress; rats; HIF-1α; PGC-1α

Introduction

In Diabetes mellitus, chronic hyperglycemia produces multiple biochemical disorders, and diabetes-induced oxidative stress could play a role in the onset and progression of the disease (20, 37). Oxidative stress in diabetes occurs due to a hyperproduction of reactive oxygen species (ROS), such as O_2^-, OH^-, and H_2O_2, or a deficiency in the antioxidant defense system. The literature shows the association between oxidative stress induced by hyperglycemia, reproduction, and pregnancy repercussions. Thus, human studies that explore responsible mechanisms for these effects 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 an appropriate experimental model (33). Induction of experimental diabetes using a beta (β)-cytotoxic drugs such as streptozotocin (STZ) is well-characterized (66). This antibiotic agent is derived from Streptomyces achromogenes and causes degranulation and necrosis of β-pancreatic cells; i.e., the animal becomes deficient in insulin production. Depending on the animal strain, the dose, the route of drug administration, and the period of life in which STZ is administered in rats, different glucose intensities are found from mild diabetes (glucose between 120 and 200/300 mg/dL) (8, 19, 38, 48, 56, 61) to severe diabetes (blood glucose above 200/300 mg/dL) (16, 9, 13, 17, 51, 63).

Higher levels of reactive oxygen species (ROS) cause oxidative damage in all mitochondrial components disturbing the ATP production. As a result, there is cell death and disruption of calcium homeostasis, leading to oxidative stress. The increased production of ROS in a hyperglycemic environment has been attributed to protein glycation and glucose auto-oxidation (47). Mitochondria are the principal source of ROS in cells as a result of defectively coupled electron transport (34). Mantena et al. demonstrated that type 2 diabetes patients presented disorders in the electron transport chain, overproduction of ROS and lipoperoxide, or a deficiency in antioxidant defense systems (36).

Palmeira et al. verified that hyperglycemia induces adaptations in the transcriptional control of cellular energy metabolism, including oxidative metabolism and inhibition of mitochondrial biogenesis (44, 45), which is defined as the growth and division of pre-existing mitochondria (26). Among the activators of mitochondrial biogenesis, there is a nuclear receptor coactivator, named PGC-1α (Peroxisome proliferator-activated receptor-γ coactivator), which plays a key role in the regulation of cellular and developmental O_2 homeostasis (24). Studies have indicated that PGC-1α serves as a key regulator of mitochondrial biogenesis in mammals (49, 67). It has been shown that PGC-1α-induced

mitochondrial biogenesis causes increased O_2 consumption, leading to a decrease in intracellular O_2 availability, which could characterize hypoxia status.

Hypoxia-inducible factors (HIFs) are among the well-defined transcription proteins that are regulated by the intracellular redox state (21). HIF-1, the major O_2 homeostasis regulator, is a heterodimer composed of two subunits: the oxygen-sensitive HIF-1α and constitutively expressed HIF-1β. Under normoxic conditions, HIF-1α is degraded by the proteasome. Under hypoxic conditions, HIF-hydroxylases are inactive and HIF-1α is stabilized, which allow the formation of a transcriptionally-active heterodimeric protein. This complex can translocate to the nucleus to permit the activation of genes essential for cellular adaptation to low O_2 conditions, including the transcriptional activation of erythropoietin, vascular endothelial growth factor, glucose transporter 1 and glycolytic enzymes (2, 55). These responses enhance O_2 delivery to the cells and facilitate ATP production, which are important for embryonic development (15, 23). However, in the presence of diabetes, hyperglycemia and hypoxia have an important pathophysiological role in diabetic complications due to poor tissue response to low oxygen tension (4, 43).

Considering that diabetes causes hyperglycemia, oxidative stress, and hypoxia, we hypothesized that a combination of these variables is associated with repercussions throughout the life of diabetic rats and their offspring. Thus, the objective of the present study is to evaluate markers related to hyperglycemia, oxidative stress, mitochondria biogenesis, and hypoxia induced by diabetes at different moments in the life of rats and in their offspring.

MATERIALS AND METHOD

Experimental animals

The local Committee of Ethics in Animal Experimentation approved all the experimental protocols used (Process number: 937/2012). 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 experiments. Male and female normoglycemic rats were mated to obtain offspring.

Induction of diabetes

At birth (Day 1), 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 (group D) (56). 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 mild diabetes group; the animals given citrate buffer and presenting glycemia lower than 120 mg/dL were included in the control group.

Experimental groups

The offspring were randomly distributed in different groups according to their euthanasia and the presence (D) or absence (C) of hyperglycemia:

GROUPS	
Control (C)	**Mild Diabetes (D)**
Euthanasia on Day 5 of life (5C)	Euthanasia on Day 5 of life (5D)
Euthanasia on Day 15 of life (15C)	Euthanasia on Day 15 of life (15D)
Euthanasia on Day 30 of life (30C)	Euthanasia on Day 30 of life (30D)
Euthanasia on Day 60 of life (60C)	Euthanasia on Day 60 of life (60D)
Euthanasia on Day 90 of life (90C)	Euthanasia on Day 90 of life (90D)
Euthanasia of dams on Day 21 of pregnancy (at term C)	Euthanasia of dams on Day 21 of pregnancy (at term D)
Euthanasia from fetuses at term (Newborn - NB0_C)	Euthanasia from fetuses at term (Newborn – NB0_D)
Euthanasia from offspring with 5 days of life (Newborn Day 5 - NB5_C)	Euthanasia from offspring with 5 days of life (Newborn Day 5 - NB5_D)
Euthanasia from offspring with 15 days of life (Newborn Day 15 - NB15_C)	Euthanasia from offspring with 15 days of life (Newborn Day 15 - NB15_D)

Animals given citrate buffer or STZ presenting 5 and 15 days of life and offspring with 5 and 15 days of life

The female rats (C and D groups) were weighed and 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 blood glucose and insulin levels, and oxidative stress markers in washed erythrocytes (superoxide dismutase - SOD, glutathione peroxidase - GSH-Px, thiol groups and malonaldehyde - MDA). Liver samples were quickly collected and washed with phosphate buffer saline (0.01 M, pH 7.4) to evaluate markers related to oxidative stress (SOD, GSH-Px, catalase, thiol groups and MDA) and gene (RT-PCR) and protein (Western Blotting) expression of peroxisome proliferator-activated receptor-coactivator (PGC-1α) and hypoxia-induced factor (HIF-1α).

Animals with 30, 60, and 90 days of life

A glucose solution was administered into the stomach of the rats through a gastric catheter at a final dose of 2.0 g/kg body weight for evaluation of oral glucose tolerance test (OGTT). Blood samples were obtained from a cut tail tip for glycemic determination (glucose oxidase) at 0, 15, 30, 60 and 120 minutes (min) after glucose administration (11). Subsequently, the animals were anaesthetized to collect blood and liver samples for measurement of oxidative stress status, hypoxia and mitochondrial biogenesis markers.

Mating period and pregnancy

Adult female rats (C and D) were mated overnight with non-diabetic males. The morning when sperm was found in the vaginal smear was designated as Day 0 of pregnancy. The mating protocol was followed for 15 consecutive days, i.e., approximately three estrous cycles (de Souza et al., 2010). On days 0, 7, 14, and 20 of pregnancy, the dams were weighed and blood samples were collected from the tail for blood glucose determination. On Day 17, the oral glucose tolerance test (OGTT) was performed to confirm the diabetic status of the animals during pregnancy. On Day 21 of pregnancy, all rats were anesthetized with 50 mg sodium thiopental/kg bw. Blood and liver samples were collected from the dams and fetuses to determine the same markers as described above. Other groups of dams (C and D) were assembled to obtain offspring on days 5 and 15 of life to evaluate the same markers.

Laboratory procedures

Blood glucose and insulin levels

Blood samples were collected from the tail and glucose levels were assessed by a One-Touch Ultra glucometer (Johnson & Johnson®). After anesthesia with sodium thiopental, blood samples were collected without anticoagulant, maintained in ice for 30 min and then centrifuged at 263 × g for 10 min at 4°C. The supernatant was collected and stored at −80°C for analysis of insulin levels using Ultra-Sensitive Rat Insulin ELISA Kit (Crystal® Chem Inc., Chicago, Illinois, U.S.A.).

Oral glucose tolerance test (OGTT) test

On Day 17 of pregnancy, the animals were submitted to an oral glucose tolerance test (OGTT).

Oxidative stress markers in erythrocytes washed

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 infranatants were used for determination of SOD and GSH-Px activities, thiol groups (SH), and malonaldehyde (MDA) levels as described by de Souza et al. (13).

Oxidative stress markers in Liver

Liver samples were quickly collected and washed with phosphate buffer saline (0.01 M, pH 7.4) for determination of oxidative stress markers.

Hepatic MDA levels, SOD and GSH-Px activities were determined using commercialized kits (Cayman® Chemical Co., Ann Arbor, Michigan, U.S.A.).

Catalase activity was determined following decreases in the initial H_2O_2 concentration (20 mM used as the initial substrate) at 240 nm and 25°C, over a time frame of 120 seconds (s). Briefly, 1µL of supernatant isolated from rat liver homogenates was placed in a cuvette and diluted to a final volume of 1 mL with phosphate-buffered saline (0.1M, pH 7). The decrease in absorption at 240 nm for 120 s, after adding 21 uL H_2O_2, was observed. CAT activity was calculated using a molar extinction coefficient (ε = 0.0436 mM/cm) and expressed as U/mg protein (1).

Thiol group levels in liver homogenates was evaluated by the method described by Jollow et al. who used a colorimetric technique, which is based on developing a yellow color when DTNB was added to compounds containing sulfydryl groups. The absorbance was read at 412 nm (25).

Protein extraction and Western blotting analysis for HIF-1α and PGC-1α in liver

The frozen liver samples from all groups were mechanically homogenized in 50 mM Tris–HCl buffer pH 7.5, 0.25% Triton X-100 and EDTA using a Polytron homogenizer (Kinematica®, Lucerne, Switzerland) for 30 s at 4°C. Following centrifugation of the homogenate, the protein was extracted from the supernatant and was quantified as described by Bradford (1976). Equal amounts of protein (70 ug) from liver samples were heated at 95°C for 5 min in the sample-loading buffer and were then subjected to SDS–PAGE under reducing conditions and transferred to nitrocellulose membranes (Sigma Chemical® Co., St. Louis, Missouri, U.S.A.). The blots were blocked with 3% bovine serum albumin in TBST (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 hour and probed overnight with the primary antibody anti HIF-1 α (1:800; ab51608; Abcam™) and anti PGC-1α (1:1000; ST1202-1SET; EMD Millipore). Mouse anti-β-actin antibody (1:1,000; sc-81178, Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.) served as loading control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies, the blots were detected with use of chemiluminescence (Amersham ECL Select Western Blotting Detection Reagent, GE Healthcare, UK). HIF-1α, PGC-1α and β-actin protein expression was quantified by densitometric analysis of the bands and was expressed as integrated optical density (IOD). The HIF-1α and PGC-1α expression was normalized to the β-actin values. Normalized data are expressed as the mean ± standard deviation (SD).

RNA extraction and RT-PCR for HIF-1α and PGC-1α in liver tissue

Total RNA from frozen liver samples was extracted using TRIzol® (Invitrogen, São Paulo State, Brazil) according to the manufacturer's instructions. The Kit High-Capacity cDNA Reverse Transcription (Applied Biosystems, Brasil, Cat. Number 4374966) was used for the synthesis of 20 µL complementary DNA (cDNA) from 3000 ng of whole RNA. HIF-1α and PGC-1α levels (assay: nm_024359.1 and nm_031347.1, respectively, Applied Biosystems, Waltham, Massachusetts, U.S.A.) (Chart 1) were determined by RT-PCR. The amplification conditions were as follows: enzyme activation at 50°C for 2 min, denaturation at 95°C for 10 min, cDNA products were amplified by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Gene expression was quantified in relation to their respective control (C) group after normalization by an internal control, beta (β)-actin (nm_031144.2) by the 2-ΔΔCt method, described by Livak and Schmittgen (32).

Chart 1. Primer Sequences for real time-PCR

Primer	Sequence 5' to 3'
HIF-1α	TACACACAGAAATGGCCCAGTGAG
PGC-1α	TGGAACTGCAGGCCTAACTCCTCCC
β-actin	CCACACCCGCCACCAGTTCGCCATG

Statistical analysis

Data were presented as mean ± standard deviation (SD). For data presenting a normal distribution, t test and Tukey´s Multiple Comparison test were used. A Gamma distribution test was performed for data presenting abnormal distribution. $P < 0.05$ was our limit of statistical significance.

RESULTS

Figures 1 and 2 show the blood glucose levels and body weights of the different groups, respectively. It was observed a hyperglycemic status on days 5, 30, and 90 of life in the diabetic rats as compared with the control group (Figure 1A). During the pregnancy period, the diabetic dams presented increased blood glucose levels on days 0 and 7 of pregnancy in relation to those of control dams (Figure 1B). With respect to body weight, the diabetic rats presented retarded weight gain as compared with the control group from day 5 to day 90 of life (Figure 2A). During pregnancy (Figure 2B), no difference was observed. The offspring from diabetic dams presented no difference in blood glucose levels and body weight gain relative to their ages compared with the control rats (Figure 1C and 2C).

On days 15, 60 and 90 of life, the diabetic rats presented lower levels of serum insulin as compared with those of control group (Figure 3A). The offspring of diabetic dams with 15 days of life presented increased insulin levels (Figure 3B).

Figure 4 shows the curves of the oral glucose tolerance test (OGTT) on days 30, 60, and 90 of life and on day 17 of pregnancy in the control and mild diabetic rats. The group 30D presented higher glycemia on time points 0, 15 and 30 min after an overloaded glucose administration as compared with those of the control group. The group 60D presented higher glycemia on all estimated time points and the group 90D presented altered glycemia on 15, 30 and 60 min. On day 17 of pregnancy, the diabetic dams presented increased glycemia on 0, 15, 30 and 60 minutes from the OGTT in relation to those of the control rats.

The superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities as well as thiol group concentration (SH) in washed erythrocytes were increased in diabetic rats on day 5 of life as

compared with their respective controls, and the MDA concentration during early life showed no difference related to the control group. However, the MDA levels of diabetic dams at term pregnancy were increased as compared with MDA levels of the control dams (Figure 5).

In liver, the glutathione peroxidase (GSH-Px) activity and thiol group concentration (SH) through the life of mild diabetic rats presented no difference from control group. It was observed a higher MDA concentration on day 5 of life. On day 30 of life, there was increased catalase activity, and at term pregnancy, it was verified lower SOD and catalase activities in the mild diabetic dams as compared with those of control rats (Figure 6)

No difference was observed in HIF-1α and PGC-1α gene (Figure 7) and protein (Figure 8) expression in liver samples of control and mild diabetic rats on different moments of life.

At birth, the newborns from D dams presented decreased thiol group levels in washed erythrocytes samples and on day 5 of life, the newborns presented increased thiol group concentrations as compared with the control group (Figure 9).

In liver samples, the offspring from mild diabetic dams with 15 days of life presented increased SOD and GSH-Px activities and increased thiol group levels. On days 5 and 15 of life, it was observed decreased MDA concentration and increased catalase activity in offspring of dams D as compared with those of control rats (Figure 10).

In Figures 11 and 12, no difference was verified in HIF-1α and PGC-1α gene and protein expression of liver sampled of offspring from mild diabetic dams compared with those taken from control rats.

DISCUSSION

In the present study, it was observed that the administration of streptozotocin (STZ) at birth caused beta (β)-pancreatic cells damage in diabetic rats, confirmed by hyperglycemia. These rats presented lower insulin levels, changes in oral glucose toletance test (OGTT) and a high rate of glycosylated hemoglobin (HbA1c) (data not shown). Scaglia et al. showed that during neonatal period the pancreas undergoes physiological changes (52), and events can also be identified in other organs, such as liver, kidneys, and central nervous system (6, 42, 60). This pancreatic remodeling is due to increased replication and apoptosis rates of β-cells between day 13 and 17. These data show that in physiological conditions, the organism has a dynamic β-cell mass, maintaining glucose homeostasis (31). In contrast to our study that performed the diabetes induction at birth, Bonner-Weir et al. administered

STZ (90 mg/bw intraperitoneally) on day 2 of life in mice. On Day 4 of life, the animals presented hyperglycemia and reduced numbers of β-cells. On postnatal day 10, the animals became euglycemic, and partial regeneration of pancreatic β-cells was evident. After 6 weeks, hyperglycemia returned and decreased β-cell volume was observed, therefore suggesting that these cells are able to partially regenerate (3). This study confirm our results, which showed that rats given STZ showed hyperglycemia on day 5 of life through destruction of β-cells by the action of β-cytotoxic drug. On 30 and 90 days of life, we also found increased levels of blood glucose, suggesting a failure in the regeneration of β-pancreatic cells. In addition, we observed in our study that the rats with mild diabetes presented low serum insulin levels on days 15, 60, and 90 of life, and this could also be explained by changes in the regeneration of these cells. Consequently, a decreased insulin levels lead to weight reduction in diabetic animals throughout life, since insulin is a peptide hormone with a high degree of homology with IGF-1 (insulin growth factor 1).

An increased glucose levels were observed on days 0 and 7 of pregnancy, these results partly corroborate previous studies performed in our laboratory, which showed increased blood glucose levels on days 0 and 14 of pregnancy (57, 58). Similarly Desoye and Mouzon verified that pregnant woman with gestational diabetes present hyperglycemia from 24 to 26 weeks of pregnancy, which is equivalent to day 14 in pregnant rats (14). However, when an overload of glucose was administered to these rats, there was a pancreatic stimulus for synthesis and secretion of insulin. Despite peaks of hyperglycemia on days 0 and 7 of pregnancy associated with glucose intolerance in the diabetic rats, we suggest measuring glucose in isolation is not a good marker for these assessments. Even with an unfavorable intrauterine environment in the presence of glucose intolerance, it was verified no influence on blood glucose levels and weight of the offspring from diabetic dams. The offspring from diabetic dams with 15 days of life presented hyperinsulinemia. These finding suggests that the intrauterine environment hyperstimulates fetal β-pancreatic cells, leading to an increase in the insulin synthesis and secretion (52) and subsequently a normalization of blood glucose level.

Yadav et al. showed that erythrocytes are vulnerable to oxidative stress due to high concentration of polyunsaturated fatty acids, ferrous ions, and molecular oxygen (68). The first line of defense against oxidative damage is the enzymatic antioxidants: superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) (10, 22). In this study, we found that five days after β-cytotoxic drug administration, the animals presented hyperglycemia, causing the mobilization of antioxidant enzymes that were able to control oxidative stress. Evidence for this result is found in unchanged MDA

levels in the neonatal period up to adulthood. Studies showing the antioxidant status in the neonatal period of animals that received β-cytotoxic drug are not found in the literature. However, Tupe et al. found that type 2 diabetes adult patients showed higher oxidative damage, and reduced GSH level in erythrocytes (62).

In the pregnancy period, diabetic rats presented oxidative stress. A study performed by Lappas et al. showed that pregnancy increases the metabolic activity of placental mitochondria, leading to the ROS generation, as well as changes in antioxidant activity (29). During diabetic pregnancy, researchers have observed increased levels of lipoperoxidation (40) and an excess amount of superoxide is produced in response to hyperglycemia (41). Corroborating the findings, Dallaqua et al. and Spada et al. verified that mild diabetic dams presented lipoperoxidation, confirmed by increased MDA levels in washed erythrocytes (7, 58). In the present study, when the same markers were analyzed in liver, it was demonstrated no mobilization in antioxidant enzymes in diabetic rats on day 5 of life contrary to results obtained in red blood cells. These results suggest that, in this period, the animals present an immaturity in the antioxidant defense system, or deficiency in this defense mechanism, leading to oxidative stress and confirmed by increased MDA levels. Ferreira et al. observed metabolic changes in hepatic mitochondria in animals with streptozotocin-induced diabetes and in Goto-Kakisaki rats (spontaneously diabetic (18). These authors noted alterations were age-dependent, indicating the occurrence of metabolic adaptations to hyperglycemia. When mitochondria were isolated from livers of diabetic rats at 9 weeks, Palmeira et al. observed decreased activity of respiratory chain, and those isolated from animals at 6 months showed increased respiratory control (45). This investigation shows that the "age" factor interferes in respiratory activity. Such early adaptive responses of mitochondrial function to diabetic stress may reflect changes in gene expression induced by hyperglycemia as a mechanism for cell adaptation to glucose toxicity (50). In our study, when offspring from diabetic dams was assessed, there was observed an increased catalase activity and thiol group concentration on day 15 postnatal. These findings corroborate Makni et al. who induced mild diabetes on day 2 of pregnancy with alloxan (120 mg/bw) and found that mothers showed low GSH, catalase, and SOD activity and increased MDA concentrations, leading to oxidative stress in the liver, while their offspring with 16 days of life showed the same results of maternal antioxidant enzymes (35). Therefore, we suggest that, in our model of mild diabetes induction, the liver showed more sensitivity to hyperglycemic insults compared to washed erythrocytes or other explanation might be a slowness of the response to oxidative insults.

The literature shows that with the aging process, diabetic status causes mitochondrial dysfunction and the accumulation of oxidative damage, such as, damage to DNA, lipids, and proteins

may be partly responsible for mitochondrial dysfunction (27). Mitochondria are the principal energy sources of the cell that convert nutrients into energy through cellular respiration (65). In addition, the mitochondria organelle suffers most from oxidative insults. At the molecular level, the oxidative dysfunction displayed in type 2 Diabetes mellitus subjects may be explained by coordinated decrease in the expression of genes involved in lipid oxidation and mitochondrial metabolism (46). PGC-1α is mainly expressed in tissues (heart, skeletal muscle, liver, brown adipose tissue, and brain) with high-energy oxidative capacity (39, 49). Studies show that an increased expression of PGC-1α in beta cells and the liver plays a role in type 2 diabetes and is supported by experiments showing reversal of diabetes and hepatic steatosis in mice fed a saturated fat-rich diet (12). However, hepatocytes isolated from PGC-1α–deficient mice exhibit diminished mitochondrial respiration rates (30), providing one mechanistic explanation for the reduced capacity to oxidize hepatic fatty acids. However, in this study, mild diabetic rats showed no change in PGC-1α gene and protein expression through their lives; neither did their offspring. In accordance with the different results found in the literature, we observed that the marker chosen for mitochondrial biogenesis analysis was not suitable, since the glycemic intensity was not able to cause the activation of this co-activator.

Ornoy et al. demonstrated that hyperglycemia induces hypoxia (43). As a result, HIF-1α was chosen for analysis in our study. HIF-1α is a transcription factor that promotes the expression of genes involved in glucose uptake and anaerobic respiration (54). Numerous factors have been shown to stabilize HIF-1α. However, mitochondrial-derived ROS and succinate are key modulators of this hypoxic signal (53). Aging-dependent defects in cell-to-cell communication or fluid circulation from the blood to the cell might lead to hepatic oxygen diffusion and vice versa (5). In this study, it was observed no change in the expression of this transcription factor in the diabetic rats and their offspring. However, However, Kang et al. analyzed the HIF-1α expression in the liver of male normoglycemic with 6, 12, 18 and 24 months of life and showed that livers were able to up-regulation HIF-1 activity (28). Apart from hypoxia, glucose also affects the HIF-1α expression and activation (59). In contrast, HIF-1α regulates the expression of enzymes involved in the process of glycolysis, GLUT-1 and GLUT-3, which mediate cellular uptake and glycolysis (64). We expected to detect a change in HIF-1α and PGC-1α gene and protein expression after administering beta citotoxic drug, but this model was not able to cause changes in these markers. Thus, the markers chosen to study hypoxia and mitochondrial biogenesis were not adequate for the proposed model.

CONCLUSION

The model proposed in this study for diabetes induction during the neonatal period caused abnormal glucose and oxidative stress throughout the lives of diabetic rats. The antioxidant status in washed erythrocytes was more efficient to control oxidative damage than the liver. However, the liver of offspring from diabetic dams was more responsive to oxidative damage due to the increased activity of antioxidant enzymes, which resulted in the decreased lipoperoxidation. Therefore, our results demonstrated that glycemic alterations in different moments in the life generate an stimulus to antioxidant defense in these rats and interfere in maternal intrauterine environment leading to consequences in their offspring in the first days of life.

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Disclosure

All authors declare that there is no duality of interest associated with their contribution to this article.

Author contribution

Author contributions: B.D., Y.K.S., F.Q.G., and I.L.I. performed experiments; B.D., and D.C.D. drafted manuscript and analyzed data; B.D., D.C.D., and T.R. analyzed oxidative stress data; J.R., F.D., and S.F. performed and analyzed protein expression data; M.V.C.R. read the manuscript.

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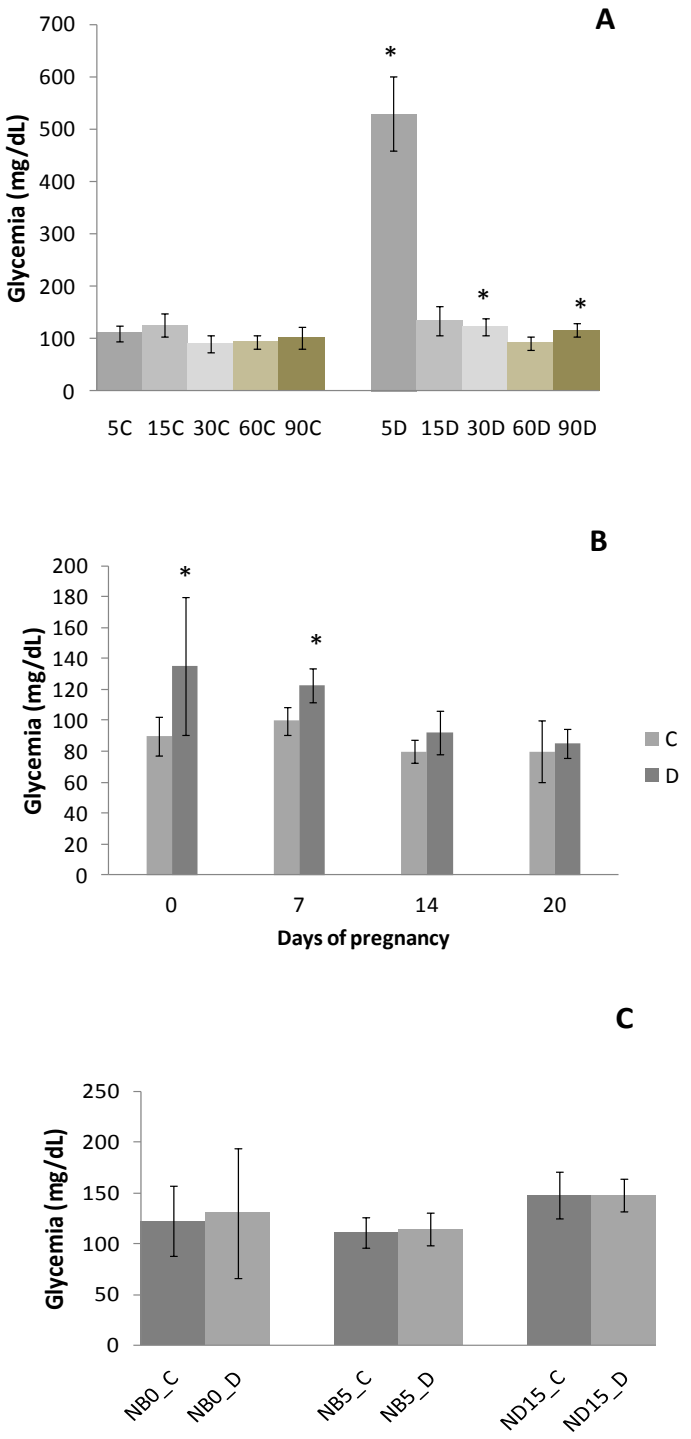

Figure 1. Blood glucose levels from control (C, n=12) and mild diabetic (D, n=12) rats in different life moments and in their offspring.

Values are expressed as mean ± standard deviation (SD).

*p<0.05-Significantly different from control group (Gamma distribution)

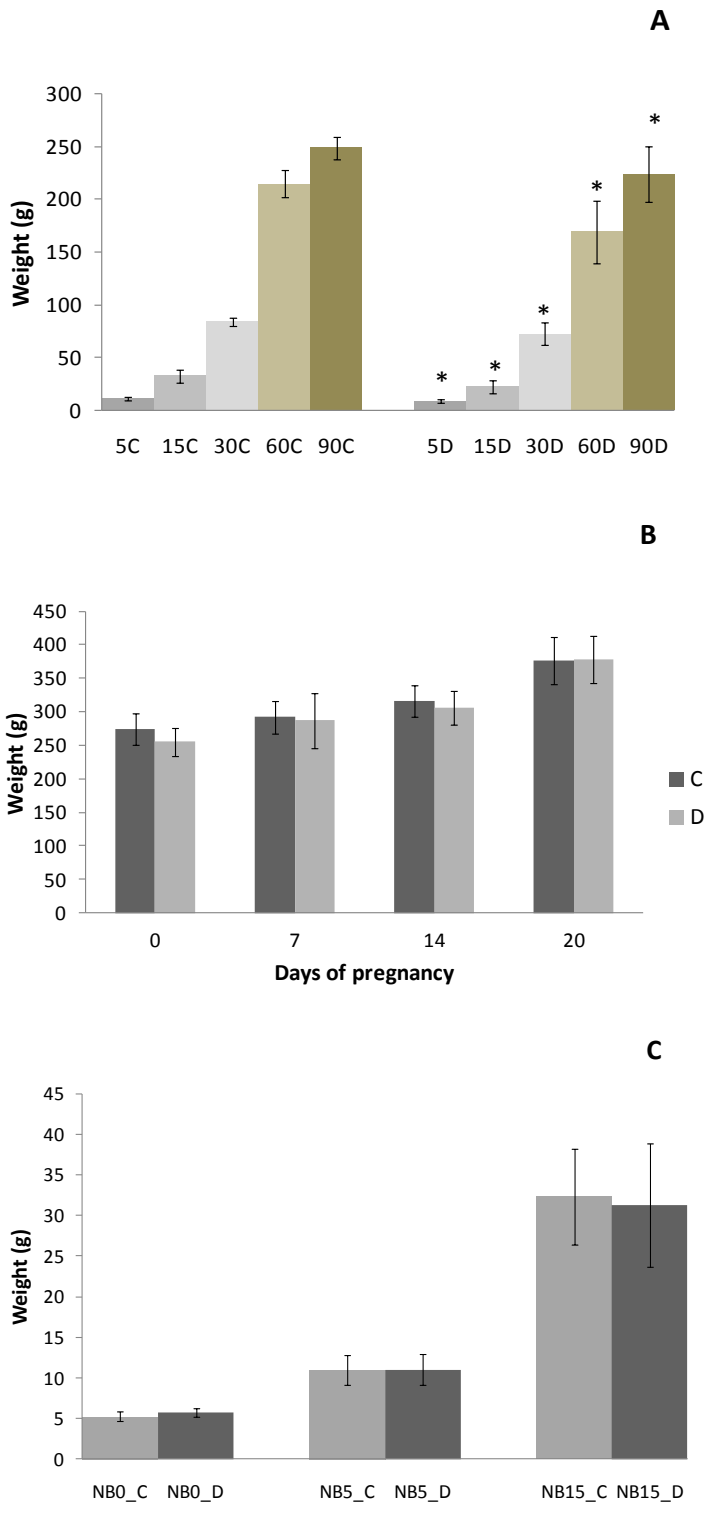

Figure 2. Body weight from control (C, n=12) and mild diabetic (D, n=12) rats in different life moments and in their offspring. Values are expressed as mean ± standard deviation (SD).

*p<0.05-Significantly different from control group (Gamma distribution)

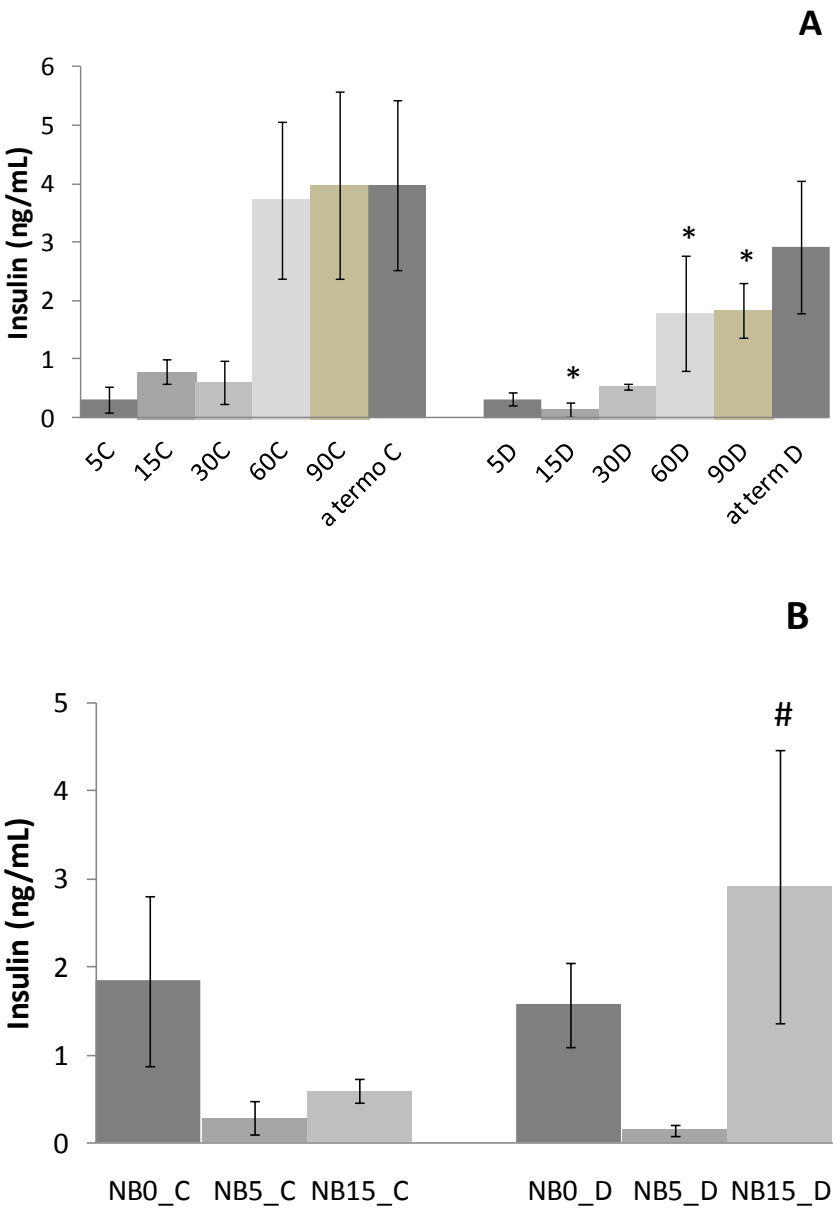

Figure 3. Insulin levels from control (C, n=5) and mild diabetic (D, n=5) rats in different life moments and in their offspring.

Values are expressed as mean ± Standard deviation (SD)

*p<0.05-Significantly different from control group (Gamma distribution)

#p<0.05-Significantly different from control group (t Test)

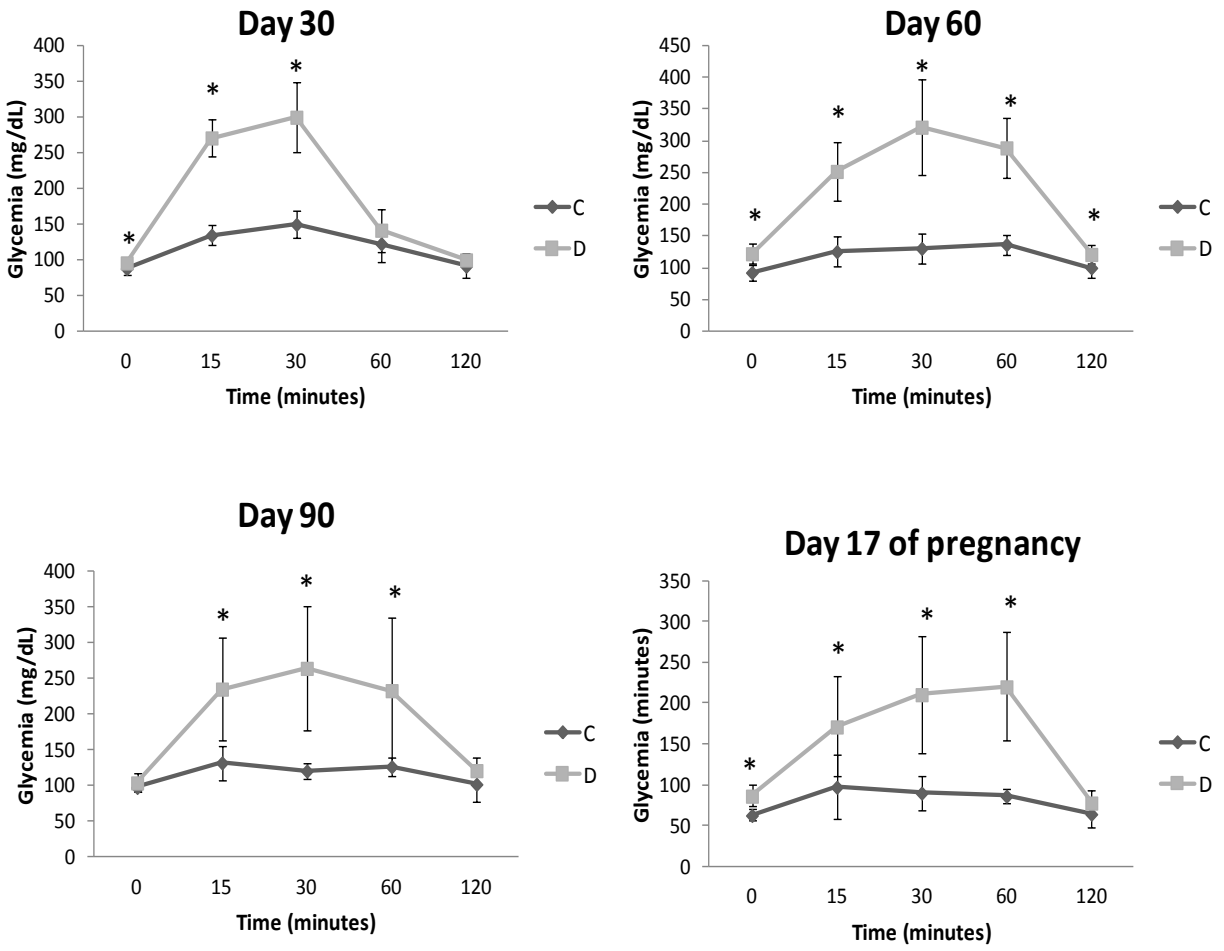

Figure 4. Oral glucose tolerance test (OGTT) in different moments of life from control (C, n=10) and mild diabetic (D, n=10) groups.

Values are expressed as mean ± standard deviation (SD).

*p<0.05 - Significantly different from control group (Gamma distribution test)

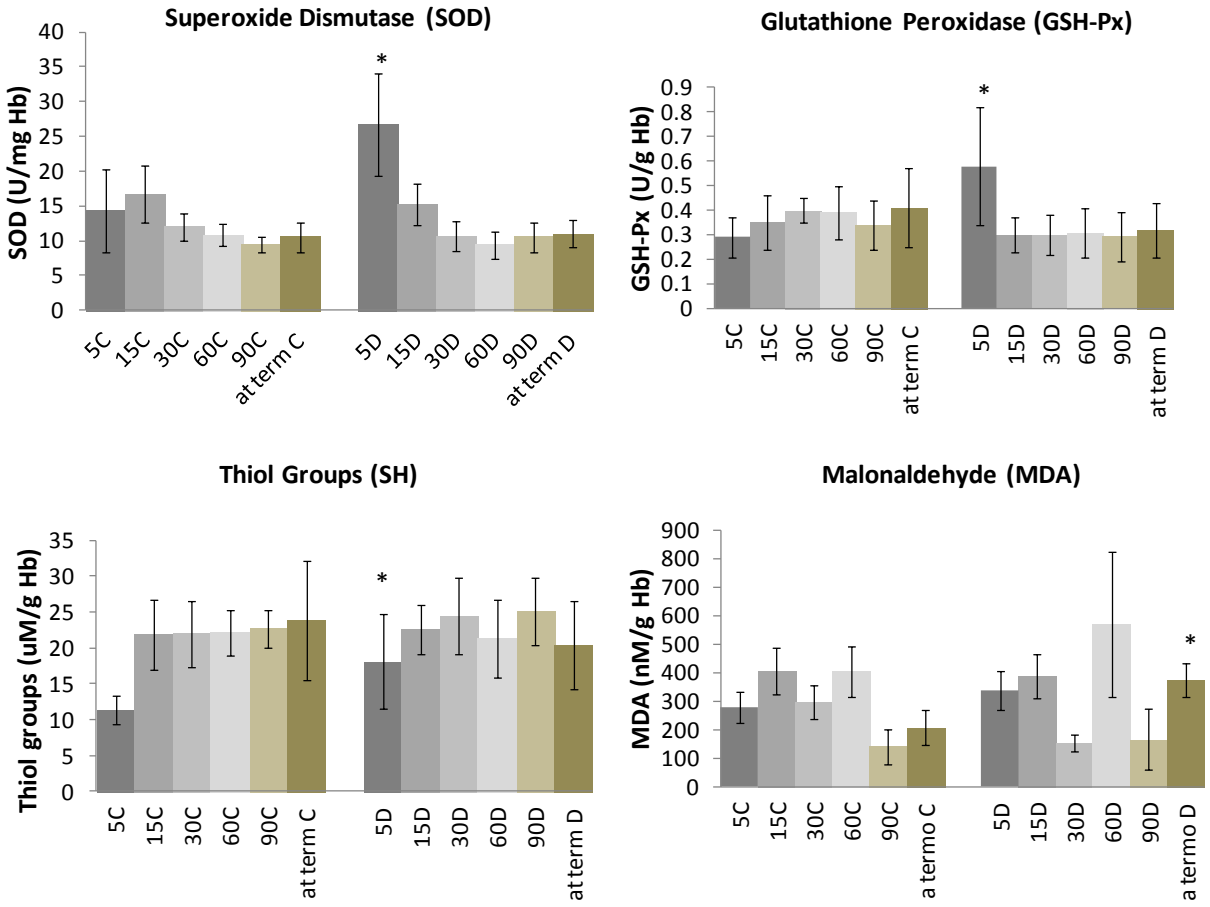

Figure 5: Oxidative stress profile of washed erythrocytes sampled from control (C, n=10) and mild diabetic (D, n=10) rats in different moments of life.

Values are expressed as mean ± standard deviation (SD).

*p<0.05 - Significantly different from control group (Gamma distribution test for SOD and MDA; Tukey's Multiple comparison test for GSH-Px and Thiol groups).

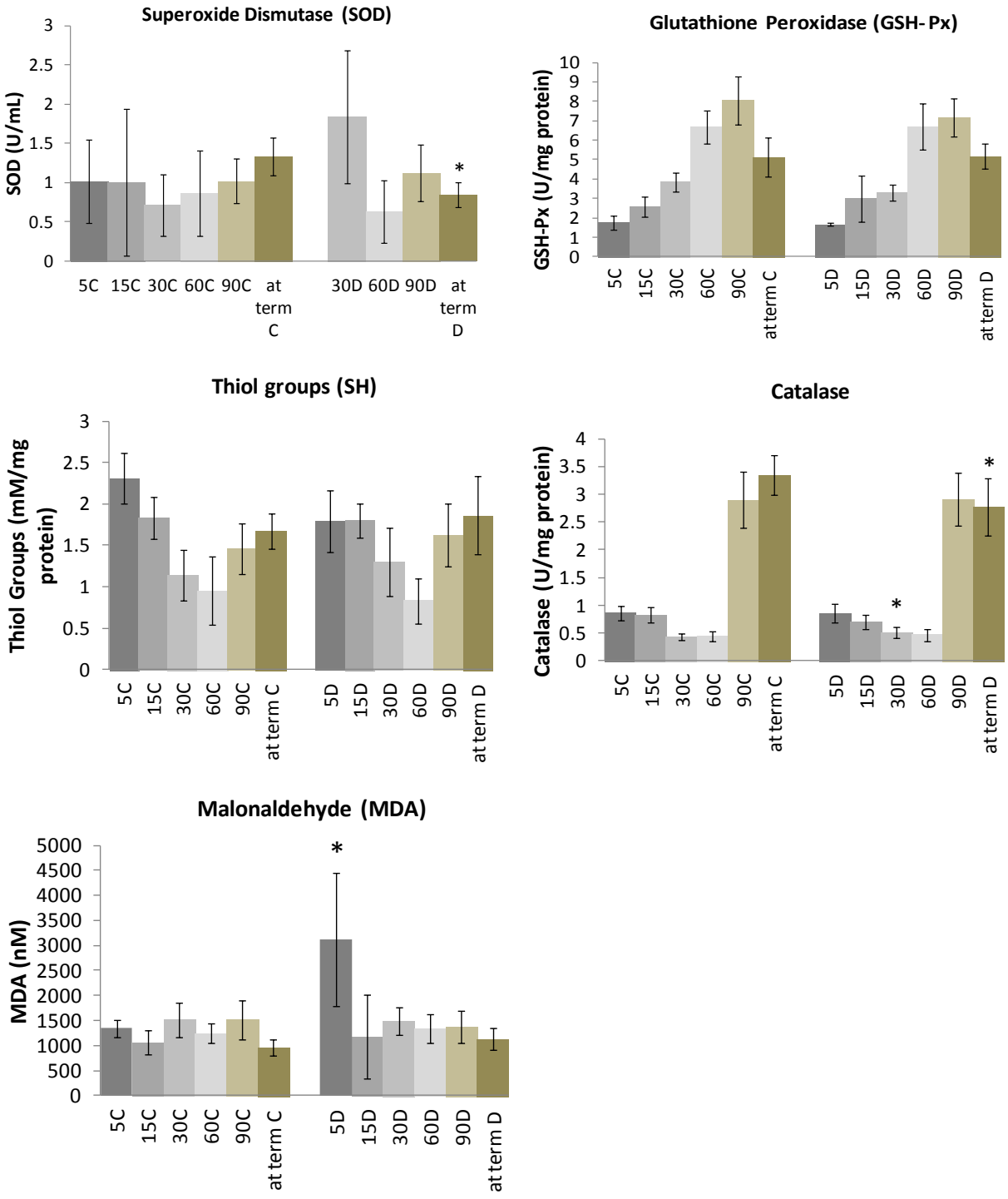

Figure 6. Oxidative stress profile of liver sampled from control (C n=8) and mild diabetic (D n=8) rats in different moments of life. Values are expressed as mean ± standard deviation (SD).

*p<0.05 - - Significantly different from control group (Gamma distribution test for catalase and MDA; Tukey's Multiple comparison test for GSH-Px, SOD and Thiol groups).

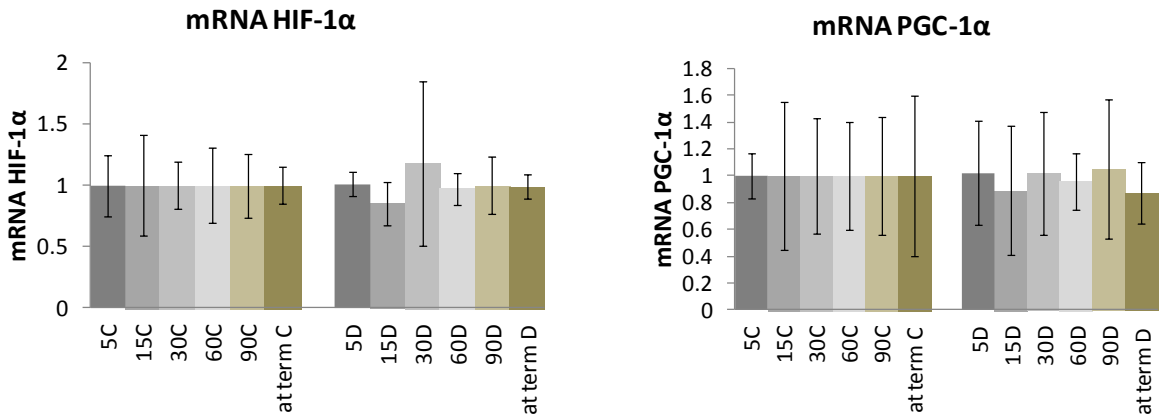

Figura 7. Gene expression of HIF-1α and PGC-1α of liver sampled from control (C n=6) and mild diabetic (D n=6) rats in different moments of life.

Values are expressed as mean ± standard deviation (SD).

p>0.05 – no significant statistically difference from control group.

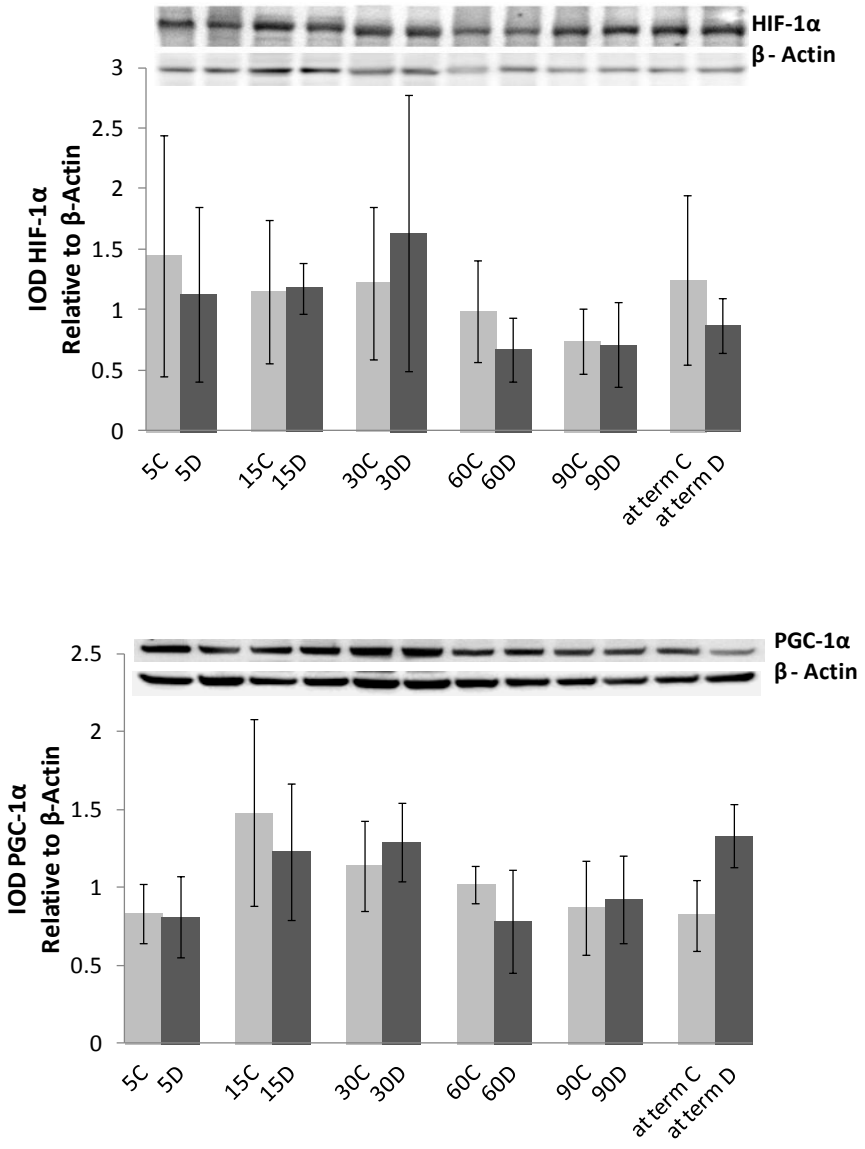

Figure 8. Protein expression of HIF-1α and PGC-1α of liver sampled from control (C n=6) and mild diabetic (D n=6) rats in different moments of life.

Values are expressed as mean ± standard deviation (SD).

p>0.05 – no significant statistically difference from control group.

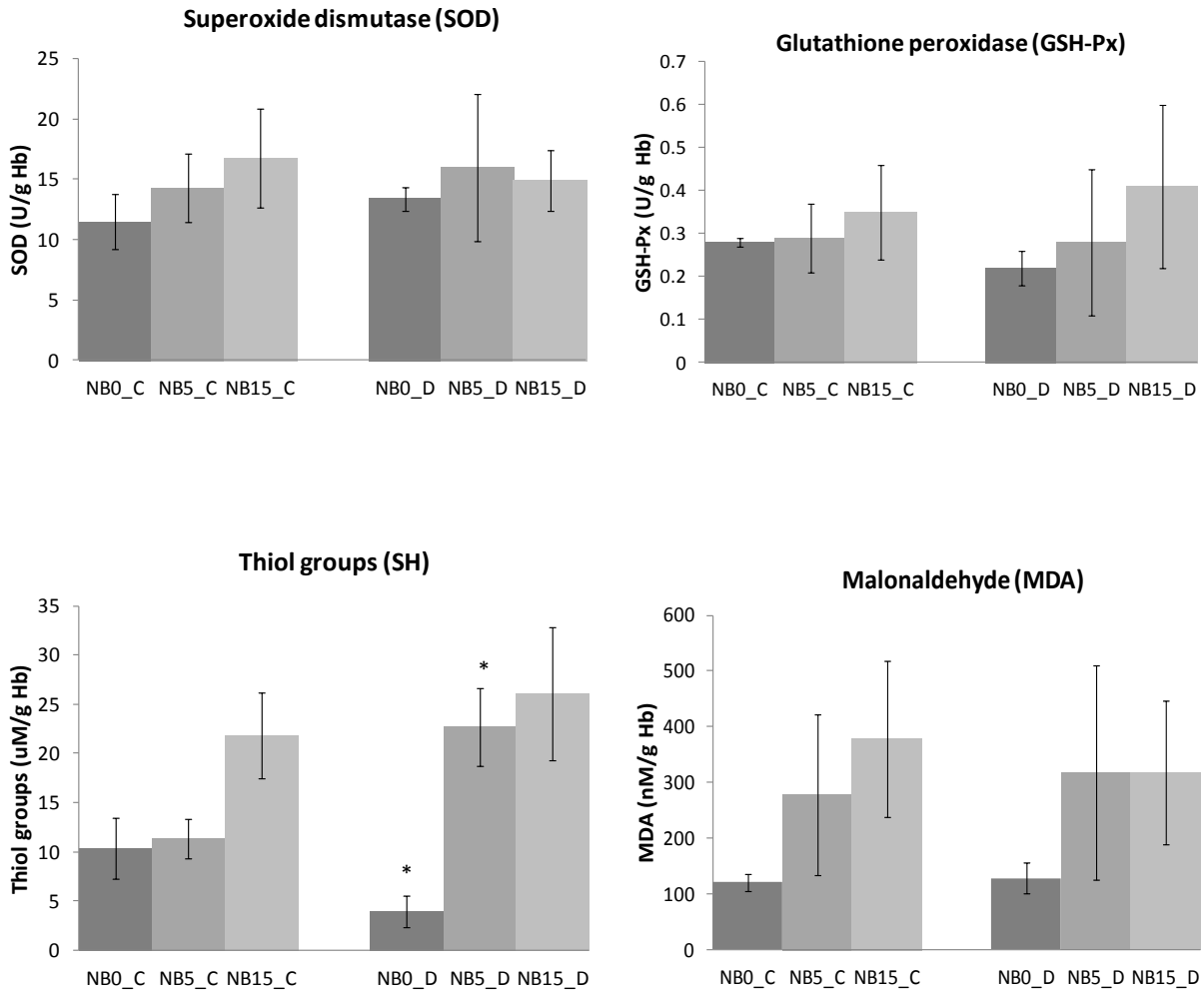

Figure 9. Oxidative stress profile of washed erythrocytes sampled of offspring in different moments of life from control (C n=12) and mild diabetic (D n=12) rats.

Values are expressed as mean ± standard deviation (SD).

*p<0.05 - Significantly different from control group (t test).

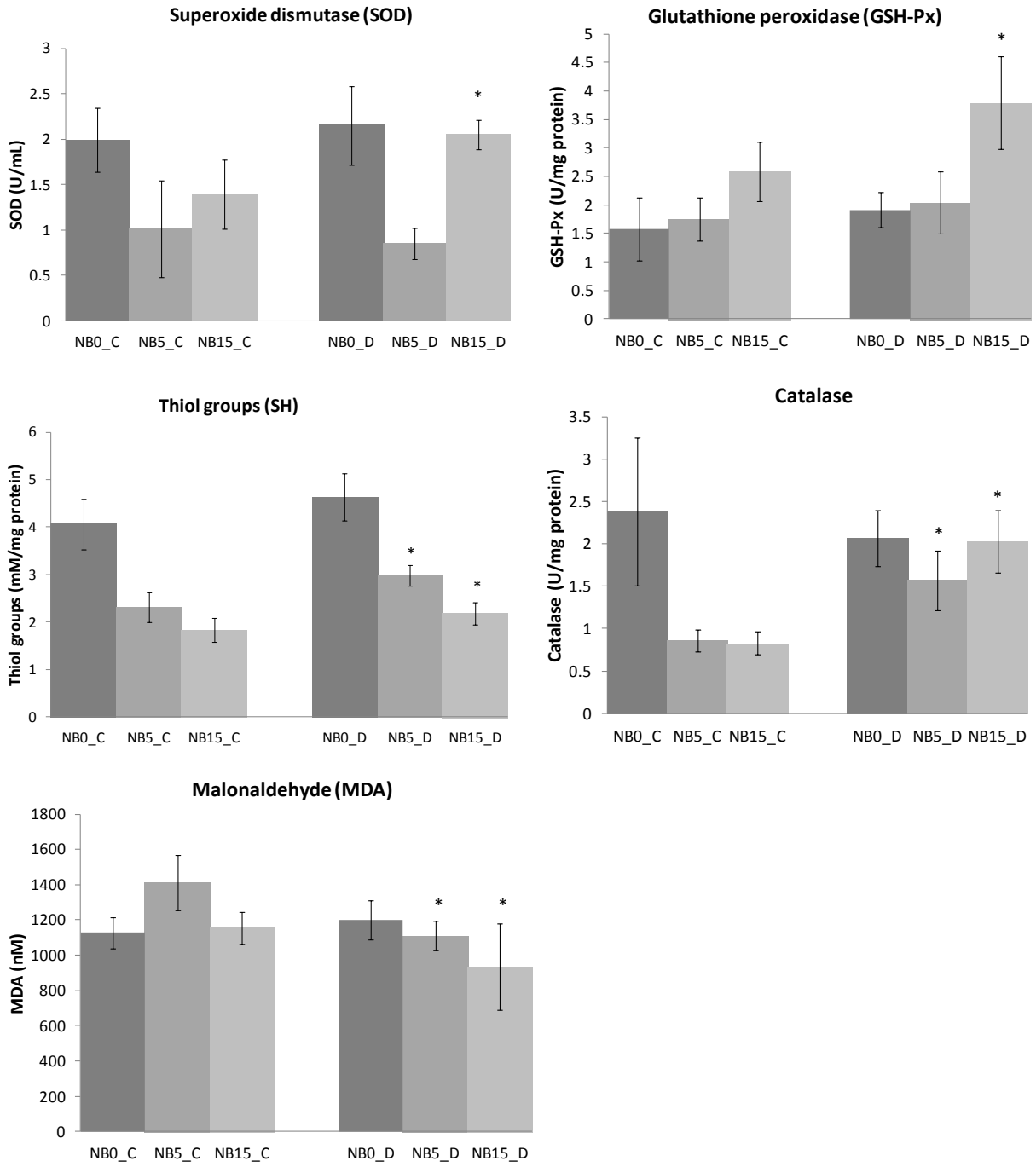

Figure 10. Oxidative stress profile of liver sampled of offspring in different moments of life from control (C n=8) and mild diabetic (D n=8) rats.

Values are expressed as mean ± standard deviation (SD).

*p<0.05 - Significantly different from control group (t test).

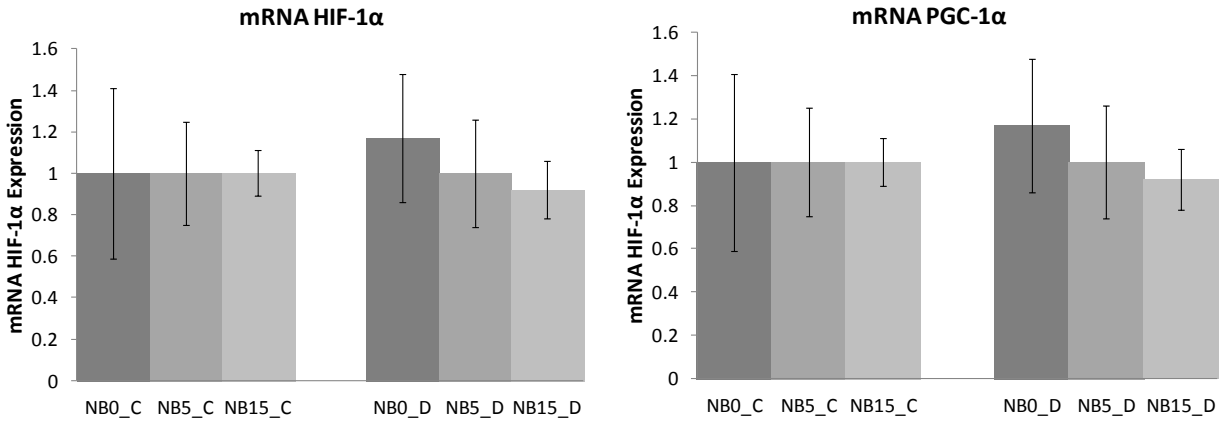

Figure 11. Gene expression of HIF-1α and PGC-1α in liver sampled from offspring from control (C n=6) and mild diabetic (D n=6) rats.

Values are expressed as mean ± standard deviation (SD).

p>0.05 – no significant statistically difference from control group.

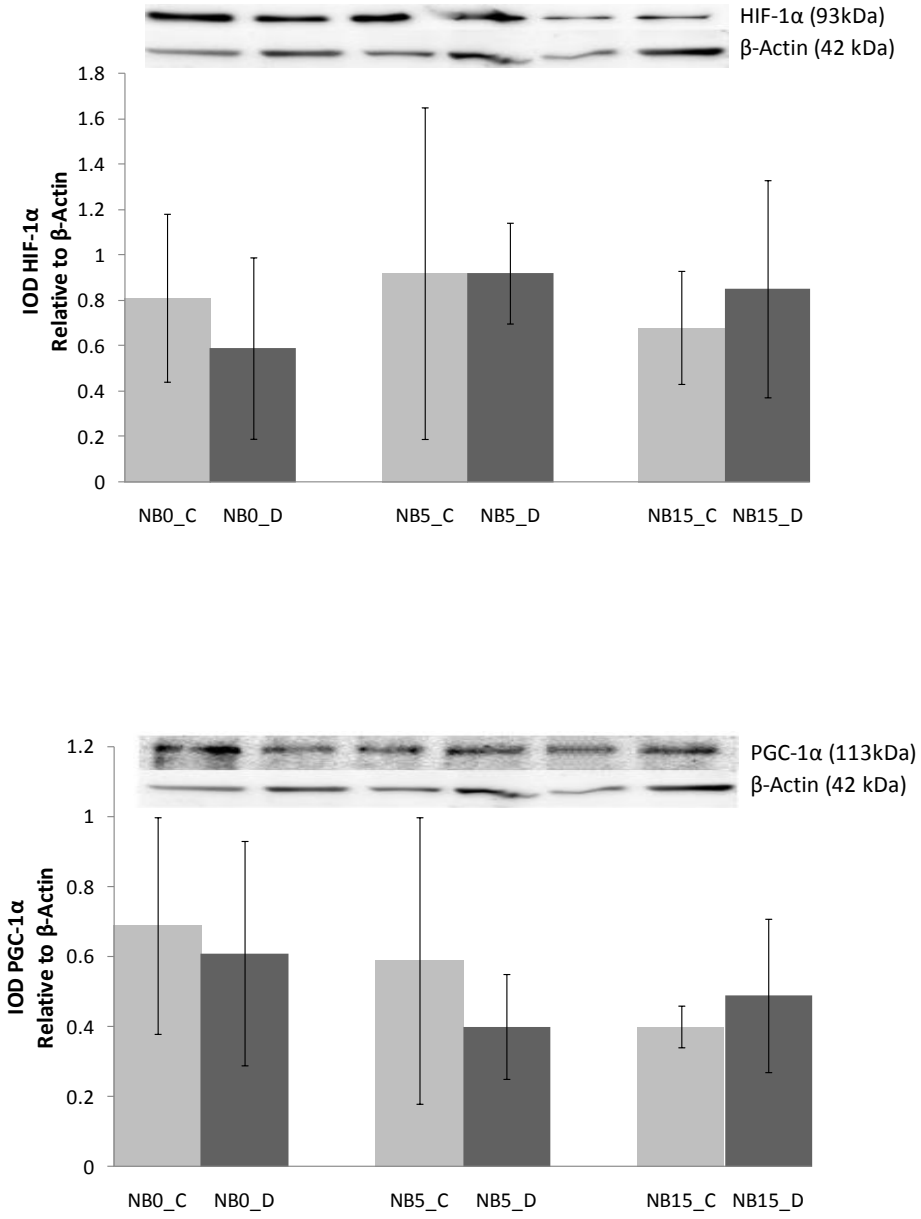

Figure 12. Protein expression of HIF-1α and PGC-1α in liver sampled from offspring from control (C n=6) and mild diabetic (D n=6) rats.

Values are expressed as mean ± standard deviation (SD).

p>0.05 – no significant statistically difference from control group.

Anexos

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

unesp

FMB

CEEA Ética

Comissão de Ética em Experimentação Animal

Criada através da Portaria DFM nº 30 de 26/04/99

Certificado

Certificamos que o **(Protocolo CEEA 937/2012 "Estudo dos marcadores de hipoxia e de estresse oxidativo em diferentes momentos da vida de ratas e em seus descendentes no quadro diabético"**, a ser conduzido por **Bruna Dallaqua**, orientada pela Profª. Drª. Débora Cristina Damasceno, com a colaboração do Prof. Titular Antonio Carlos Cicogna, Profª Drª Célia Regina Nogueira, Emilio Herrera, Franciane Quintanilha Gallego, Iracema de Mattos Paranhos Calderon, Isabela Lovizutto Iesse, Profª Titular Marilza Vieira Cunha Rudge, Rebeca Germano Serrano, Tiago Rodrigues, Yuri Karen Sinzato, com Apoio Técnico de Dijon Henrique Salomé de Campos, Loreta Casquel de Tomasi, Maria Teresa de Sibio e Sandro José Conde , está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os "ratos" são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEEA em 26/04/2012.

Profª Drª Maria Rosa Bet Moraes Silva
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

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Anexo 1

Anexo 2

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Bruna Dallaqua <brunadallaqua@gmail.com>

E-00004-2015 Manuscript Received

1 mensagem

edwyer@the-aps.org <edwyer@the-aps.org> 4 de janeiro de 2015 21:33
Responder a: edwyer@the-aps.org
Para: brunadallaqua@gmail.com

Dear Ms. Dallaqua:

On 4th Jan 2015, the manuscript record, E-00004-2015, entitled "INFLUENCE OF DIABETES ON OXIDATIVE STRESS STATUS IN DIFFERENT LIFE MOMENTS OF RATS AND THEIR OFFSPRING" by Bruna Dallaqua, Yuri Sinzato, Franciane Gallego, Isabela Iessi, Tiago Rodrigues, Jaqueline Rinaldi, Flavia Delela, Sergio Felisbino, Marilza Rudge, and débora Damasceno has been sent to the Editor-in Chief for assignment.

The manuscript type is Research Article.

If any author has not seen this work, or was not aware the submission, please contact Ed Dwyer in the APS office, edwyer@the-aps.org.

Thank you for submitting to the journal.

Editorial Staff