

## Effects of the Inbreeding Reproduction on the Obesity Phenotypes Over Generations

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### Abstract

**Background:** The literature describes an increase in milk intake during the rat lactation period when the litter size is reduced, which induces an obesity condition in adulthood. However, there are no reports in this obesity model concerning the heritability of obesity phenotypes.

**Objectives:** This study aimed to evaluate in males rats, the effects of a consanguineous reproduction on obesity phenotypes produced by over-nutrition during the lactation period in the litter size reduced, over 5 generations.

**Materials and Methods:** Litters were reduced to 2 males and 2 females after birth and the consanguineous mating was performed in adulthood. A normal litter containing 8 males was used as no consanguineous group to compare the results with those of the reduced litters concerning to follows obesity phenotypes evaluated: Lee index, body mass, food intake, retroperitoneal fat deposit, length of the small intestine, goblet cells number, as well as for the glucose, cholesterol, triglycerides, LDL, HDL and VLDL levels.

**Results:** The obesity morph phenotypes were significantly increased ( $p < 0.05$ ) and maintained over generations being followed with opposite changes in the LDL, HDL, and VDL levels, while glucose, cholesterol and triglycerides were reduced.

**Conclusion:** We conclude that the effects detected were maintained over generations using the inbreeding reproduction associated to reduction in size litter which were an increase in the morphological and some of the metabolic obesity phenotypes as well as the reduction in the glucose, cholesterol and triglycerides levels.

### Keywords

Consanguinity, Rat, Obesity Phenotypes, Reproduction

### Introduction

The World Health Organisation [1] reported that in 2016, 39% women and 18% children and adolescents aged 5-19 were overweight or obese. An analysis of 450 nationalities, from representative surveys carried out in 144 countries, showed that the prevalence of obese children <5 years of age increased from 4.2% in 1990 to 6.7% in 2010 and is expected to reach 9.1% in 2020 [2, 3]. Therefore, obesity is a global problem of public health, especially in circumstances of social calamity, when the health system exhausts its operational capacity, as observed in the current pandemic produced by the COVID-19.

In humans, studies have been tried to understand the relationship between maternal-fetal nutrition interaction and metabolic syndrome by the metabolic imprinting process, also denominated genomic imprinting [4-6]. In general, studies about the genetic of obesity aim to answer the following question: can metabolic changes be transmitted from one generation to another?

On the other hand, animal models have been developed to be used in studies about obesity and in this context, the use of the reduction of animals by litter [7] seems to be a realistic model closest to what occurs with humans regarding the number of children by couple concerning fertility. Studies on human fertility reveal that the fertility rate (expressed by live births per woman) taken in a period of 5 years, from 1950 until 1955 was 4.45 and from 2015 until 2020 was estimated in 2.25 live births per woman. For example, in Brazil in the same periods, fertility reduced from 6.1 to 1.74 live births per woman according to the United Nations [8].

Therefore, in experimental studies, when the number of rats is reduced from 8 (a standard litter) to 3 or 4 animals (reduced litter) the pups have hyper nutrition during the lactation period and this condition leads to obesity in adulthood [9]. In humans, an association between the family genetic background and the modern lifestyle, in which a lot of low nutrition quality food is offered during childhood, seems to predispose the children to obesity in adulthood [10-13].

Furthermore, reports also describe that the base of the metabolic imprinting for obesity is the genetic background, which may be produced by epigenetic mechanisms. In fact, the epigenetic mechanisms have received more attention in human studies, as well as in the experimental models of obesity [14,15].

Therefore, assuming that the reduction of rat number by litter increases their food intake during the lactation period induce an obesity condition in adulthood, our hypothesis in this investigation was that the association of this experimental model with a consanguineous reproduction could stabilize some of these phenotypes of the obesity along the generations produced.

Therefore, we aimed to evaluate the effects of consanguineous reproduction associated to known study model of obesity produced by reduction in the number of animals by litter, on the obesity phenotypes over 5 generations.

## Materials and Methods

### Experimental design

The sample size was defined based on a pilot experiment performed in our laboratory using three animals in which a quantitative morphological parameter (number of goblet cells) from the small intestine was evaluated. The results were transformed into the square root and submitted to analysis of variance test to obtain the mean, the minimum difference between the means and the standard deviation of the mean. These values were used to determine the n sample in the Bioestat 5.0 software of the public domain obtained from the

Mamirauá Institute in Brazil [16] (<https://www.mamiraua.org.br/downloads/programas/>) adopting a significance level of  $\alpha = 0.05$  and a test power of 80%. The result indicated a minimum number of four animals per repetition. However, we decided to use three litters (six males and six females) for each generation. Because only males were investigated in this experiment, the final n sample for each generation evaluated was six animals that were submitted to all analyses performed in the present work.

### Ethics in animal research

The University Committee for Ethics in Animal Research approved all experiments in the study with the protocol number 19741/2014. All animals used in this investigation (Wistar Rats) were obtained from the UEPG animal house (NAEVI) and were kept under conventional conditions with a 12 h light/ dark cycle (lights on at 06:30 am/lights off at 6:30 pm), at room temperature between 23° and 25° celsius, and received food (nutrition-balanced ration from Nuvital, Brazil) and water ad libitum. In each animal box, a PVC tube (25 cm X 10 cm) was placed to increase animal welfare along the experimental period.

### Obtaining the normal litter group (C)

From a family denominated A, three adult male rats were mated with three females from a family denominated B. All rats had a mean weight of 300 g and three months old when were mated in the 1:1 ratio (male: female). After being born, the pups were reduced to eight animals by litter which includes males and females. At the weaning eight males were jointed in a box to form the control group (normal litter size) which was submitted to the same analysis performed by the reduced litters as mentioned in the next topics. In this experiment, the rats from the control group, were used to establish a start reference point to compare the parameters obtained between a size normal litter with those reduced size litter produced in each generation. On the other hand, this group will not be necessary because our aim in the present investigation focused in the reduced litter size and consanguineous reproduction. The use of only one control group also consider the ethic principle of 3R, that is, the reduction of the number of experimental animal as son as possible.

### Obtaining the reduced litters from zero up to 5 generations (G0-G5)

Three adult male rats from the A family were mated with three female rats from the B family in the 1:1 ratio (male:female) and after birth, the pups were reduced randomly on the third day into two males and two females by each litter, which were denominated G0. When these animals (G0) reached 90 days of life the first inbreeding was performed by mating one female from each litter with her brother. These matings generated a second-generation (denominated G1) in which pups were reduced randomly on the third day after being born into two females and two males by each litter and thus successively reduced litters were produced until G5. However, in the experiments, only males were used totaling n=6 per generation evaluated and n=8 for the normal litter (control group). For this report, we will present most of the

results obtained for the C, G1, G3 and G5 groups, while for some parameters, we will present the results obtained for the G5 group.

### Estimative for lee index, body mass, food intake and retroperitoneal fat deposit

The rat body mass mean was taken using an electronic balance on 30, 60 and 90 days of life and was expressed in grams. Food consumption was measured two times/week during all the experimental period using an electronic balance and was expressed as a mean of food intake by each rat, in grams. On the 90th day, the rats were anesthetized with halothane and the length from head to the caudal region was measured to estimate the Lee Index, which was calculated as the ratio between the cubic body mass (measured in grams) and skull-caudal length (measured in centimeters) according to Bernardis and Patterson [17]. For the fat deposit estimative, the abdominal region was opened by laparoscopy and the retroperitoneal fat from both sides of the body was taken being used an electronic balance to determine fat pad mass, which result was expressed in grams.

### Estimative for small intestine length and number of goblet cells

The small intestine was collected and the total length was taken using a measuring tape and was expressed in centimeters (cm). For estimative of goblet cell number, a sample of the small intestine (jejunum region) was collected from control and G5 group and immersed in 2% paraformaldehyde, dehydrated in crescent ethanol concentration from 70% up to 100%, immersed in 3 baths of xylol and embedded in paraffin. Longitudinal semi seriated sections of 5  $\mu$ m were obtained and submitted to PAS to detect neutral mucin and stained with alcian blue, pH 4,0, to detect acid mucin. The results were expressed by the number of goblet cells obtained in 20 villi counted in each animal for group evaluated.

### Obesity phenotype blood parameters

When all animals reach 90 days of life they were anesthetized using and injection with xylazine (15 mg/kg) and ketamine (50 mg/kg). Four milliliters of blood were collected in micro tube, using the cardiac puncture method. To avoid the interference of the circadian variation, the blood samples were taken in the morning period. After that, the blood samples were incubated for 10 minutes at 37 °C, centrifuged at 3000 rpm and the supernatant was transferred to another clean micro tube and stored under -20 °C. After that, 300 micro-liters were submitted to spectrophotometry analysis using enzymatic staining from Wiener Lab.® (Rosario, Argentina) according to the manufacturer's instructions. The samples were analyzed at 505 nm and 37°C for the determination of the concentration of the following molecules: glucose, total cholesterol, triglycerides, high-density lipoprotein (HLD), very low-density lipoprotein (VLDL), and cholesterol low-density lipoprotein (LDL-C) which was calculated from the formula of Friedwald:  $LDL-C = total\ cholesterol - HDL-C - (triglycerides/5)$ . The results obtained from 6 male rats of each generation were expressed as mg/dL.

### Statistical analysis

The parameters were analyzed by Graphpad prism software 3.0. Lee Index, Body Mass, Food intake, blood lipids, and glucose profile, small intestine length and retroperitoneal fat deposit were submitted to ANOVA with the Tukey post-test, and the number of goblet cells was submitted to Student t-test at the significance of  $P < 0.05$  level.

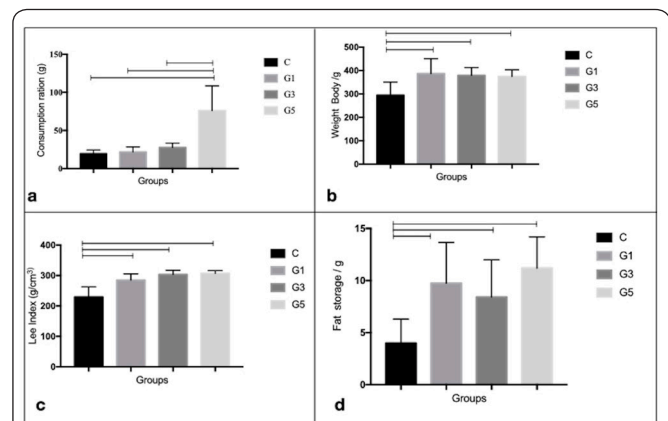
## Results

### The morphological obesity phenotype was maintained along 5 generations

An increase ( $P < 0.05$ ) in the food consumption was observed for the G5 group when compared to G3, G1, and C, respectively (Figure 1a). This finding was confirmed by the increase in the weight body (Figure 1b), Lee index (Figure 1c) and retroperitoneal fat deposit (Figure 1d) observed for the G5, G3, and G1 groups when compared to C group ( $P < 0.05$ ).

### The blood obesity phenotypes were significantly changed along 5 generations

Figure 2a shows that the glucose level decreased for the G1 and G3 groups, being significant for G5 when compared to C group ( $P < 0.05$ ). Total cholesterol level was reduced ( $P < 0.05$ ) for the G1, G3 and G5 groups when compared to C group (Figure 2b). The triglyceride levels also decreased ( $P < 0.05$ ) for the G1, G3, and G5 groups when compared to C group (Figure 2c). Concerning lipoproteins LDL and HDL,

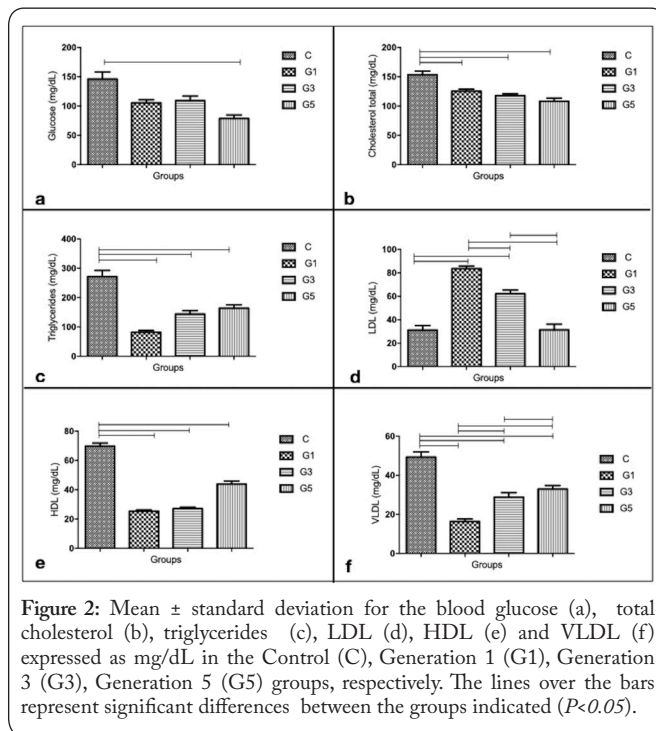


**Figure 1:** Mean  $\pm$  standard deviation for the food consumption (a), body weight (b), Lee Index (c) and retroperitoneal fat deposit (d), measured in the Control (C), Generation 1 (G1), Generation 3 (G3), Generation 5 (G5) groups, respectively. The lines over the bars represent significant differences between the groups indicated ( $P < 0.05$ ).

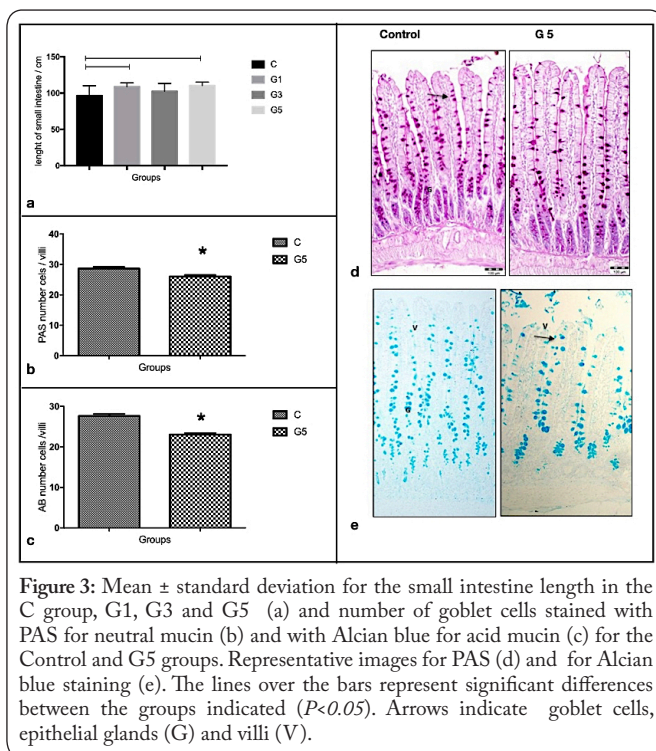
the results demonstrated an increase ( $P < 0.05$ ) in LDL for the G1 and G3 groups when compared to C group and G5, respectively, (Figure 2d) while the HDL showed a decrease ( $P < 0.05$ ) for the G1, G3, and G5 groups when compared to C group. The VLDL levels were reduced ( $P < 0.05$ ) for the G1, G3, and G5 groups when compared to C group.

The length of the small intestine increased while the number of the goblet cells was reduced. The figure 3a shows an increase in the length of the small intestine ( $P < 0.05$ ) for the





G1 and G5 compared to the C group. The figures 3b and 3c show a reduction ( $P < 0.05$ ) of the number of the goblet cells producing neutral and acid mucin, respectively, for the G5 group compared to the C group. Representative images of the reduction in the goblet cells submitted to PAS reaction and Alcian blue staining are presented in the Figures 3e and 3d, respectively



## Discussion

The suckling phase is considered one of the “windows of development” in which a reduction in the number of rats per

litter leads to an increase in milk intake, imprinting metabolic imprinting and producing an obesity condition in the adulthood [18,19].

However, there are few studies concerning transmissibility of the metabolic or morph phenotypes changes into successive generations, using the obesity model produced by reduction of size litter. Therefore, in the present study, we carried out experiments to evaluate the effects of a consanguineous reproduction on obesity phenotypes produced by over-nutrition using reducing the number of rats per litter size from one to the next generation.

We demonstrated that the values for weight body, Lee index, retroperitoneal fat deposit, and length of the small intestine were higher in the rats of first-generation (G1) and remained increased after a consanguineous reproduction. These results indicate the transmissibility of the obesity characteristics for G3 and G5. In an F1 generation of mice, where the imprinting metabolic used was a higher calories diet from gestation up to adulthood phase, also demonstrated an increase in % body fat. However, in this imprinting model, the only metabolic parameter significantly increased in the F1 was the glucose level [9].

On the other hand, our results showed that the glucose, cholesterol, and triglycerides did not follow the increase observed for the morph phenotypes weight body, fat storage, food consumption or length of intestine. Although they seem discrepant results, we consider that they are perfectly plausible when compared to results found in the literature. For example, in the obesity model based on a high-fat diet [20, 21] any increase in the glucose level was demonstrated, however, in the obesity model using a high carbohydrate diet (higher glucose levels) or for a cafeteria diet a reduced level for glucose was found [22-24] which is similar with our results using the consanguineous reproduction from reduced size litter.

In addition, the literature also demonstrates different results concerning molecules involved in adiposity regulation. It is known that the abdominal fat deposits [25] increase the risk to develop metabolic syndrome due to their relationships with the lipolysis and lipogenic enzymes such as the 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11  $\beta$ -HSD1) as well as an increase in TNF- $\alpha$  and IL-6 [21, 22], and a reduction in adiponectin levels [26, 27]. However, adiponectin level was increased in the F1 generation in the obesity model produced by a diet with higher calories [9] demonstrating that there are ambiguous results in the literature for molecules involved in the regulatory physiology of the adiposity and also in the concentration of other molecules considered as adiposity markers.

In our study, we expected that the total cholesterol and triglycerides levels could be higher when compared to the control group or with other obesity model studies already described [26]. Despite this, the literature also describes controversial results for profile lipids according to the obesity model used. For example, an increase in triglycerides concentration but no difference in cholesterol concentration were observed in obesity produced by a high-fat diet [28], and for a similar study using the small litters, but not a consanguineous reproduction, although all detectable fatty

acids were elevated in the serum of the pups at weaning compared to controls, a significant down regulation of insulin receptor substrate-1 (Irs-1), protein kinase B (Akt2), and glucose transporter 4 (Glut4) at the protein level in epididymal fat of small litters were observed at adulthood, indicating differences in the results according to the tissue studied [29].

Nevertheless, in our study, significant changes were found for the LDL, HDL, and VLDL levels over generations. A decrease in the VLDL level was found when compared to the control group which may be also associated with the increase in LDL and with the reduced levels of HDL. Therefore, in our study, the lipoproteins LDL and HDL seem to have some roles in the changes found in our model of obesity used.

The literature also indicates that the lipids profiles have a lot of contradictory results according to the model of obesity considered, and it seems that the variations may be dependent on other factors such as the model of study, sex, age, nutritional status, or strains. It already was demonstrated that in inbred strains such as C57Bl6 and AKR mice [30] the genetic susceptibility to variations in the profile lipids in an obesity condition is lower while in SWR/J and A/J mice [31, 32] are more resistant to induction of obesity. The variations in the metabolic phenotypes profile of lipids results, described in the literature, concerning to different experimental approaches and obesity models used, maybe also affected by the heterozygosity level involved in the control mechanism of the metabolism [33, 34].

Furthermore, the circadian variation for the cholesterol, triglycerides, and glucose levels can be an additional factor to contribute to the different results found inside and among the literature and that obtained in our present study [35-38] Therefore, the circadian rhythms must be considered for the evaluation of the metabolic parameters, especially, the hour of the day in which are collected the blood samples. In our experiment, to avoid the interference of the circadian variation, the blood samples were taken in the morning period which is not clearly informed in the major of the manuscripts concerning to metabolic molecules evaluated.

On the other hand, it is also known that in obesity there is a significant change in the intestinal microbiota and that the lipopolysaccharides may pass across the epithelial barrier and produce an inflammatory process in the connective tissue, conducting in an increase in insulin resistance [39]. To control the invasion of toxins of the microbiota, the goblet cells produce different types of mucin that have a protective function [30]. Because of this, the evaluation of the goblet cell number using PAS and Alcian blue staining was also evaluated in our experiment comparing the C group with the last generation produced (G5).

In our study, we expected that the number of goblet cells should be increased in response to the obesity condition, however, it was found that there is a significant decrease in the number of the goblet cells that produce neutral and acid mucin on the G5 group. This result is different from our previously reported [40] using the monosodium glutamate (MSG) obesity model where the hypothalamic obesity increased the number of goblet cells. However, it is important to know that the reduction of the number of the goblet cells observed in our

present study can be related to the circadian variation and/or with differentiation process which is different along with the times of the day which was not evaluated in the present study. Our results also demonstrated an increase in the length of the small intestine, which corroborates a previous report using the MSG obesity model [41] in which was demonstrated that several organs in obesity conditions seem to have their growth reduced but not the intestine.

In addition to maintaining the morphological phenotypes over generations, the present study demonstrated that the consanguineous reproduction using the reduction of litter size as an strategy to produce an obesity condition, also induced some variations in metabolic levels of cholesterol, triglycerides and glucose and, although this is not completely understood, they differs from those found in the literature for non-consanguineous reproduction used in different models for the obesity studies.

## Conclusion

We conclude that the effects detected were maintained over generations using the inbreeding reproduction associated to reduction in size litter which were an increase in the morphological and some of the metabolic obesity phenotypes as well as the reduction in the glucose, cholesterol and triglycerides levels.

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## Conflict of Interest

The authors declare no conflict of interest.

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