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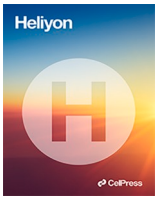

Research article

Effect of transgenerational diabetes via maternal lineage in female rats

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A R T I C L E I N F O

Keywords:
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Diabetogenic tendency
Macrosomia
Pregnancy
Rodents

A B S T R A C T

Aim: To investigate the transgenerational effect of maternal hyperglycemia on oxidative stress markers, lipid profile, glycemia, pancreatic beta (β)-cells, and reproductive outcomes in the F2 adult generation. Additionally, to expand the knowledge on transgenerational diabetes the F3 generation at birth will be evaluated.

Methods: On day 5 of postnatal life female *Sprague-Dawley* rat newborns (F0 generation) were distributed into two groups: Diabetic (Streptozotocin-STZ, 70 mg/kg body weight, subcutaneous route) and Control rats. Adult female rats from the F0 generation and subsequently the F1 generation were mated to obtain the F2 generation, which was distributed into F2 generation (granddaughters) from control (F2_C) and diabetic (F2_D) rats. Oral Glucose Tolerance Test (OGTT), the area under the curve (AUC), blood biochemical analyses, and pancreatic morphology were analyzed before pregnancy. Reproductive outcomes were performed at the end of pregnancy. At birth, the glycemia and body weight of F3_C and F3_D rats were determined. $p < 0.05$ was considered significant.

Results: F2_D had higher body weight, triglyceride levels, and percentage of insulin-immunostained cells, contributing to glucose intolerance, and insulin resistance before pregnancy. At day 21 of pregnancy, the F2_D showed increased embryonic losses before and after implantation (84.33 and 83.74 %, respectively). At birth, F3_D presented hyperglycemia, and 16.3 % of newborns were large for pregnancy age (LGA).

Conclusion: Diabetes induction since the neonatal period in the first generation (F0) led to transgenerational (F2 and F3 generations) changes via the maternal lineage of female rats, confirming the relevance of control strictly the glycemia all the time.

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Summary sentence

Pre-gestational hyperglycemia via the maternal lineage in the F0 generation caused important changes in glycemic and lipid metabolism, reproductive changes in the F2, and increased birth weight and hyperglycemia in the F3 generation.

1. Introduction

The endocrine portion of the pancreas is termed the pancreatic islet and corresponds to a specialized micro-organ and is mainly composed of alpha (α), beta (β), and delta (δ) cells. The β-cells synthesize and secrete insulin, α-cells are responsible for glucagon synthesis, and δ-cells secrete somatostatin [1]. Thus, endocrine cells play an essential role in maintaining glucose homeostasis, and their imbalance can lead to diseases such as *Diabetes mellitus* (DM).

International Diabetes Federation (IDF) reports that 537 million adults are diabetic person (1 in 10). DM is a chronic metabolic disease characterized by high blood glucose levels, compromising the function of different organs and systems [2]. Evidences show that hyperglycemia increases the production of reactive oxygen species (ROS) due to reductant entry into the mitochondrial electron transport chain [3]. For this, there are antioxidant enzymes, such as superoxide dismutase (SOD), that dismutate the superoxide anion (O_2^-), and catalase (CAT) and glutathione peroxidase (GSH-Px), which catalyze the decomposition reaction of hydrogen peroxide (H_2O_2). The defense enzymatic system neutralizes and regulates ROS levels to maintain physiological homeostasis. Therefore, the imbalance between oxidant and antioxidant levels may constitute a stress signal that activates specific redox-sensitive signaling pathways, leading to oxidative stress [4]. Oxidative stress leads to impaired glucose uptake in muscle and fat cells and decreases insulin secretion from β-cells [5,6], further damaging the hyperglycemic condition.

Hyperglycemia during the gestational period can also contribute to changes in fetal programming. It can alter development and metabolism that, associated with epigenetic factors, possibly cause metabolic disorders in adult offspring [7], such as the increased risk for overweight, obesity, DM Type 2, Gestational *Diabetes mellitus* (GDM), and insulin resistance [8,9]. According to intergenerational studies, the hyperglycemic intrauterine environment led to reduced glucose tolerance, high serum concentration of total cholesterol, triglycerides, VLDL-c, insulin resistance, glucose intolerance, and percentage of insulin-immunostained cells in adulthood F1 generation from diabetic rats [10]. In addition, these rats presented embryofetal losses at the end of pregnancy [11].

Literature about diabetes and its complications in studies of insulin-producing cells, insulin levels, and the presence or absence of glucose tolerance or diabetes over generations is limited. Therefore, our goal is to investigate the transgenerational effect of the hyperglycemic environment (pre-gestational diabetes) on markers of oxidative stress, lipid profile, glycemia, pancreatic β-cells, and reproductive outcomes in the F2 generation in adulthood. Additionally, we intend to evaluate the glycemia and body weights in the F3 generation at birth to expand the knowledge of transgenerational diabetes to better understand the mechanisms involved in developing future prophylactic therapies and develop effective treatments. We hypothesize that maternal hyperglycemia in the F0 generation can lead to oxidative stress and metabolic changes in adult females of the F2 generation, leading to damage to the intrauterine environment and also affecting the next generation (F3 generation), thus perpetuating the diabetogenic tendency via the maternal lineage in these animals.

2. Methods

2.1. Ethics Committee on the Use of Animals

Procedures and animal handling were made according to the Brazilian College of Animal Experimentation guidelines and International Guiding Principles for Biomedical Research Involving Animals proclaimed by the Society for the Study of Reproduction. The local Ethics Committee for the Use of Animals of our Institution approved all the methods adopted in this study (Protocol CEUA Number: 1375/2021).

2.2. Animals

Females of the F2 (granddaughters) and F3 (great-granddaughters) generations originated from diabetic rats (F0) (Sprague Dawley) that received beta (β)-cytotoxic drug (Streptozotocin, STZ, Sigma-Aldrich®, USA) for diabetes induction were used. The diabetes-induced outcomes on mothers (Generation F0) [12] and daughters (Generation F1) [10,13] have been previously published. All animals were maintained at the local laboratory under controlled conditions of humidity (50 ± 10 %), temperature (22 ± 2 °C), and light/dark cycle (12 h) in polypropylene cages lined with wood shavings. Filtered water and regular chow were offered *ad libitum*. As a form of environmental enrichment, paper balls were used in the cages [14].

2.3. F0 generation (parental animals) and F1 generation (daughters)

The experimental sequence of the animals for induction of mild diabetes using STZ (Sigma Aldrich®, 70 mg/kg body weight, subcutaneous route) was performed on day 5 of postnatal life [10]. For the non-diabetic (control) group, the female pups received citrate buffer (vehicle) in the same volume and administration route as the diabetic group (both groups as Parental generation = F0). Blood glucose levels were determined by the Oral Glucose Tolerance Test (OGTT) as inclusion and exclusion criteria on day 90 of life for these adult female rats [12]. The inclusion criterion was glycemia ≥200 mg/dL at any time of the OGTT for the diabetic animals and

glycemia <140 mg/dL as a limit value during the test for the control rats [12]. Female rats at postnatal day (PND) 5 were injected with streptozotocin [10] to induce β-cell necrosis, reproducing glycemic levels similar to type 2 DM or GDM. These rats show higher glycemic levels, a lower percentage of full-term pregnancy, and a higher percentages of inadequate fetal weight [18].

On day 120 of life, control, and mildly diabetic female rats were mated with normoglycemic male rats to obtain their daughters (F1 generation) through vaginal delivery. In adulthood (120 days), these daughters from both groups were mated following the same protocol as the parental generation to obtain their granddaughters (F2 generation) following both diabetic and control rat lineages. After birth, the female granddaughters continued with their mothers from lactation until the weaning period (30 days of life), considering four female and four male pups per mother (litter adjusted to 8 pup/litter). On postnatal day 10, the female pups were weighed. After weaning, two females per mother (litter) were randomly selected to compose the experimental groups corresponding to their mothers, and the male pups were used in other studies.

3. Procedures performed on F2 generation (female granddaughters)

After weaning, the rats were distributed into experimental groups (Fig. 1):
F2_C: Generation F2 from control rats;
F2_D: Generation F2 from mild diabetic rats.

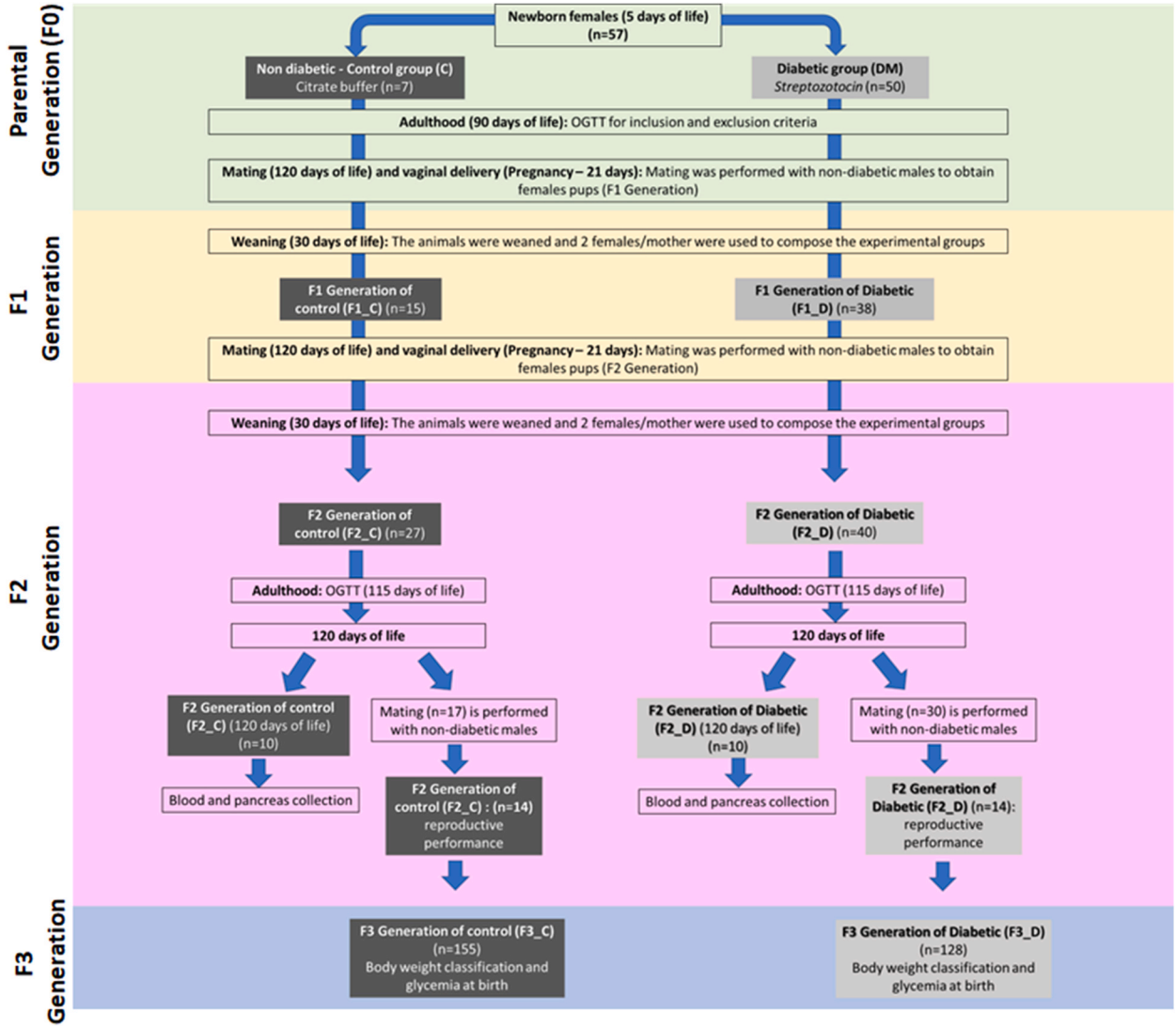

Fig. 1. Experimental sequence of the study.

3.1. Oral Glucose Tolerance Test (OGTT) and area under the curve (AUC) calculation

On day 115 of life, the rats were submitted to the OGTT. After 6 h of fasting, a drop of blood was collected by venipuncture in the rats' tails for glycemic determination (time 0). The rats were then given a glucose solution (0.2 g/mL) intragastric (*gavage*) at a 2.0 g/kg body weight dose. The blood glucose levels were determined after 30, 60, and 120 min (min) of the glucose solution [11]. These measurements were also used to mathematically estimate the total area under the curve (AUC) using the trapezoidal method [14].

3.2. Anesthetic procedure, glycemia, and laparotomy

On day 120 of life, the F2 fasted rats were weighed. A small drop of blood was removed from the tail extremity of each rat (n = 10 animals, from five different mothers (F1), using two females (F2) per litter) for glycemic determination. Following, these animals were anesthetized with sodium thiopental (Thiopentax - 120 mg/kg body weight, Cristália®, Brazil, intraperitoneal route). After confirmation of the signs of the anesthetic procedure following the instructions of the Institutional Veterinarian, the animals were decapitated to obtain blood samples. These samples were collected to evaluate the activity of antioxidant enzymes, thiobarbituric acid reactive species (TBARS), serum insulin, triglyceride, and cholesterol measurements. The animals were then submitted to laparotomy for the collection of the pancreas.

3.3. Evaluation of biochemical profile

From here, all procedures were performed with the blinded investigator for the biochemical analyses. The whole blood samples were centrifuged at 1575 × *g* for 10 min at 4 °C. After this procedure, the serum was obtained and stocked in a freezer at −80 °C until the insulin determination (Crystal Chemical® - Code: 90060, United States), triglyceride (Wiener lab® - Code 1780105, Rosário, Argentina), and cholesterol (Wiener lab® - Code 1220114, Rosário, Argentina) determinations by spectrophometry following the instructions of the manufacturers according to each specific commercial kit. Very low-density lipoprotein (VLDL)-cholesterol values were determined from the triglyceride concentrations [15].

3.4. Homeostatic Model assessment (HOMA) and TyG index

Insulin resistance (HOMA-IR) and beta-cell function (HOMA-β) were calculated. For HOMA-IR, the value was calculated using the following equation: [fasting insulin (μU/mL) × fasting glucose (mmol/L)]/22.5. HOMA-β expressed the beta-cell function and was calculated using the following equation: [20 × fasting insulin (μU/mL)/(fasting glucose (mmol/L) − 3.5]. For the TyG index, the value was calculated using the following equation: TyG = Ln [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2] [16].

3.5. Oxidative stress analyses

The procedures used for oxidative stress parameters followed the methodology described by Sinzato et al. (2023) [17]. For this, all biomarkers were estimated in washed erythrocytes. Thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), reduced thiol groups (-SH), antioxidant activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were performed. The blood samples were in heparinized tubes and were centrifuged at 1200 rpm (185×*g*) for 10 min at 4 °C. The plasma was discarded, the erythrocytes were washed in 2 mL of phosphate saline buffer (PBS, pH 7.4) and centrifuged at 1575×*g* at 4 °C for 1 min, and the supernatant was discarded. This procedure was repeated three times, and then the washed erythrocyte samples were divided into two aliquots: an aliquot was diluted (1:20) into purified water for the measurement of TBARS and –SH levels and another aliquot was diluted into stabilizing solution of 2.7 mM EDTA and 0.7 mM 2-mercaptoethanol (1:20) for H_2O_2, SOD, GSH-Px, and CAT measurements. The hemoglobin (Hb) concentration was determined using Drabkin's solution [17].

3.6. Immunohistochemical analyses of the pancreas

Five pancreas/groups were selected for immunohistochemical analysis, considering a pancreas from the F2 generation coming from different mothers (F1 generation). The pancreas samples were weighed and fixed in 4 % formaldehyde for 24 h and then conditioned in 70 % ethanol. The fragments were processed and included in paraffin. Sections of 5 μm were obtained using a rotary microtome, which was incubated with a polyclonal antibody anti-insulin (Abcam®, Code: ab8304, USA). Antigen retrieval was performed in the Elite Bistro (Johnson Becker®, USA) pressure cooker with citrate solution (pH 6.0) for 20 min. Endogenous peroxidase (peroxidase inhibitor-containing hydrogen peroxide and 15 mM sodium azide - Dako®, USA) blockade was performed for 40 min at room temperature. For the blockade of non-specific proteins, Protein Block (0.25 % casein in PBS, containing carrier protein and 15 mM sodium azide) was used for 30 min in an oven at 27 °C. Dilution for the primary antibody was 1:10.000 (incubation for 2 h). After incubation of the primary antibody, the secondary antibody (Histofine®, Germany) was added for 30 min in an oven at 27 °C. For the development of peroxidase, the chromogen DAB (3,3-diaminobenzidine) was used for 3 min at room temperature. Then, the slides were counterstained in Harris's hematoxylin and mounted. The images were captured using the computerized image system (Software KS-300, version 3.0, Zeiss®, Germany), integrated into digital camera image (CCD-IRIS/RGB, Sony®, China), and microscope (DMR, Leica®, Brazil). Ten islets were randomly selected for analysis for each pancreas, totaling 50 islets/group. 40x magnification was used for all analyses. For morphological analysis of pancreatic islets, islets were randomly selected throughout the pancreas (head, body, and tail). These islets

had at least six cells and there was no maximum cell limit stipulated. The percentage of insulin-positive cells in the pancreatic islet was determined by the ratio between the number of immunolabeled cells and total cells using ImageJ (NIH free access). The value obtained was multiplied by 100 [12].

3.7. Mating and pregnancy

On day 120 of life, other F2_C and F2_D rats were mated for 15 consecutive days, comprising three estrous cycles. For this, three females were placed in the presence of a normoglycemic male overnight. The following day (7–9 am), the males were removed, and the vaginal smear was performed. The presence of spermatozoa on the slides confirmed the pregnancy diagnosis, which was considered gestational day zero (D0) [12]. These rats were weighed on the first and last days of pregnancy to assess maternal weight gain.

3.8. Laparotomy and reproductive outcomes

On day 21 of pregnancy, the rats of both groups were anesthetized with sodium thiopental (Thiopentax®, Cristália, Brazil, intraperitoneal route - 120 mg/kg body weight) and decapitated for exposure of the uterine horns for count of the implantation, reabsorption (embryonic deaths), and fetus (alive or dead) numbers. For indirect counting of the oocyte, the ovaries were removed for the corpora lutea number. The rate of embryonic losses before implantation (pre-implantation losses) was calculated by: [(Number of corpora lutea − Number of Implantation)/(Number of corpora lutea)] × 100. The rate of embryonic losses after implantation (post-

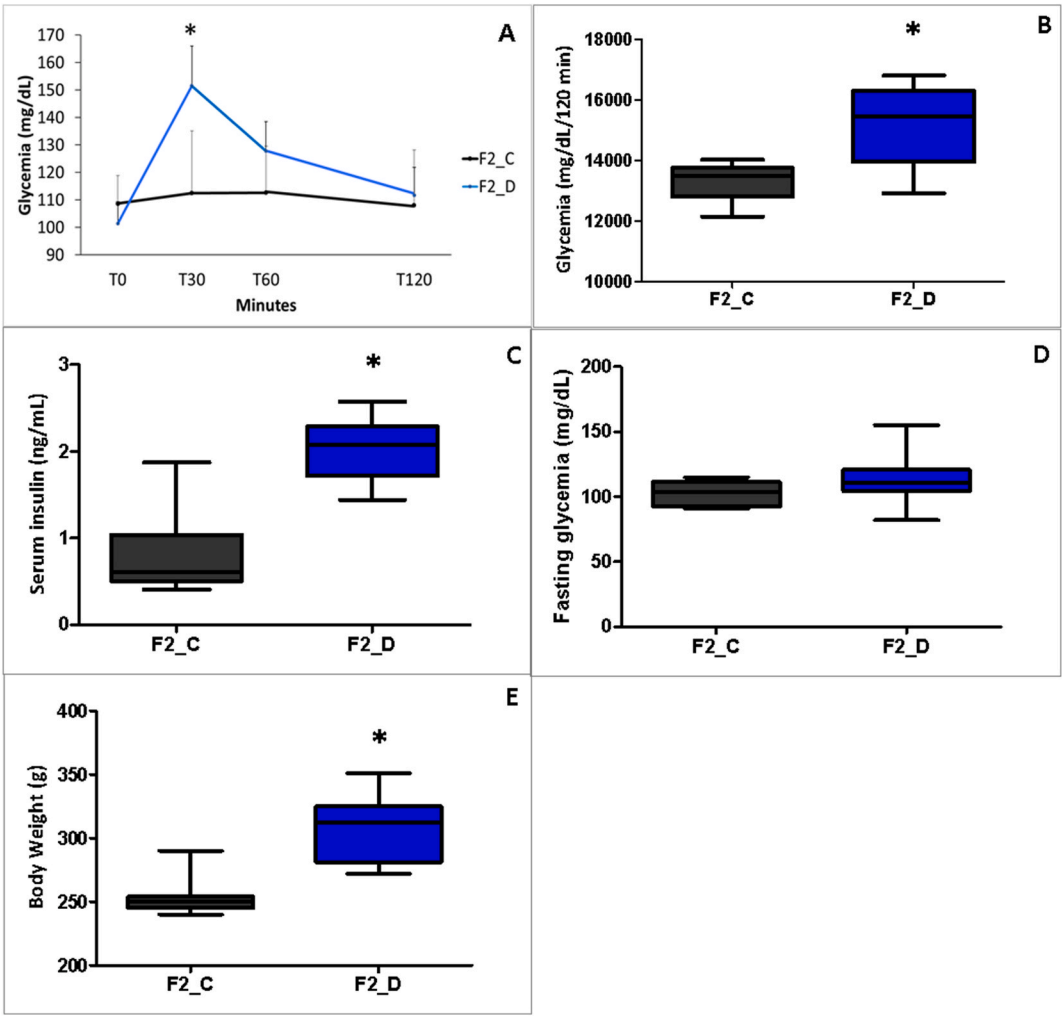

Fig. 2. Glycemic profile and body weight of the F2 generation. A - Oral Glucose Tolerance Test (OGTT) on day 115 day of life. B - Area under the curve (AUC) on day 115 of life. C - Serum insulin concentration (ng/mL) on day 120 of life. D - Fasting glycemia (mg/dL) on day 120 of life. E – Body weight (g) on day 120 of life. Data expressed as mean ± standard deviation (n = 8 animals/group). *p < 0.05- compared to the F2_C group (Gamma Distribution Test for OGTT; Student t-test for the area under the curve, serum insulin, fasting glycemia, and body weight).

implantation losses) was determined by: [(Number of Implantation − Number of live fetuses)/(Number of implantation)] × 100] [18].

5. F3 generation (Great granddaughters): Blood glucose level, fetal body weight, body weight classification, and sex ratio.

At birth, all the F3 generation female newborns were sexed, weighed, and classified as small (SGA), adequate (AGA), and large for gestational age (LGA) [18]. To determine blood glucose, two female newborns (F3 generation)/mother (F2 generation) totaled 24 female newborns (generation F3). The placental efficiency was calculated by using the ratio between the fetal and the placental weight [18].

4. Statistical analysis

To calculate the sample size (n), a randomized design with the factorial design of the Research Support Services Office (EAP) of Botucatu Medical School was used. Some 10 animals (that is correct if they always came from different original F0 mothers/groups have been established for each group). For IHQ, the calculation was estimated in five animals/groups, with a minimum of 10 islets/animal/pancreas. Data were shown as mean ± standard deviation (SD). F2 generation: T-test was used for the rats on day 120 of life-related to body weight, the area under the curve, serum insulin, fasting blood glucose levels, antioxidative enzymatic activities, TBARS, total cholesterol, and VLDL, triglyceride levels; HOMA-IR, HOMA-β, and TyG indexes, islet area, maternal weight gain, corpora lutea, implantation, and embryonic death numbers; placental weight and efficiency index. For OGTT, Gamma Distribution was used. Poisson was performed for the immunohistochemical analyses of insulin. F3 generation: the embryonic losses before and after implantation were analyzed for contingency analysis, and the Chi-square test evaluated the fetal body weight classification. A minimum confidence limit of 95 % ($p < 0.05$) was considered for all statistical comparisons.

5. Results

5.1. F2 generation

In this study, 27 females were from control rats (F2_C), of which 10 were used to compose the F2_C group on day 120 of life, and 17 were used for mating. From diabetic rats, 40 females ordered the F2_D: 10 rats were used on day 120 of life, and 30 were used for mating (Fig. 1).

Fig. 2 shows the glycemic profile and body weight. During the OGTT, the F2_D rats displayed hyperglycemia 30 min (min) after the glucose overload (Fig. 2A). An increased circulating glucose level was observed in the F2_D rats compared to the F2_C group during 120 min of the OGTT (Fig. 2B). On day 120 of life, the F2_D group had higher serum insulin concentrations than the F2_C group (Fig. 2C). Regarding fasting blood glucose, there was no difference between the groups (Fig. 2D) ($p > 0.05$). The F2_D rats had a higher body weight on day 120 of life (Fig. 2E) compared to the F2_C group. The weight (g) of the pancreas was greater in the F2_D group (0.86 ± 0.15) when compared to the F2_C ($p < 0.05$).

The biochemical parameters are shown in Table 1. On day 120 of life, the F2_D rats had higher TyG index, VLDL-cholesterol, and triglyceride levels; increased HOMA-IR and HOMA-β indexes, and a decreased total cholesterol level compared to the F2_C rats ($p < 0.05$).

Regarding oxidative stress data, F2_D female rats showed a lower activity of the antioxidant enzymes, as SOD (Fig. 3A) and GSH-Px (Fig. 3C), decreased H_2O_2 (Fig. 3E) and –SH (Fig. 3F) levels. There was no difference in TBARS concentration (Fig. 3B) and catalase enzyme activity between groups (Fig. 3D).

About the morphological data of the endocrine pancreas of these animals, no significant statistical difference was found in the area of the pancreatic islets (Fig. 4A) ($p > 0.05$), as observed in the photomicrographs of group F2_C (Fig. 4 B) and F2_D (Fig. 4C). A higher percentage of insulin-positive cells in the pancreas of F2_D rats was observed compared to the F2_C rats (Fig. 4D, E, and 4F) ($p < 0.05$).

On day 21 of pregnancy, parameters of maternal reproductive outcomes of F2 dams were shown in Table 2. In group F2_C, 17 female rats were placed to mate; 3 did not mate, and the animals with a positive pregnancy diagnosis reached full-term (14 rats). For the F2_D group, 30 female rats were placed to mate. Of these, 20 rats had a positive pregnancy diagnosis, 14 rats had full-term pregnancy. There were no differences regarding the maternal body weight gain, number of corpora lutea and implantation, and placental weight (Table 2). There was a lower number of live fetuses and a higher number of embryonic deaths, pre-and post-implantation losses, and placental efficiency in the F2_D group when compared to the F2_C group (Table 2) ($p < 0.05$).

Table 1
Biochemical parameters of F2_C (granddaughters of control rats) and F2_D (granddaughters of diabetic rats) rats at 120 days of life.

	F2_C (n = 7)	F2_D (n = 8)
Total cholesterol (mg/dL)	**58.06 ± 4.46**	**26.99 ± 9.03***
VLDL (mg/dL)	**5.51 ± 2.77**	**15.06 ± 5.81***
Triglycerides (mg/dL)	**25.84 ± 12.68**	**75.34 ± 29.07***
HOMA-IR	**4.25 ± 1.66**	**12.06 ± 2.4***
HOMA-β	**151.10 ± 67.17**	**545.7 ± 228.28***
Tyg index	**7.18 ± 0.51**	**8.08 ± 0.43***

Data expressed as mean ± standard deviation.
*$p < 0.05$- compared to the F2_C group (T test).

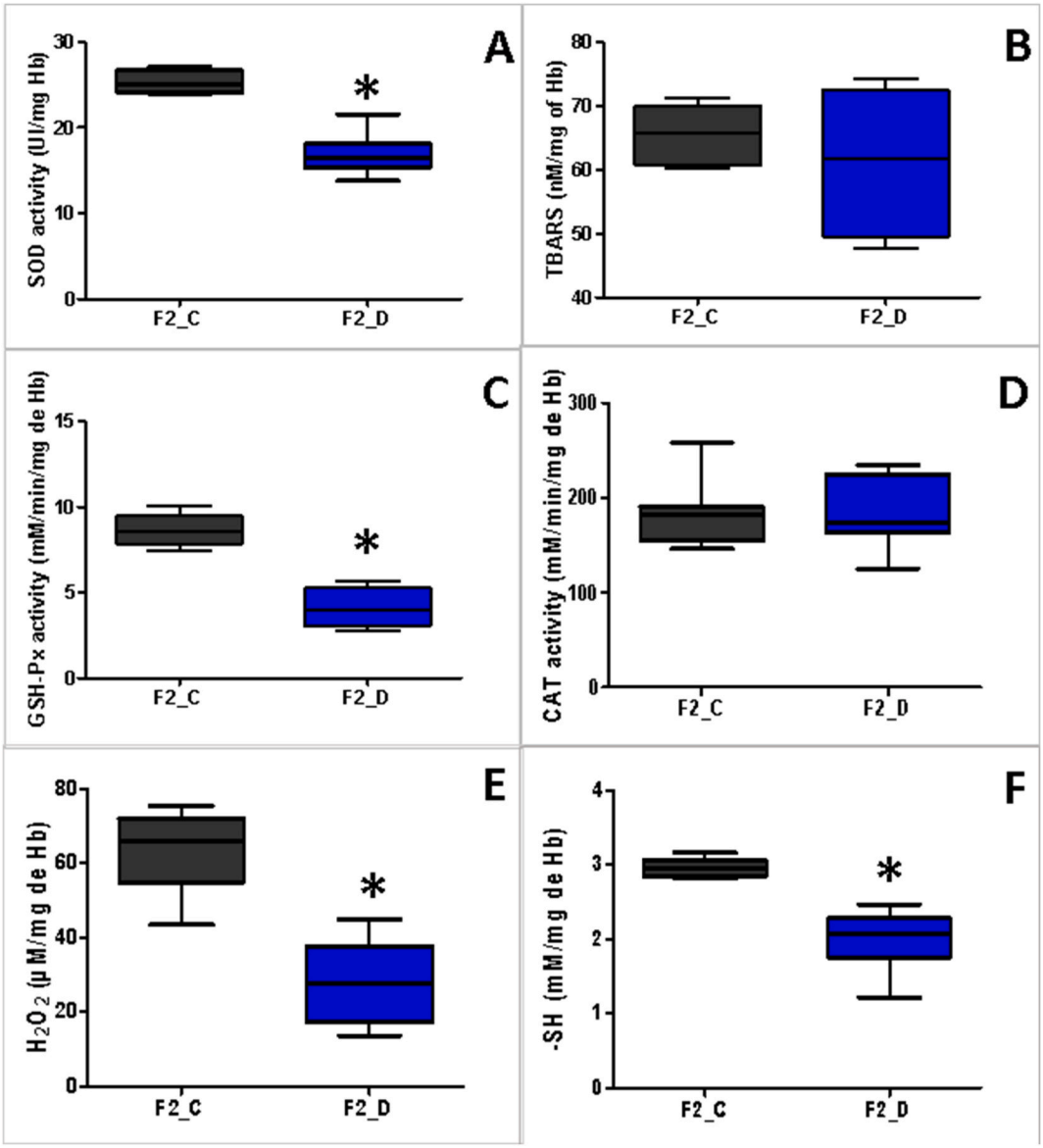

Fig. 3. Oxidative stress markers on day 120 of life of F2 Generation (Granddaughters). A- SOD, superoxide dismutase activity; B- TBARS, thiobarbituric acid reagent substance concentration; C- GSH-Px, glutathione peroxidase activity; D-CAT, catalase activity; E– H_2O_2, hydrogen peroxide levels; (F) –SH, reduced thiol group levels. Values are expressed as mean ± SD, standard deviation (n = 10 rats/group). *$p < 0.05$—compared to the F2_C (T-test).

5.2. F3 generation data

F3_D newborns had greater fetal body weight (Fig. 5A) and glycemic levels (Fig. 5C), a decreased percentage of fetuses considered adequate for gestational age (AGA), and an increased percentage of fetuses considered as large for gestational age (LGA) (Fig. 5B) than the F2_C group ($p < 0.05$). There was no difference between the proportion of males and females in the experimental groups (Fig. 5D) ($p > 0.05$).

6. Discussion

Previous results in our laboratory have shown that maternal diabetes causes changes in metabolism [10] in the endocrine pancreas [13] off adult female rats and also impairs reproductive outcomes in these rats [11]. Our results demonstrated that rats' hyperglycemia via the maternal lineage led to transgenerational effects, causing changes in the F2 and F3 generations. In adulthood, the F2 rats (granddaughters) of the diabetic generation (F2_D) presented greater body weight and abnormal glucose homeostasis, confirmed by

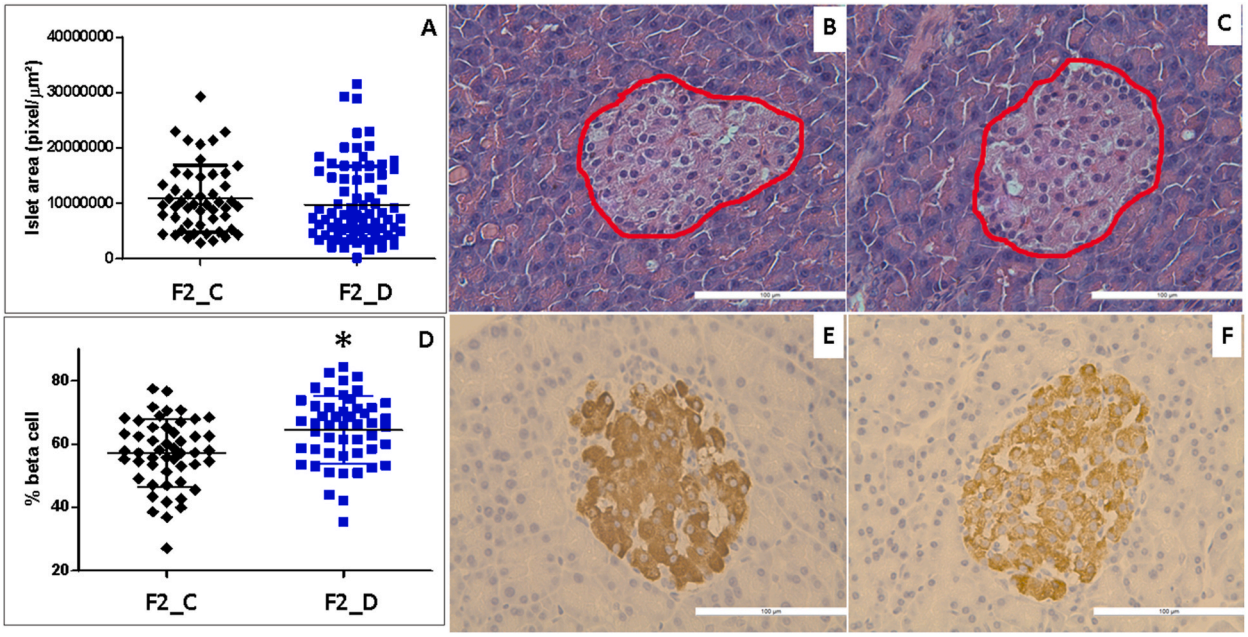

Fig. 4. Pancreatic islet morphology on day 120 of life. A- Pancreatic islet area (square millimeters). B- Representative photomicrograph of the pancreatic islets (circled in red) of F2_C rats (Hematoxylin & Eosin staining - H&E). C– Representative photomicrograph of the pancreatic islets (circled in red) of F2_D rats (H&E). D- Ratio of the number of labeled pancreatic beta cells to the total number of cells (labeled and unlabeled) by anti-insulin antibody from F2 rats. E– Photomicrograph of pancreatic islet immunostained for insulin from F2_C group. F- Photomicrograph of pancreatic islet immunostained for insulin from F2_D group. Magnification: 40×. Data presented as mean ± standard deviation (n = 50 islets/group).
$p < 0.05$- compared to the F2_C group (Poisson's Distribution). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Reproductive parameters on the 21 days of pregnancy of the F2_C (granddaughters of control rats) and F2_D (granddaughters of diabetic rats) rats.

	F2_C	F2_D
Number of mated females	17	30
Female rats with a positive diagnosis for pregnancy (%)	82,35 (14)	66,66 (20)
Number of female rats with at term pregnancy (%)	100 (14)	70 (14)
Maternal weight gain (g)	119.0 ± 26.0	100.3 ± 45.1
Corporea Lutea	13.9 ± 1.7	13.8 ± 1.8
Implantation	13.4 ± 1.5	11.4 ± 3.8
Live fetuses	12.8 ± 1.3	9.1 ± 4.6*
Pre-implantation loss (%)	3.4	21.7*
Post-implantation loss (%)	4.0	26.2*
Placenta weight (g)	0.58 ± 0.13	0.57 ± 0.1
Placental efficiency	9.17 ± 1.92	9.80 ± 1.78*

Values expressed as mean ± standard deviation (SD) for lutea corporea, implantation, live fetuses, placenta weight, and placental efficiency (n = 14 rats/group).
*$p < 0.05$ compared to the F2_C group (T-Test).
Values are expressed in percentages (%) for pre and post-implantation loss (n = 14 rats/group).
*$p < 0.05$ compared to the F2_C group (Analysis of Contingency).

the Oral Glucose Tolerance Test (OGTT). Moreover, the F2_D rats displayed hypertriglyceridemia and a higher percentage of insulin-immunostained cells related to peripheral insulin resistance, contributing to glucose intolerance. These findings confirm an abnormal metabolism similar to human DM2. The changes found before pregnancy directly reflected on the reproductive performance of the F2_D rats, such as a decreased percentage of rats with a positive pregnancy diagnosis, a lower number of rats with full-term pregnancies, and higher rates of embryonic losses before and after implantation at the end of pregnancy. Furthermore, the diabetogenic tendency remained in the next generation, as great-grandchildren newborns (F3_D), which showed hyperglycemia and macrosomia, confirming the transgenerational diabetes-induced damages in rats.

Diabetes in adulthood induced by transgenerational diabetes in both clinical studies and laboratory animals may cause changes

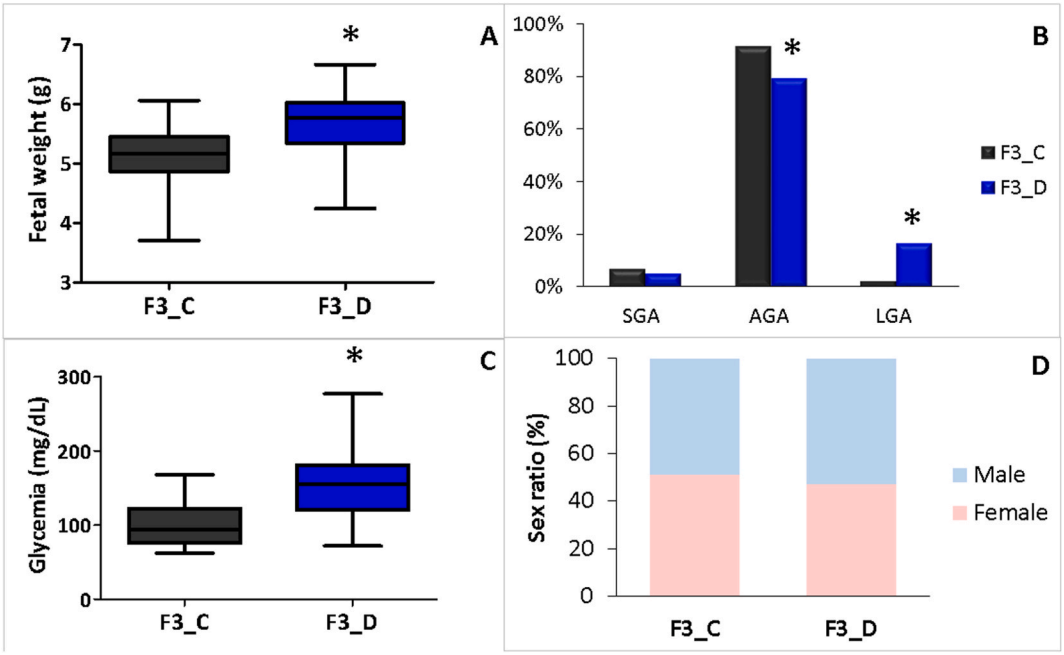

Fig. 5. Data on F3 generation (great-granddaughters) at birth. A- Fetal body weight (g). B– Fetal body weight classification. C– Glycemia (mg/dL). D– Sex ratio percentage (%) (n = 28 female newborns/group, 2 female newborns/mother for glycemia) (n = 155 newborns for F3_C, and n = 128 newborns for F3_D for fetal body weight, fetal body weight classification, and sex ratio). Values expressed as mean ± standard deviation (SD)
$p < 0.05$—compared to the F3_C group (T-test for fetal body weight and glycemia).
*$p < 0.05$—compared to the F3_C group (Chi-square test for fetal body weight classification).

throughout the individual's life. Therefore, the body weight assessed at birth is essential to verify the transgenerational effect of diabetes. The present study demonstrated that F2_D rats showed abnormal body weight in adulthood. Regardless of whether the newborn is classified as small (SGA) or large (LGA) for gestational age, consequences occur in adult life due to abnormal growth to compensate for the birth weight [19]. Sinzato et al. [11] showed that female F2_D rats were born SGA. Ong et al. [20] verified that individuals born SGA have two pathways to follow: 1) No changes or 2) Rapid growth, called catch-up. The short-term benefits of rapid and accelerated growth are resistance to infections and childhood survival [21]. However, intrauterine restriction-induced catch-up growth in rats increases the adipocyte size in the retroperitoneal muscle, which increases the levels of circulating triglycerides and HOMA-IR, indicating the effect of catch-up growth on fat accumulation and abnormal metabolism [19,22].

In addition to the hyperglycemic maternal environment from the F0 generation, the catch-up observed in F2_D rats might also have influenced it since, in our study, the F2_D rats presented metabolic complications on day 120 of life. Although fasting glycemia had no change, F2_D rats had hyperinsulinemia and higher TyG and HOMA-IR indexes, indicating insulin resistance in these animals, similar to other studies in our laboratory [10]. Clinical studies demonstrate that fasting hyperinsulinemia and insulin resistance are substantial risk factors for the progression of diabetes [23,24]. Our laboratory has shown that maternal diabetes causes lipid and glycemic abnormalities and higher fasting insulin in daughters from diabetic rats, confirming insulin resistance by increased TyG index [10]. Besides abnormal glucose metabolism, the endocrine pancreas of adult granddaughters was also compromised. The HOMA-β index and the percentage of immunostained cells for insulin observed an improved function of β-cells. This suggests that β-pancreatic cells had been working at high capacity as a compensatory mechanism against insulin resistance to achieve glucose homeostasis. The β-cell is about to go into exhaustion, failing to synthesize and secrete insulin and progressing to diabetes, as explained by Weir & Bonner-Weir [25], who describe five stages of evolving β-cell dysfunction during progression to diabetes. This could explain the differences observed in the percentage of insulin-positive cells in the F1 and F2 generation of diabetic rats, demonstrating the evolutionary process from glucose intolerance to diabetes.

Epidemiological studies show that decreased HDL cholesterol levels are related to a high risk of developing DM2 [26,27]. HDL-cholesterol can modulate glucose concentration through insulin secretion by pancreatic β-cells and stimulate glucose uptake in skeletal muscle [28–31]. In our study, the adult female F2_D rats (120 days of life) had lower serum levels of total cholesterol in the pre-pregnancy period, suggesting that this reduction might be related to lower levels of HDL-cholesterol in these animals, also contributing to hyperglycemia. Furthermore, F2_D rats showed higher serum triglyceride levels, which are associated with diabetic dyslipidemia [32–34] and can be considered a marker of type 2 DM [35]. Excessive release of unesterified fatty acids by adipocytes under insulin resistance and/or deficiency seems to be a significant driver of dyslipidemia in diabetes and insulin resistance [36]. Thus, our results indicate that the diabetes status of the F0 generation in female rats led to fetal programming in F1 generation females and, consequently, F2 generation female rats, leading to severe impairments in these animals' different generations.

In this study, although the activity of antioxidant enzymes is lower in F2_D rats, they are managing to act; that is, they are being consumed to maintain reduced H_2O_2 levels without leading to oxidative stress. Although these animals did not present oxidative stress in their blood, more studies need to be carried out to verify whether there is the presence of oxidative stress or changes in the activities of antioxidant enzymes in tissues related to hyperinsulinemia and insulin resistance, such as the endocrine pancreas, liver and muscle.

To observe whether the metabolic changes in the pre-gestational period harmed the pregnancy of these animals, the F2_D rats were subjected to mating. In this study, the F2_D rats had a decreased percentage of rats with a positive pregnancy diagnosis, a lower percentage of rats with full-term pregnancies, and higher rates of embryo losses before and after implantation at the end of pregnancy. Previous studies have shown that daughters from diabetic rats (F1 generation) also show changes in maternal reproductive outcomes. However, different results were observed in F2_D and F1_D dams, such as fetal body weight and placental efficiency [11]. The increased percentage of LGA fetuses might be because placental efficiency is also higher in the F2_D group, contributing to a greater nutrient exchange to their fetuses (F3 generation).

The F3 generation of diabetic rats (F3_D) presented hyperglycemia at birth. Increased blood glucose stimulates the fetal pancreas to synthesize and secrete insulin. Besides its hypoglycemic hormone, insulin acts as a growth hormone [37], suggesting that the endocrine pancreas of the F3_D rats is being upstimulated due to the increased blood glucose level, consequently synthesizing and secreting more insulin. This contributed to a rise in the number of F3_D fetuses classified as LGA at birth.

This study shows the effects of diabetes in F2 and F3 generation in vivo animals using different biomarkers to identify the diabetogenic process. However, there are some limiting factors, such as the evaluation of the presence of oxidative stress in other tissues, the lack of carrying out immunohistochemical analysis of alpha and delta cells in F2 generation, and alpha, beta, and delta-cells in F3 generation, and the other limitation fact is related to the absence of calculation of Lee index of the newborns for assessment of body mass index.

7. Conclusion

In conclusion, maternal hyperglycemia, existing since the neonatal period of the F0 generation, was capable of negatively programming the females of future generations, such as hyperinsulinemia, insulin resistance, and glucose intolerance in the F2 generation. Additionally, macrosomia and hyperglycemia in the F3 generation. Thus, the transgenerational effect of diabetes contributes to the increased number of diabetic people worldwide. Then, raising awareness about lifestyle changes, such as regular exercise and a balanced diet, might help interrupt this cycle and improve the health of individuals with diabetes in future generations.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article. Raw data that support the findings of this study are available from the corresponding author, FQG, upon reasonable request.

Code availability

Not applicable.

Declarations

The procedures and handling of animals used in this study are under the guidelines provided by the Code of Ethics for the Care and Use of Animals from the Institutional Animal Care and Use Committee (IACUC) and the National Council for the Control of Animal Experimentation (CONCEA) and authorized by the Ethics Committee on the Use of Animals (CEUA) of the Botucatu Medical School – FMB/UNESP (Protocol No. 1375/2021).

CRediT authorship contribution statement

Franciane Quintanilha Gallego: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Vinícius Soares Barco:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Yuri Karen Sinzato:** Writing – original draft, Investigation. **Verônyca Gonçalves Paula:** Writing – review & editing, Investigation. **Maysa Rocha de Souza:** Writing – original draft, Methodology. **Larissa Lopes da Cruz:** Writing – original draft, Methodology. **Sayon Roy:** Writing – review & editing, Investigation. **José Eduardo Corrente:** Writing – review & editing, Formal analysis. **Débora Cristina Damasceno:** Writing – review & editing, Writing – original draft, Methodology, Investigation.

Declaration of competing interest

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

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