Systems Biology in Reproductive Medicine

ISSN: 1939-6368 (Print) 1939-6376 (Online) Journal homepage: https://www.tandfonline.com/loi/iaan20

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To cite this article: Thaigra Sousa Soares, Ana Paula Andreolla, Carolina Abreu Miranda, Eduardo Klöppel, Luhara Silva Rodrigues, Rafaianne Queiroz Moraes-Souza, Débora Cristina Damasceno, Gustavo Tadeu Volpato & Kleber Eduardo Campos (2018) Effect of the induction of transgenerational obesity on maternal-fetal parameters, Systems Biology in Reproductive Medicine, 64:1, 51-59, DOI: 10.1080/19396368.2017.1410866

To link to this article: https://doi.org/10.1080/19396368.2017.1410866

Published online: 11 Dec 2017.

SYSTEMS BIOLOGY IN REPRODUCTIVE MEDICINE
2018, VOL. 64, NO. 1, 51–59
https://doi.org/10.1080/19396368.2017.1410866

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RESEARCH ARTICLE

Effect of the induction of transgenerational obesity on maternal-fetal parameters

Thaigra Sousa Soares [a,b], Ana Paula Andreolla[a], Carolina Abreu Miranda [a,b], Eduardo Klöppel[a,b], Luhara Silva Rodrigues[a], Rafaianne Queiroz Moraes-Souza[a,b], Débora Cristina Damasceno [b], Gustavo Tadeu Volpato [a,b], and Kleber Eduardo Campos [a,b]

[a]Laboratory of System Physiology and Reproductive Toxicology, Institute of Biological and Health Sciences, Federal University of Mato Grosso (UFMT), Barra do Garças, Mato Grosso State, Brazil; [b]Gynecology, Obstetrics and Mastology Graduate Course, Laboratory of Experimental Research on Gynecology and Obstetrics, Botucatu Medical School, Univ Estadual Paulista_Unesp, Botucatu, São Paulo State, Brazil

ABSTRACT

Maternal obesity can cause complications for both women and their offspring for generations. Therefore, we intended to verify the repercussions of induction of transgenerational obesity on biochemical parameters, reproductive performance, and congenital anomaly frequency in Wistar rats. Female rats were used from successive generations. The female rats of parental generation (F_0, n=10) were mated to obtain their offspring (F_1 generation). F_1 female rats received a monosodium glutamate (MSG) solution to induce obesity (n=07) or vehicle (control, n=06) during the neonatal period. These adult female rats were classified as normal or obese using the Lee Index, mated, and delivered offspring (F_2 generation), which were also evaluated for obesity using the Lee Index in adult life (F_2MSG, n=13, born from obese dams) or non-obesity status (F_2Control, n=12, born from control dams), and were mated in adulthood. During pregnancy, glycemia and an oral glucose tolerance test (OGTT) were analyzed. At term pregnancy, the females were sacrificed for serum biochemical profile, maternal reproductive outcomes, and fetal development. In F_2MSG rats, body weight gain at early pregnancy, glycemia by OGTT, total cholesterol, high-density-lipoprotein, and alanine transaminase activity were higher compared with those of F_2Control rats. F_2MSG rats also presented a lower implantation number and gravid uterus weight, increased pre-implantation loss and anomaly frequency in their fetuses (F_3 generation) compared with those of F_2Control rats. Therefore, even without significant changes in body weight gain, obesity was established at the end of pregnancy of Wistar rats using other biomarkers. Additionally, these rats showed multiple adverse reproductive outcomes, confirming the deleterious effects that lead to obesity.

ARTICLE HISTORY
Received 4 July 2017
Revised 12 November 2017
Accepted 13 November 2017

KEYWORDS
Fetus; obesity; rat; transgenerational

Introduction

Obesity and its related comorbidities have become one of the main conditions negatively impacting public health, representing an alarming global problem. The epidemic proportions of obesity are mainly resulting from lifestyle changes, including increased high calorie intake and decreased physical activities [Flegal et al. 2015]. Obesity and being overweight have also increased in women of reproductive age [McDonald et al. 2010], and have increased strongly in populations with low and average incomes, especially in urban areas of developed countries [Nelson et al. 2010]. During pregnancy, obesity can cause complications for both women and their offspring, which may result in stillbirth and congenital anomalies [Begum et al. 2011]. The impaired maternal intrauterine environment may induce critical changes in fetal growth and development, contributing to the risk of developing disease in later life [Barker 2007; Gluckman et al. 2008].

Clinical and experimental studies show that maternal obesity induced intrauterine changes may influence the fetal organism leading to metabolic adaptations and/or complications [Gluckman et al. 2008], such as glucose intolerance, obesity, and metabolic syndrome in adult life [Barker 2007; Campos et al. 2007; Desai et al. 2013]. Obese women present an increased risk of birth defects in their offspring [Stothard et al. 2009]. In experimental models, obesity is related to an abnormal biochemical profile, with obese rats presenting with increased serum triglyceride levels and glucose intolerance in their offspring [Chen et al. 2014].

CONTACT Prof. Dr. Gustavo Tadeu Volpato gtvolpato@gmail.com Laboratório FisioTox, Instituto de Ciências Biológicas e da Saúde - UFMT, Rodovia Valdon Varjão, 6390, Barra do Garças – MT.

Studies regarding the transgenerational effects of obesity are being conducted to evaluate the offspring outcomes after maternal and/or paternal exposures of their respective parents or ancestors [Vickers 2014; Hanafi et al. 2016]. However, no study of transgenerational obesity, pregnancy, and teratogenesis in rats has yet been conducted. Therefore, we began to test our hypothesis that obesity might negatively affect the maternal and fetal organisms in future generations. The objective of the present study was to verify the repercussions during the induction of transgenerational obesity on biochemical parameters, reproductive performance, and congenital anomaly frequency in Wistar rats.

Results and discussion

Four generations of rats were used to study the repercussions of inducing transgenerational obesity in female rats as shown in Figure 1: F_0 (parental generation); F_1 (obesity induction); F_2 (for maternal study); and F_3 (for fetal study). The F_1 rats showed 0% obesity compared to the control group and 100% obesity compared to the monosodium glutamate (MSG) group, and all F_1 rats became pregnant. The F_2 dams in Table 1 showed maternal obesity biomarkers, and the F_2MSG group presented an increased body weight gain in the first week of pregnancy, total percentage of obesity by Lee index classification at days 0 and 21 of pregnancy, and increased periovarian adipose tissue weight at term pregnancy, but also showed decreased weight gain in the third week of pregnancy and gravid uterus weight compared to the F_2Control group. Similarly, women that become pregnant with higher body weight can present a lowering of this parameter until the end of the gestational period. Moreover, pregnant women with

Table 1. Obesity biomarkers during pregnancy of adult offspring from obese and non-obese dams.

	Groups	
	F_2Control *(n=12)*	F_2MSG *(n=13)*
Weight gain in pregnancy (g)		
1st week	16.9 ± 1.8	24.1 ± 6.5*
2nd week	19.8 ± 3.4	20.8 ± 4.4
3rd week	81.9 ± 4.9	55.9 ± 10.8*
Total body weight gain	118.6 ± 13.2	95.9 ± 15.1
Gravid uterus weight (g)	76.2 ± 2.5	51.9 ± 10.2*
Positive obesity (%)[a]		
Day 0	0.0	100.0*
Day 21	8.3	100.0*
Periovarian adipose tissue weight (g)	3.3 ± 0.6	5.2 ± 0.6*

Data shown as mean± standard error of mean (SEM); *p<0.05 - compared to F_2 Control group (Student´s t test; [a]Fisher´s Exact test).

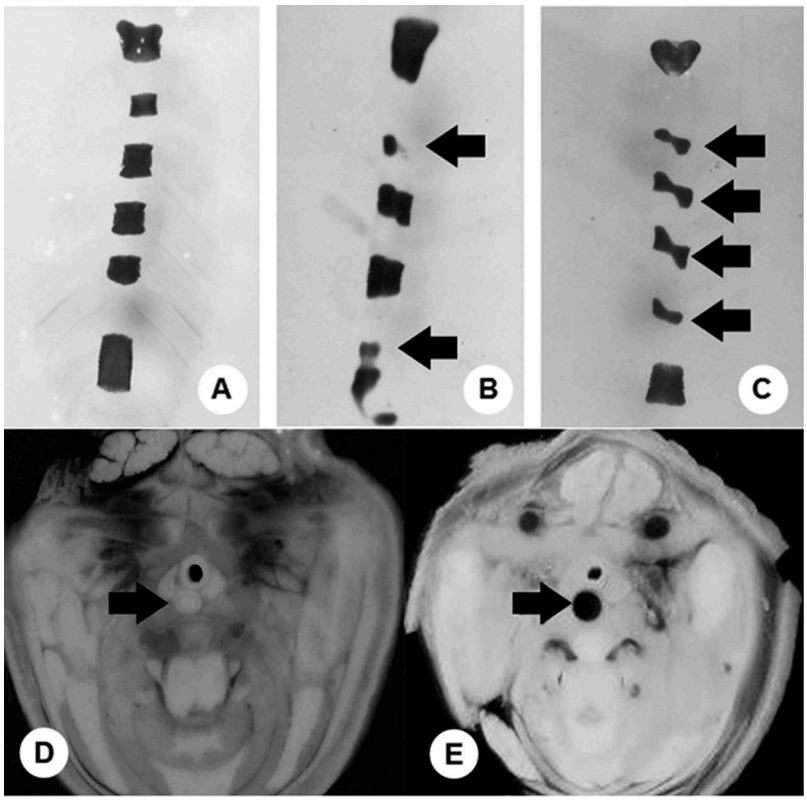

Figure 1. Experimental design. F_0: Healthy female rats mated to obtain F_1; F_1 (obese rats): Female newborn induced to obesity by monosodium glutamate (days 2, 4, 6, 8, and 10 of life) and mated (90 days old); F_2: Female offspring from obese rats and mated (90 days old); F_3: Fetuses of F_2 generation.

morbid obesity may present lower or no body weight gain [Rasmussen et al. 2010; Thangaratinam et al. 2012].

The Lee index is an anthropological biomarker for obesity classification used in experimental studies [Bernardis and Patterson 1968; Fernandes et al. 2012]. The higher periovarian fat weight and 100% obesity of F_2MSG rats are consistent with obesity in our study. Several different biological mechanisms for obesity development, such as epigenetic factors influencing phenotype parameters, even in the absence of genetic or environmental alterations [Desai et al. 2015], affect metabolic pathways and promote obesity throughout generations [Armitage et al. 2008].

The early pregnancy period in the F_2MSG group showed greater body weight gain, and this was expected to be maintained throughout the pregnancy. However, even with obesity confirmation, a lesser body weight gain in the last week of pregnancy was verified, which is directly related to the embryo loss at the beginning of pregnancy. This finding is associated with the reduced gravid uterus weight shown in this study. Moreover, the anthropometric parameters are considered obesity biomarkers [Novelli et al. 2007]. The intrabdominal adipose tissue accumulation (periovarian and periepididymal tissue) is often referred to as visceral obesity in rats and is traditionally associated with this syndrome [Hishikawa et al. 2005; Tchernof and Després 2013]. The increased visceral fat supports studies using MSG-induced obese rats [Von Diemen et al. 2006; Suárez-Román et al. 2016], as MSG neonatal treatment leads to a series of endocrine disturbances, such as an inhibitory regulatory effect of the adrenal glands, which increases glucocorticoid levels and fat mass [Perello et al. 2003; Moreno et al. 2006; Von Diemen et al. 2006; Zubiría et al. 2014].

Blood glucose was increased in early pregnancy in F_2MSG rats compared with those of control dams. Moreover, blood glucose levels at fasting and 120 minutes (min) timepoints in the oral glucose test tolerance (OGTT) were higher compared with those of F_2Control rats. At 30 and 60 min of OGTT, glycemia was increased in both groups compared to fasting glycemia in the same group, but the glycemia was increased at 120 min compared to fasting glycemia only in the F_2MSG group. The area under the curve (AUC) of OGTT was also increased in F_2MSG rats in relation to that of F_2Control rats (Table 2).

Obesity is strongly associated with an imbalance in glucose homeostasis [Hotamisligil et al. 1993], which is related to abnormal insulin secretion and/or action that leads to glucose intolerance [Campos et al. 2007]. Although our study showed no differences in serum glucose on specific days during the gestational period, the transgenerational obesity model showed higher blood glucose levels in OGTT and AUC, suggesting a classical glucose intolerance [Foti et al. 2005; Campos et al. 2007]. The impairment of glucose tolerance is dynamic by a considerable number of inducing factors, and shown in other studies using transgerational obesity model in rats, by obesogenic diet in the parental generation [Hanafi et al. 2016], and in mice using a combination of high chaloric feeding and low dose streptozotocin treatment in the previous generation [Wei et al. 2014] or a low protein diet inducing altered glucose homeostasis from F1 to F3 mice [Frantz et al. 2011]. In all these studies, including this one, glucose tolerance alteration provoke changes in uterus homeostasis in the parental generation. Pregravid obesity is associated with a systemic inflammatory state that contributes to worsening of pregnancy and imbalance of glucose homeostasis. Moreover, glucose is the main energy source for development and fetal growth in later pregnancy can be impaired responding to glucose uptake from the maternal organism [Herrera and Ortega-Senovilla 2010]. Glucose intolerance and insulin resistance are related to a decrease of insulin-stimulated IR, IRS1, and IRS2 tyrosine phosphorylation, and associated to the substrates PI-3K in liver and muscles. In adipose tissue, a decreased association of insulin-stimulated IRS1 tyrosine phosphorylation and IRS1/PI-3K is evident. Both effects impair GLUT-4 translocation in cell membranes [Foti et al. 2005; Prada et al. 2007].

Table 3 presents maternal biochemical parameters. F_2MSG rats presented a decreased total cholesterol and high-density lipoprotein (HDL-c). The reduced total

Table 2. Glycemic levels and oral glucose tolerance test (OGTT) during pregnancy of adult offspring from obese and non-obese dams.

	Groups	
	F_2Control *(n=12)*	F_2MSG *(n=13)*
Glycemic level on pregnancy (mg/dL)		
Day 0	99.1 ± 7.1	119.2 ± 4.5*
Day 7	107.2 ± 7.8	115.8 ± 3.9
Day 14	97.7 ± 10.7	98.0 ± 3.7
Day 21	94.6 ± 3.4	88.0 ± 3.4
Glycemic level on OGTT (mg/dL)		
Fasting timepoint	69.3 ± 2.6	82.0 ± 2.1*
30 min timepoint	113.4 ± 8.4#	119.6 ± 4.7#
60 min timepoint	97.0 ± 3.1#	100.3 ± 4.2#
120 min timepoint	65.3 ± 2.7	91.3 ± 5.1*#
Area under the curve (mg/dL/120min)	10765.7 ± 349.7	12067.5 ± 368.8*

OGTT: oral glucose test tolerance. Data shown as mean± standard error of mean (SEM); * $p<0.05$ - compared to F_2 Control group (Student´s t test); # $p<0.05$ – compared to fasting timepoint (ANOVA followed by Dunnett's test).

Table 3. Maternal serum biochemical parameters, triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL-c), very low-density lipoprotein (VLDL-c), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), at 21st day of pregnancy of adult offspring from obese and non-obese dams.

	Groups	
	F_2 Control *(n=12)*	F_2 MSG *(n=13)*
TG (mg/dL)	120.6 ± 7.9	146.2 ± 13.3
TC (mg/dL)	133.5 ± 11.2	100.0 ± 4.7*
HDL-c (mg/dL)	50.7 ± 4.7	35.7 ± 3.9*
VLDL-c (mg/dL)	24.1 ± 1.6	27.9 ± 1.8
ALT (U/L)	62.5 ± 7.2	44.9 ± 3.4*
AST (U/L)	106.0 ± 10.5	109.4 ± 11.1

Data shown as mean± standard error of mean (SEM). * $p<0.05$ - compared to F_2 Control group (Student´s t test).

Table 4. Maternal reproductive outcome of adult offspring from obese and non-obese dams.

	Groups	
	F_2 Control *(n=12)*	F_2 MSG *(n=13)*
Pregnant rats	12	13
At term pregnancy	12	9
Corpora lutea		
Total (N)	152	197
Mean ± SD	12.67 ± 0.40	15.15 ± 1.01*
Implantation		
Total (N)	145	106
Mean ± SD	12.08 ± 0.43	8.15 ± 1.69*
Live fetuses		
Total (N)	134	101
Mean ± SD	11.17 ± 0.41	7.77 ± 1.65
Dead fetuses		
Total (N)	2	0
Mean ± SD	0.17 ± 0.17	0.00 ± 0.00
Resorptions		
Total (N)	9	5
Mean ± SD	0.45 ± 0.46	0.38 ± 0.18
Litter size[b]	11.33	11.22
Live birth index (%)[c]	98.53	100.00
Pre-implantation loss (%)[a]	4.61	46.19*
Post-implantation loss (%)[a]	7.58	4.72

Data shown as mean±standard deviation (SD) and proportions (%)[a]. * $p<0.05$ - compared to F_2 Control group (Student´s t test;[a]Fisher´s Exact test); [b] Litter size: Total number of pups delivered (live and still-born)/Number of dams that delivered; [c] Live birth index: (Number of pups born alive/Total number of pups born) x 100.

cholesterol and HDL-c levels are related to the fluctuating anabolism during the first two thirds of pregnancy by fat deposit accumulation. During the last third, a catabolic status is observed when fat deposit breakdown is enhanced [Herrera and Ortega-Senovilla 2010]. Serum lipid changes during the pregnancy can affect the outcomes at the end of the gestational period [Scifres et al. 2014]. Although there is a classic increase of triglyceride levels in the obesity state [Knopp et al. 1970], our study showed pregnancy preferentially induced catabolism of carbohydrates and lipids for fetal development, confirmed by unchanged blood glucose levels and decreased serum triglycerides. The tendency for the hypoglycemic state found in late pregnancy is compensated by the uptake of circulating triglycerides, leading to an impaired lipoprotein lipase activity in maternal tissues [López-Luna et al. 1991; Desai et al. 2013]. Moreover, the increase of hepatic enzymes in blood are important indicators of liver injury, like alanine aminotransferase activity (ALT) [Milinković et al. 2005]. ALT was reduced in F_2MSG rats, showing a value within a normal range [Messias et al. 2010], suggesting no hepatic change.

The adult female offspring born to obese rats (F_2MSG) presented reduced implantation and increased embryonic loss before implantation in relation to those of F_2Control rats (Table 4). Suárez-Román et al. [2016] also verified a lesser implanted embryo number and Campos et al. [2008] observed a reduced fertility rate in MSG-induced obese rats. Similarly, obese women are three times more probable to undergo low fertility compared with women presenting with an adequate body mass index [Rich-Edwards et al. 1994], which is also related to insulin resistance and hyperandrogenemia [Dağ and Dilbaz 2015]. Similar to humans, obesity also induces reduced implantation and pregnancy rates, impairs reproductive functions by affecting both ovaries and endometrium, mainly by changes in luteinizing hormone and androstenedione levels [Dağ and Dilbaz 2015]. Then, excess body fat impairs female reproductive functions in humans and in laboratory models [Hall et al. 2005]. It is suggested that a mechanism is the transmission by altering the epigenome through somatic or germ cells [Bazzano et al. 2015]. An abnormal maternal intrauterine environment directly affects the fetus and their germ cells, which are the origin of the next generation [Aiken and Ozanne 2014]. However, the exact mechanisms by which excess body fat interferes with reproductive function are still not fully understood.

In the F_2MSG group, it was verified that nine rats finished at term pregnancy, nine of them finished at term pregnancy. No differences in fetal and placental weights, or placental efficiency were observed (Table 5). Moreover, the total number of fetuses with anomalies showed no difference between the experimental groups. The rate of fetuses with incomplete ossification of the sternebra, abnormally shaped sternebra, and a distended trachea were increased in the F_3MSG group compared with those of control rats (Table 6; Figure 2).

In a study of pre-gestational obesity in rats, maternal obesity led to an embryotoxic effect and reduced somite numbers [Suárez-Román et al. 2016]. The changes found in F_3MSG newborns, such as incomplete ossification of sternebrea and abnormal sternebrea, are considered variations, which were

Table 5. Fetal and placental weights, and placental efficiency of adult offspring from obese and non-obese dams.

	Groups	
	F_2 Control *(n=12)*	F_2 MSG *(n=09)*
Fetal body weight (g) Mean ± SE	5.16 ± 0.04	5.08 ± 0.03
Placental weight (g) Mean ± SE	0.42 ± 0.01	0.41 ± 0.01
Placental efficiency Mean ± SE	12.65 ± 0.18	13.07 ± 0.22

Data shown as mean± standard error (SE). $p > 0.05$: non-significant statistical difference.

Table 6. Fetal anomaly frequency of adult offspring from obese and non-obese dams.

	Groups	
	F_3 Control *(n=134)*	F_3 MSG *(n=101)*
External anomalies		
Number fetuses examined (litter)	134 (12)	101 (9)
Total number of fetuses (%) with alteration	1 (0.7%)	2 (2.0%)
Mean % fetuses with alteration per litter (mean ± SE)	0.8 ± 0.8	2.0 ± 1.3
Hidropisia	0 (0.0 %)	1 (1.0 %)
Gastrosquises	1 (0.7 %)	1 (1.0 %)
Skeletal anomalies		
Number fetuses examined (litter)	65 (12)	53 (9)
Total number of fetuses (%) with alteration	30 (46.1%)	28 (53.8%)
Mean % fetuses with alteration per litter (mean ± SE)	45.6 ± 7.4	54.0 ± 5.5
Incomplete ossification of cranius	1 (1.5%)	1 (1.9%)
Supranumerary rib	4 (6.1%)	5 (9.4%)
Wavy rib	2 (3.1%)	0 (0.0 %)
Costela reduzida	1 (1.5%)	0 (0.0 %)
Sternebra agenesis	2 (3.1%)	0 (0.0 %)
Unossified sternebrae	13 (20.0%)	8 (15.1%)
Incomplete ossification of sternebrae	8 (12.3%)	18 (34.0%)*
Abnormally shaped sternebrae	14 (21.5%)	25 (47.2%)*
Visceral anomalies		
Number fetuses examined (litter)	62 (12)	48 (9)
Total number of fetuses (%) with alteration	21 (33.9%)	23 (47.9%)
Mean % fetuses with alteration per litter (mean ± SE)	32.4 ± 7.9	47.3 ± 8.7
Distended Trachea	0 (0.0%)	13 (27.1)*
Extended artery Anonymous	2 (3.2%)	0 (0.0%)
Kidney agenesis	1 (1.6%)	0 (0.0%)
Calice extended	1 (1.6%)	0 (0.0%)
Hydroureter	18 (29.0%)	16 (33.3%)
Hydronephrosis	0 (0.0%)	2 (4.2%)
Sinuosis Ureter	1 (1.6%)	0 (0.0%)
Ectopic testicle	1 (1.6%)	0 (0.0%)

Data shown as mean± standard error (SE) and proportions (%).* $p<0.05$: compared to F_3 Control group (Fisher´s Exact Test).

merely observed and represent no teratological effect. However, tracheal abnormality is classified as a malformation, leading to functional consequences [Solecki et al. 2003]. Human and animal studies show similar outcomes in an inadequate intrauterine environment, which leads to an increased susceptibility to obesity in later life [Vickers 2014]. The results of this study improve the understanding of the transgenerational biological mechanisms to the metabolic disorder transmission.

As the main limiting factor of our study, we highlight the difficulty of mating in F_1 obese dams. Due to the reduced number of at term pregnant obese dams, the F_2 female pups collected to F_1 pregnant obese dams were 1 to 3 even though the final data collected were satisfactory. The experimental rat systems constantly evolve and the appropriate methodologies are the best way to collect better data [Iannaccone and Jacob 2009]. The use of a transgenerational obesity model to study reproductive parameters can be a starting point to further research, using treatment to decrease the obesity index and improve the reproductive biomarkers, or to comprehend the action mechanism of pre-implantation loss with evaluation of blastocyste or uterine morphometry.

Obesity induces biological changes in the maternal pregnancy period as the fetus develops. It is important to evaluate health conditions before considering pregnancy, aiming at reducing the abnormal maternal intrauterine environment-induced negative effect on their offspring, which may interfere with fetal programming. When heritable, obesity affects the embryo, its perinatal development, and the adult life of these offspring. So, even without relevant changes in body weight gain at term pregnancy, the female rats born to obese dams presented with abnormal glucose tolerance and triglyceride levels at the end of pregnancy, and their obesity was established by other biomarkers. Additionally, multiple adverse reproductive outcomes and impaired fetal development were demonstrated, showing the deleterious effects that may lead to transgenerational obesity.

Materials and methods

Parental generation rats (F_0)

Wistar rats weighing about 200 grams (g) (90 d of life) were adapted for seven days in the vivarum of the Laboratory of System Physiology and Reproductive Toxicology (FisioTox). The rats were kept in collective cages in controlled conditions of temperature (22 ± 2°C), light (12h light/dark cycle), and relative humidity (60 ± 5%). The animals were fed laboratory chow 3.0 Kcal/g (Presence Rat Chow®, Paulínia, Brazil) and tap water *ad libitum*. The procedures and animal handling were performed in accordance with the guidelines provided by the Brazilian College of Animal Experimentation in agreement with the International Guiding Principles for Biomedical Research Involving Animals promulgated by the Society for the Study of Reproduction. The Ethical

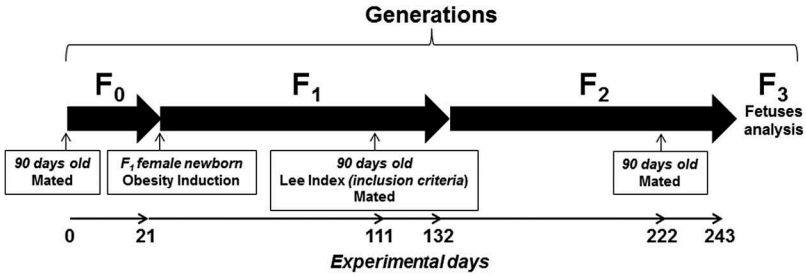

Figure 2. Main anomalies showed in F_3 fetuses. (A) Normal sternebra of rat fetuses; (B) incomplete ossification of sternebra (arrow); (C) abnormally shaped sternebra (arrow); (D) section of normal rat fetus's thorax with normal trachea (arrow); and (E) distended trachea (arrow) in fetus's thorax section.

Committee for Animal Research of the UFMT Brazil approved all protocols under process number 23108.045215/2014-39.

For offspring obtained for obesity induction, females (F_0 generation, n = 10) were caged overnight with males. Mating was confirmed by the presence of sperm in the vaginal smear the next morning. Pregnant rats were kept in individual cages during the pregnancy period (21 d), including vaginal delivery, and lactation periods (21 d). The experimental protocol followed four generations (F_0 to F_3) as shown in Figure 1.

F_1 generation rats - obese and non-obese rats

From total number of offspring, only female newborns (NB) were kept until the number of eight with their own dams (one newborn/nipple) from d 0 to the end of the lactation period (21 d). When the number of females would not reach eight, male NB were placed in the litter. This procedure was used to obtain better and equal feeding for all NB. Female offspring were randomly distributed into two groups: rats that received saline solution (0.9% NaCl) subcutaneously (sc) at postnatal d 2, 4, 6, 8, and 10 of life (Control group, n=6) and rats given a solution of monosodium glutamate (MSG - $C_5H_8NNaO_4.xH_2O$ G1626 Sigma-Aldrich®, St Louis, USA) with a dose of 4.0 mg/g body weight, sc. at the same days as those of the Control group (MSG group, n=7) [Campos et al. 2007, Suárez-Román et al. 2016]. After weaning, all female rats were transferred to collective cages (four animals per cage) and maintained until adult life (90 d of life) in controlled conditions.

At 90 d of life, the parameter of obesity was obtained by the Lee Index, defined as the cube root of body weight (g) x 10/nasoanal length (cm), for which a value equal to or less than 0.300 was classified as normal. Rats presenting values higher than 0.300 were classified as obese [Bernardis and Patterson 1968; Fernandes et al. 2012] and included in the MSG group. The exclusion criteria were applied when the Lee Index values were higher than 0.300 for rats neonatally treated with saline; or values equal or lower than 0.300 for rats neonatally treated with MSG. After the Lee Index, all female rats were mated overnight with normal males and the sperm presence in vaginal smears the next morning was defined as d zero (0) of pregnancy and included in their respective group. The pregnant rats were kept in individual cages during pregnancy (21 d) and weaning (21 d) periods.

F_2 generation rats – offspring from obese and control dams

The offspring obtained from F_1 rats is known as F_2 generation. These rats were kept in controlled conditions until adult age. At d 90 of life, all F_2 female rats were mated with normal male rats, and after the confirmation of pregnancy (d 0), the rats were classified as normal or obese by the Lee Index. During pregnancy, the rats were maintained in individual cages and in two experimental groups, following their respective F_1 generation: F_2Control - offspring from non-obese dams (n=12), and F_2MSG - offspring from obese dams (n=13).

Course of pregnancy of F_2 rats

At d 0 (early pregnancy), 7 (embryonic period), 14 (fetal period), and 20 of pregnancy (end of pregnancy – at term pregnancy), maternal body weight and glycaemia were evaluated. Blood samples were obtained by venous puncture of the tail for blood glucose concentrations by conventional glucometer (One Touch Ultra - Johnson & Johnson®, São José dos Campos, Brazil) and the values were presented in mg/dL. Total body weight gain was determined using the difference of the last and the first day of body weight measured in the pregnancy period.

After 6 h of fasting, all the rats were submitted to OGTT at d 17 of pregnancy for evaluation of glucose intolerance status. Glucose responses during the OGTT were evaluated by the estimation of the total area under

the curve (AUC), using the trapezoidal method [Sinzato et al. 2012].

At d 21 of pregnancy, the Lee Index was performed one more time and the rats were lethally anesthetized by sodium thiopental (Thiopentax®). The periovarian adipose tissue was removed and weighted. The gravid uterus was dissected to count dead and live fetuses, resorption (embryonic death), implantation sites, and corpora lutea numbers. The rate of pre-and postimplantation loss, the litter size, and live birth index were calculated [Volpato et al. 2015; Parker 2012].

Maternal whole blood samples obtained at d 21 of pregnancy were centrifuged at 1,200 x *g* to obtain serum. The serum samples were then stored at -20°C for measurement of triglyceride, total-cholesterol and high-density lipoprotein levels, and aspartate aminotransferase and alanine aminotransferase activities were determined using commercial kits (Wiener® Rosario, Argentina). The very low-density lipoprotein levels were determined by mathematical estimation [Knopfholz et al. 2014].

The weight of the F_3 generation of fetuses, following the groups as their dams (F_3Control rats from F_2Control dams, F_3MSG rats from F_2MSG dams) and their placentas from these dams were weighed to calculate the placental efficiency (fetal weight/placental weight). The fetuses were evaluated in a microscope with respect to incidence of external anomaly. After external analysis, half of the fetuses were fixed in Bodian´s solution and serial sections were prepared as described by Wilson [1965] for visceral examination. The remaining fetuses were prepared for examination of the bones by the staining procedure of Staples and Schnell [1964].

Statistical analysis

For comparison of the mean values between the experimental groups, Student's t test was used. The proportions were calculated by Fisher's exact test. ANOVA followed by Dunnett test was used to compare different timepoints during OGTT. For fetal data, litter was used as the statistical unit. Differences were considered statistically significant when $p < 0.05$.

Acknowledgments

The authors wish to thank Iuri Júnor Francisco, Biomedical Sciences graduate student, for technical assistance.

Declaration of interest

The authors report no conflicts of interest with respect to the research, authorship, and/or publication of this article.

Notes on contributors

Conception and design of the study (Figure 1) were carried out by: GTV, KEC; Analysis and interpretation of reproductive parameters (Tables 1, 4, 5, and 6; and Figure 2) were supported by: TSS, LSR, RQM-S, DCD, GTV; Analysis and interpretation of Metabolic parameters (Tables 2 and 3) by: APA, CAM, EK, DCD, KEC; The drafting of the article, critical revision, and final approval of the manuscript were supported by all authors.

ORCID

Thaigra Sousa Soares http://orcid.org/0000-0003-4402-0241
Carolina Abreu Miranda http://orcid.org/0000-0002-4402-1466
Débora Cristina Damasceno http://orcid.org/0000-0002-7003-9643
Gustavo Tadeu Volpato http://orcid.org/0000-0002-4753-3264
Kleber Eduardo Campos http://orcid.org/0000-0001-6847-4577

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