

## **Influence of maternal hyperglycemia on rat daughters and granddaughters**

**Influência da hiperglicemia materna nas filhas e netas de ratas**

**Influencia de la hiperglucemia materna en hijas y nietas de ratas**

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### **Abstract**

This study evaluated the influence of maternal diabetes on the reproductive outcomes of adult daughters during full-term pregnancy in rats. The local ethics committee approved all experimental protocols. The adult female pups of nondiabetic (control – CP) and diabetic (DP) mothers were subjected to mating with healthy male rats for reproductive analysis. On day 17 of pregnancy, both groups underwent an oral glucose tolerance test (OGTT) to evaluate glucose tolerance. At the end of pregnancy, the rats were anesthetized for laparotomy and exposure of maternal organs. Uterine horns, ovaries, fetuses, and placentas were weighed to classify fetal weight and placental efficiency.  $P < 0.05$  was considered a statistically significant limit, and Pearson's correlation was used. Higher maternal fasting blood glucose was correlated with a lower number of live fetuses and, consequently, with a lower litter weight. Thus, maternal fasting glucose during pregnancy is a relevant biomarker related to the poor reproductive outcome of daughters of rats. Furthermore, these findings show the detrimental effects of fetal programming induced by maternal diabetes in adulthood in the subsequent generation.

**Keywords:** Diabetes; Rats; Reproductive outcomes; Fetus; Pregnancy.

### **Resumo**

Este estudo teve como objetivo avaliar a influência do diabetes materno no desempenho reprodutivo de suas filhas adultas na prenhez a termo em ratas. O Comitê de ética local aprovou todos os protocolos do estudo. A prole feminina adulta de mães não diabéticas (controle – FC) e diabetes moderada (FD) foram submetidas a acasalamento com machos saudáveis para análise dos resultados reprodutivos. No 17º dia de gestação, ambos os grupos foram submetidos a um teste oral de tolerância à glicose (TOTG) para avaliação da tolerância à glicose e, ao final de prenhez, as ratas foram anestesiadas para laparotomia e exposição dos órgãos maternos. Os cornos uterinos, ovários, fetos e placentas foram pesados para classificação do peso fetal e eficiência placentária.  $P < 0,05$  foi considerado o limite estatisticamente significativo, e a correlação de Pearson foi usada. A maior glicemia materna em jejum correlacionou-se com o menor número de fetos vivos e, conseqüentemente, com menor peso da ninhada. Assim, a glicemia de jejum materna durante a gravidez é um biomarcador relevante relacionado ao comprometimento do resultado reprodutivo de filhas de ratas.

Além disso, esses achados mostram os efeitos prejudiciais da programação fetal induzida pelo diabetes materno na idade adulta da geração sucessiva.

**Palavras-chave:** Diabetes; Ratos; Resultados reprodutivos; Feto; Gravidez.

### Resumen

Este estudio tuvo como objetivo evaluar la influencia de la diabetes materna en el desempeño reproductivo de sus hijas adultas durante el embarazo a término en ratas. El comité de ética local aprobó todos los protocolos del estudio. Las crías hembras adultas de madres no diabéticas (control - HC) y moderadamente diabéticas (HD) se sometieron a apareamiento con machos sanos para el análisis de los resultados reproductivos. El día 17 del embarazo, ambos grupos se sometieron a una prueba de tolerancia a la glucosa oral (OGTT) para evaluar la tolerancia a la glucosa y, al final del embarazo, las ratas fueron anestesiadas para laparotomía y exposición de los órganos maternos. Se pesaron los cuernos uterinos, los ovarios, los fetos y las placentas para clasificar el peso fetal y la eficiencia placentaria.  $P < 0,05$  fue considerado como el límite estadísticamente significativo y se utilizó la correlación de Pearson. Una mayor glucemia materna en ayunas se correlacionó con un menor número de fetos vivos y, en consecuencia, con un menor peso de la camada. Por lo tanto, la glucosa materna en ayunas durante el embarazo es un biomarcador relevante relacionado con el deterioro del resultado reproductivo de las hijas de ratas. Además, estos hallazgos muestran los efectos perjudiciales de la programación fetal inducida por la diabetes materna en la edad adulta en la generación siguiente.

**Palabras clave:** Diabetes; Ratas; Resultados reproductivos; Feto; Embarazo.

## 1. Introduction

Diabetes mellitus (DM) is a syndrome characterized by a disturbance in insulin secretion/action. It can be classified into three main classes: Type 1 DM – is referred to as an autoimmune disease. In this DM, the pancreatic beta cells, which synthesize the hormone insulin, are destroyed by the body's lymphocytes; Type 2 DM – is referred to as exacerbated resistance to the action of insulin in peripheral tissues, and gestational DM (GDM), which appears around the week 24 of gestation with the establishment of glucose intolerance followed by diabetes (American Diabetes Association 2022). In 2021, the prevalence of diabetes between 20 and 79 years old was 10.5%, with approximately 537 million people. However, by 2045, there is a forecast for an increase to 783 million (Sun et al. 2022). Therefore, it is essential to study diabetes and its repercussions.

During pregnancy, diabetes-induced hyperglycemia alters embryonic implantation (Paula et al. 2022) and embryofetal development, compromising fetal viability (Bueno et al. 2020). Several studies have been performed to study the health-disease process in the offspring of mothers who have illnesses before and/or during pregnancy (DOHaD, 2023). When Barker (2007) evaluated the adult offspring of women who lived during the Dutch famine in World War II, he found cardiovascular problems in these individuals related to the fact that they had experienced intrauterine growth restriction. In this way, it becomes increasingly essential to describe the repercussions of the maternal intrauterine environment with the embryo-fetal, perinatal, and adult development to understand and ensure the health process of the offspring.

Therefore, it is essential to develop experimental models to induce mild diabetes. Damasceno et al. (2013) verified that, according to the dose of the beta-cytotoxic drug used, administration route, and induction period, it could modify the glycemic level. A mild hyperglycemic intensity causes changes in the numbers of implantations, live fetuses, fetal viability, birth weight, and morphological changes in the pancreas of newborns (Damasceno et al. 2011; Bueno et al. 2020; Paula et al. 2022; Sinzato et al. 2022). Furthermore, these offspring had higher glycemic intensity, confirming glucose intolerance and insulin resistance in adulthood (Paula et al. 2022). When adult female offspring became mothers, they had embryos with developmental abnormalities, fewer embryo numbers (Barco et al. 2022), and live fetuses compared to nondiabetic daughter rats (Sinzato et al. 2022).

Considering the impacts caused by intergenerational diabetes on the number of offspring (Generation F2), it is relevant to assess whether these repercussions are due to maternal glucose intolerance (Generation F1) caused by intrauterine diabetes (Generation F0 - grandparents). Our hypothesis is that exposure to maternal hyperglycemia impairs the reproductive parameters of adult female pups, interfering with glucose metabolism and fetal growth.

## 2. Methodology

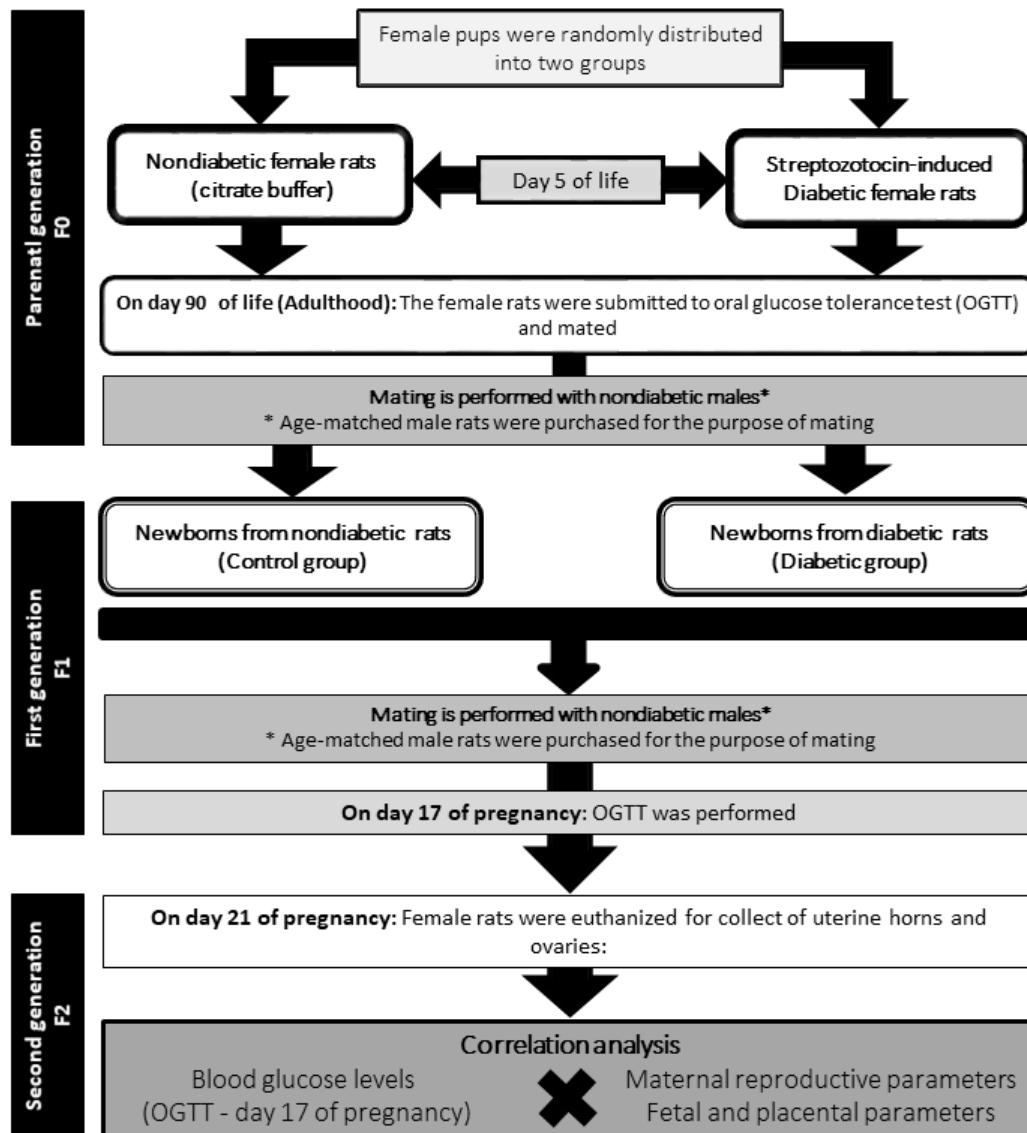
### *Experimental approach for diabetes induction (Parental generation)*

#### *Animals*

Female and male Sprague-Dawley rats weighing approximately 150 and 250 g, respectively, were acquired from the Animal Facility of the State University of Campinas (CEMIB\_UNICAMP) and kept in the vivarium of our Institution under controlled temperature conditions ( $22 \pm 2^\circ\text{C}$ ), humidity ( $60 \pm 10\%$ ), and light/dark cycle (12 h). Filtered water and feed were given ad libitum. For environmental enrichment, paper balls were used in animal cages (Simpson & Kelly 2012). The procedures and handling of animals were carried out by the guidelines provided by the National Council for the Control of Animal Experimentation (CONCEA) and authorized by the Ethics Committee for the Use of Animals (CEUA) of the Botucatu Medical School, UNESP (Protocol Number 1875-2017).

The experimental design of the study was presented in Figure 1.

**Figure 1 - Experimental design.**



Source: Authors.

### *Diabetes induction and inclusion criteria*

On postnatal day 5 (PND), the female pups (Parental Generation) were given streptozotocin (Sigma Aldrich®, United States, 70 mg/kg dose, intraperitoneal route) for diabetes induction. The nondiabetic (control) female rats received a similar volume of citrate buffer - 0.01 M pH 4.5 as a vehicle (Paula et al. 2022). The glycemia was measured in these female adult rats on PND 75 and classified by the American Diabetes Association (ADA, 2022). Following, female rats were considered diabetic when presenting glycemia  $\geq 200$  mg/dL (11.11 mmol/L) at least at a one-time point during the oral glucose tolerance test (OGTT). The rats with glycemia  $< 140$  mg/dL (7.77 mmol/L) at least three times during the OGTT were included in the control group. The female rats not accomplishing the above mentioned characteristics were anesthetized by sodium thiopental (Thiopentax®, Cristália, Brazil – 120 mg/kg dose), euthanized, and excluded from this study.

### *Mating, pregnancy, and lactation*

On PND 90 after inclusion criteria, the diabetic (D) and nondiabetic (control - C) female rats were mated as previously written. The offspring were obtained by vaginal delivery to avoid differences in maternal care between male and female pups (Beery & Francis 2011), which might result in epigenetic effects (Champagne et al. 2003). After birth, the male pups were decapitated, while female pups were kept in number from six to eight pups per litter until weaning day 22 to maintain the milk intake balanced. The excess number of females was also euthanized by decapitation. Female pups of nondiabetic (control) and diabetic rat mothers were randomly assigned to compose the experimental groups after weaning.

### *Experimental steps for female pups from the maternal environment induced by diabetes (F1 generation)*

#### *Mating and Pregnancy*

On PND 120, OC and OD rats were mated as previously described for their mothers. After confirmation of pregnant rats by sperm presence in the vaginal smear, this day was considered gestational day zero (GD0). Each pregnant rat was placed in one cage for individual care and observation.

#### *Oral glucose tolerance test (OGTT) and Area Under the Curve (AUC)*

On day 17 of pregnancy, OGTT was carried out for glucose tolerance evaluation as previously described by Gallego et al. (2019). The total glucose response was assessed by calculating the AUC using the trapezoidal method (Tai 1994).

#### *Laparotomy for obtaining pups (generation F2)*

On day 21 of pregnancy (full-term pregnancy), the OC and OD rats were anesthetized by sodium thiopental (Thiopentax®, Cristália, Brazil – 120 mg/kg dose, intraperitoneal route) and decapitated. The uterine horns, ovaries, fetuses, and placentas were removed and weighed to evaluate the maternal reproductive outcomes. The number of corpora lutea were counted of the ovaries, and used as an indirect parameter to assess the number of oocytes. The gravid uterus was dissected to count the number of alive and dead fetuses, resorptions (embryonic deaths), and implantation sites. The percentage of embryonic loss before implantation (pre-implantation loss rate), the rate of embryonic loss after implantation (postimplantation loss rate). The fetuses and placentas were weighed, and the placental efficiency was determined by the ratio between [fetal weight (g)/placental weight (g)] x 100 (Moraes-Souza et al. 2017).

### *Statistical analysis*

To calculate the sample size (n), a randomized design was made by the Research Support Office of Botucatu Medical School, Unesp, based in relation to Area Under the Curve (AUC) obtained from the OGTT glycemic values, using 90% power

and error type I of 5%, with a minimum “n” of 10 animals/group). The analyzes determined by a specialist in Biostatistics at our Institution, using SAS Software -STATISTICAL ANALYSIS SYSTEM version 9.4, 2021. Pearson's correlation test was performed to evaluate the relationship existing between the variables studied, considering a minimum confidence limit of 95% ( $p < 0.05$ ).

### 3. Results

**Table 1** - Blood glucose levels by Oral Glucose Tolerance Test performed on the day 17 of pregnancy of daughters from nondiabetic and diabetic dams.

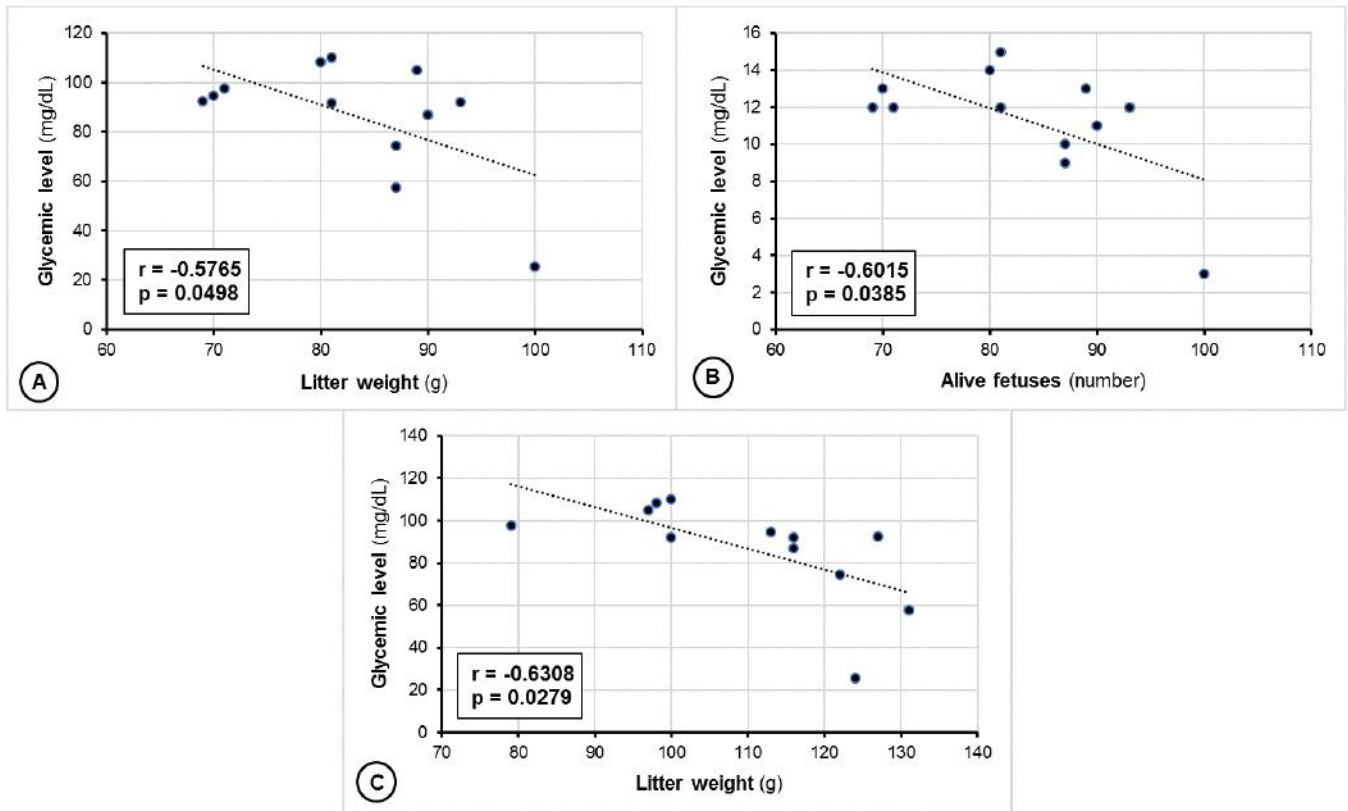
Groups	Rat	Glycemia (mg/dL)			
		Time 0	Time 30	Time 60	Time 120
Control	1	80	98	100	81
Control	2	89	97	92	95
Control	3	87	122	103	89
Control	4	81	100	97	80
Control	5	80	69	73	68
Control	6	71	79	77	71
Control	7	73	79	65	73
Control	8	69	127	121	71
Control	9	71	52	51	78
Control	10	77	91	87	78
Fdmod	11	87	131	93	68
Fdmod	12	93	116	84	86
Fdmod	13	132	189	135	103
Fdmod	14	81	100	103	82
Fdmod	15	90	116	120	80
Fdmod	16	100	124	116	96
Fdmod	17	70	113	107	77
Fdmod	18	77	136	90	85
Fdmod	19	98	81	81	84
Fdmod	20	92	122	103	85

Source: Authors.

#### *Correlation analysis between OGTT and maternal reproductive outcomes*

Figure 2 presents a correlation analysis between the oral glucose tolerance test (OGTT) and maternal reproductive outcomes. A significant negative correlation was showed between the glycemic level at 0 minutes (T0 - fasting) and AUC in the OGTT and the number of live fetuses at term pregnancy. Litter weight also had a significant negative correlation with glycemia at time points 0 (T0) and 30 minutes (T30). There were no significant correlations between maternal OGTT *versus* other parameters (maternal weight gain, number of corpora lutea, number of implantations, number of embryonic deaths, and percentage of losses before and after embryo implantation).

**Figure 2** - Correlation analysis between blood glucose levels measured at different points in the oral glucose tolerance test (OGTT) pregnant daughters from nondiabetic and diabetic dams. **2a.** OGTT and number of alive fetuses; **2b.** OGTT x litter weight (T0); **2c.** OGTT x litter weight (T30) of pregnant daughters from nondiabetic and diabetic dams.



$n = 10$  animals/group;  $p < 0.05$  - Pearson correlation test. It is important to verify in the figure that the line is descending, which represents a significant negative correlation that means one variable is inversely proportional to the other. The blue points on the graph represent the animal data. Source: Authors.

**Correlation analysis between OGTT and fetal weights, placental weights, and placental efficiency**

**Table 2** - Correlation analysis between blood glucose levels measured at different points in the oral glucose tolerance test (OGTT) and fetal and placental parameters of pregnant daughters from nondiabetic and diabetic dams.

Variables	T0	T30	T60	T120
<b>Fetal weight (g)</b>	$r = -0.08314$ $p = 0.7973$	$r = -0.5417$ $p = 0.0689$	$r = -0.02937$ $p = 0.9278$	$r = 0.3846$ $p = 0.2170$
<b>Placental weight (g)</b>	$r = 0.5464$ $p = 0.0660$	$r = 0.1879$ $p = 0.5587$	$r = 0.3252$ $p = 0.3023$	$r = 0.4487$ $p = 0.1435$
<b>Placental efficiency</b>	$r = -0.5089$ $p = 0.0911$	$r = -0.5072$ $p = 0.0924$	$r = -0.2741$ $p = 0.3886$	$r = -0.1220$ $p = 0.7038$

$n = 10$  animals/group;  $p > 0.05$  – non-significant – Pearson's correlation test. Source: Authors.

No significant correlations ( $p > 0.05$ ) were verified between the different OGTT moments analyzed in relation to fetal weights, placental weights, and placental efficiency.

It is important to note that the p values in the table were not less than 0.05, so there was no significance between the correlations.

#### 4. Discussion

In this study, the relationship between hyperglycemia in rats born to diabetic mothers and the reduction in the number of live fetuses and the weight of the litter at the end of pregnancy was verified. Furthermore, no significant relationship was demonstrated between maternal hyperglycemia, as assessed by the oral glucose tolerance test (OGTT), concerning other variables of maternal reproductive performance (maternal weight, number of corpora lutea, number of implantations, embryonic losses before and after implantation, and embryonic deaths). The hyperglycemia found in mother rats and their adult daughters was confirmed by the OGTT, which is considered the gold standard for diagnosing diabetes in women and laboratory animals.

Intrauterine hyperglycemia compromises the development of the embryo, fetus, and newborn. This impairment may be related to the stimulus of high glucose concentration for the excessive formation of reactive oxygen species (Damasceno et al. 2002; Sinzato et al. 2022). Hyperglycemia limits substrates for mitochondria and causes downregulation of mitochondrial free radical scavengers, leading to increased generation of reactive oxygen species (ROS) (Zheng et al. 2022). Associated with oxidative stress, the placenta's antioxidant activity is insufficient to protect the developing fetus fully (Yang et al. 2023). Furthermore, the excessive concentration of glucose in maternal blood favors fetal hyperglycemia, leading to oxidative stress in the fetal cells themselves (Brownlee 2001; Damasceno et al. 2002). This homeostatic alteration leads to the abnormal development and/or functioning of several fetal organs and systems (Ornoy et al. 2021), contributing to the appearance of malformations and/or infeasibility for their birth, which justifies the lower number of live fetuses found in this study.

The relationship between maternal hyperglycemia and lower litter weight may be related to the lower number of live fetuses and the fact that these fetuses are classified as small for gestational age, as verified in other studies of this research group (Sinzato et al. 2021; Kloppel et al. 2023). Although changes in the weight of fetuses and placentas from diabetic and nondiabetic rats were not significantly correlated with glucose levels during the OGTT of these mothers, it should be noted that maternal glycemia is directly associated with a higher percentage of fetuses classified as small for gestational age which shows that this classification is more efficient to demonstrate compromised fetal weight induced by maternal hyperglycemia than body weight itself, which corroborates another study by our laboratory team (Sinzato et al. 2021). However, the results found about the classification of fetal weights in this study are different from those found in the children of diabetic women who are macrosomic. In humans, children of diabetic mothers tend to have a higher birth weight, justifying the fact that maternal hyperglycemia leads to fetal hyperglycemia and hyperinsulinemia at the first moment, contributing to excessive fetal growth due to the physiological characteristics of insulin similar to those of growth hormones in the last trimester of pregnancy (Hufnagel et al. 2022). However, in rats, the main period of fetal growth is between days 18 and 21 of pregnancy, which is insufficient to significantly increase the adipose tissue mass of these fetuses in intrauterine life. However, these newborns store more hepatic triglyceride at birth, which is considered a marker of macrosomia for diabetic rats (Herrera et al. 2000). This data is a limiting factor of this study since if the authors had measured the levels of hepatic triglycerides in these newborns, there would be the possibility of discussing their macrosomia.

Some limitations can be pointed out in this study, such as the lack of biochemical and morphological determinations (lipid profile and pancreatic endocrine analysis in fetuses). This information might help to clarify the mechanisms involved in impaired fetal growth. As strengths presented by this study, the authors demonstrated that the glycemic alterations of the adult female from diabetic rats could promote adverse effects on the development and growth of their offspring, highlighting the negative impact of maternal diabetes over generations. Additionally, this study with laboratory animals emphasizes the need for tight blood glucose control before and during pregnancy to prevent disease and promote health.

## 5. Conclusion

In conclusion, maternal fasting blood glucose during pregnancy is a relevant biomarker related to impaired reproductive outcome in daughters of rats. In addition, the relationship between maternal hyperglycemia and the reduced number of live fetuses and the weight of the litter was confirmed. These findings show the detrimental effects of fetal programming induced by maternal diabetes in adulthood in the succeeding generation. Besides, insights for future research about analysis of the biochemical and pancreatic endocrine profile of the grandchildren of diabetic rats can provide valuable information for studying the transgenerational damage caused by hyperglycemia on fetal growth.

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