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RENAN DE OLIVEIRA VENCI

OVERFEEDING WITH A POOR NUTRITIONAL DIET DURING LACTATION IMPAIR THE LONG-TERM METABOLISM IN MALE WISTAR RATS

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração – Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Paulo Cezar de Freitas Mathias

Coorientador: Prof. Dr. Douglas Lopes de Almeida

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RENAN DE OLIVEIRA VENCI

LACTATION, OVERFEEDING AND A POOR NUTRITIONAL DIET: DEVELOPMENTAL CONSEQUENCES FOR THE PANCREAS

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas pela Banca Examinadora composta pelos seguintes membros:

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BIOGRAFIA

Renan de Oliveira Venci nasceu em 02/10/1993 em Maringá-PR. Possui graduação em Tecnologia em Biotecnologia pela Universidade Estadual de Maringá (UEM), turma de 2017. Atualmente é mestrando no Programa de Pós-graduação em Ciências Biológicas da Universidade Estadual de Maringá. Tem experiência na área de biologia celular e bioquímica, atuando principalmente nos seguintes temas: programação metabólica e secreção de insulina.

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APRESENTAÇÃO

Esta dissertação é composta de um artigo científico, intitulado *"Overfeeding with a poor nutritional diet during lactation impair the long-term metabolism in male wistar rats"*. O trabalho demonstra as consequências no desenvolvimento do pâncreas de uma dieta de má qualidade em grande quantidade durante a lactação. Em consonância com as regras do programa de pós-graduação em ciências biológicas, o artigo foi redigido de acordo com as normas da revista *Endocrine: International Journal of Basic and Clinical Endocrinology*, com atual fator de impacto 3,29 (QUALIS CB1: B1).

RESUMO

O início da vida pós-natal possui papel importante para a determinação da saúde metabólica na vida adulta, e o ambiente nutricional das matrizes e suas proles durante a lactação tem efeitos a longo prazo no metabolismo do indivíduo adulto. Neste contexto, hipotetizamos que a superalimentação combinada a uma dieta de baixo valor nutricional durante a lactação, pode alterar o correto funcionamento do pâncreas endócrino na vida adulta de ratos Wistar. Para isso, fêmeas foram induzidas ao cruzamento e quando constatada a prenhez, foram acondicionadas em caixas individuais até o nascimento natural das ninhadas. Após o nascimento as ninhadas foram divididas em quatro grupos experimentais: 1) Grupo NP-NL que recebeu dieta normoproteica, composta por uma ninhada de 9 filhotes; 2) Grupo NP-PO com a mesma suplementação alimentar, porém a ninhada foi reduzida à 3 filhotes machos por matriz; 3) Grupo LP-NL que recebeu dieta hipoproteica, composta por ninhada de 9 animais e 4) Grupo LP-PO com dieta hipoproteica, porém com ninhada reduzida à 3 animais por matriz. Aos 90 dias, os animais foram eutanasiados e avaliados os parâmetros biométricos, bioquímicos e morfofisiológicos do pâncreas endócrino. Os animais LP-PO apresentaram um perfil biométrico semelhante ao grupo controle (NP-NL). No Teste intravenoso de Tolerância à Glicose (ivGTT), a dieta hipoproteica elevou a glicemia ($p<0.01$). A análise KITT, através do resultados obtidos pelo Teste Intraperitoneal de Tolerância a Insulina (ipITT), demonstrou que a prole de mães submetidas a dieta hipoproteica são mais sensíveis à insulina ($p<0.0001$) e a interação com o fator redução de ninhada aumenta a sensibilidade à insulina ($p<0.0001$), fazendo com que o grupo LP-PO seja o mais afetado. Os grupos NP-PO, LP-NL e LP-PO demonstraram reduções no tamanho das ilhotas pancreáticas comparado ao grupo controle ($p<0.05$). Mesmo com um fenótipo de peso normal, o grupo LP-PO apresentou disfunções morfológicas no pâncreas endócrino e intolerância à glicose juntamente à alta sensibilidade a insulina.

Palavras-chave: DOHaD; dieta hipoproteica; redução de ninhada; programação metabólica.

ABSTRACT

The early life nutritional environment affects the development of metabolism, mainly in the maturation of the central nervous systems (CNS) and endocrine organs; we investigated the effects of maternal low-protein diet combined with postnatal early overfeeding on the offspring's pancreatic beta-cell function in later life. Only male rats were used; delivery was considered post-natal-day 0 (PN0). Wistar rats' dams were divided into control (NP) or low-protein diet (LP). LP dams remained on the diet until PN14, after which all animals were supplied with the control diet. At PN2, litters were adjusted to 9 (normal litter - NL) or 3 (postnatal overfeeding -PO) pups, resulting in 4 experimental groups: NP-NL, NP-PO, LP-NL and LP-PO. Litters were weaned on PN21. At PN83, a batch of animals from all experimental groups, the Glucose Decay Constant index (KITT) was calculated from the results of Intraperitoneal Insulin Tolerance Test (ipITT), one week after, the animals underwent surgery for cannula implantation, followed by intravenous glucose tolerance test (ivGTT). At PN90, animals were euthanized and tissues collected. LP-PO animals' present a biometric profile similar to the control (NP-NL) group. In the ivGTT, LP maternal diet elevated the glycemia ($p<0.01$). KITT analysis showed that LP maternal diet animals are more sensible to insulin ($p<0.0001$) and postnatal overfeeding enhances the sensibility to insulin ($p<0.0001$), making the LP-PO group the most affected. All groups demonstrated reductions in pancreatic islet size compared to control group ($p<0.05$). Even with a normal weight phenotype, the postnatal overfeed offspring of mothers who received the low-protein diet presented morphological disorders in the endocrine pancreas with glucose intolerance and high insulin sensitivity.

Key-words: DOHaD; low-protein maternal diet; early postnatal overfeeding; metabolic programming; glucose homeostasis.

overfeeding with a poor nutritional diet during lactation impair the long-term metabolism in male wistar rats

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ABSTRACT

Purpose: The early life nutritional environment affects the development of metabolism, mainly in the maturation of the central nervous systems (CNS) and endocrine organs; we investigated the effects of maternal low-protein diet combined with postnatal early overfeeding on the male offspring's pancreatic beta-cell function in later life. **Methods:** Only male rats were used; delivery was considered post-natal-day 0 (PN0). Wistar rats' dams were divided into control (NP) or low-protein diet (LP). LP dams remained on the diet until PN14, after which all animals were supplied with the control diet. At PN2, litters were adjusted to 9 (normal litter - NL) or 3 (postnatal overfeeding - PO) pups, resulting in 4 experimental groups: NP-NL, NP-PO, LP-NL and LP-PO. Litters were weaned on PN21. At PN83, a batch of animals from all experimental groups, the Glucose Decay Constant index (KITT) was calculated from the results of Intraperitoneal Insulin Tolerance Test (ipITT), one week after, the animals underwent surgery for cannula implantation, followed by intravenous glucose tolerance test (ivGTT). At PN90, animals were euthanized and tissues collected. **Results:** LP-PO animals' present a biometric profile similar to the control (NP-NL) group. In the ivGTT, LP maternal diet elevated the glycemia ($p<0.01$). KITT analysis showed that LP maternal diet animals are more sensible to insulin ($p<0.0001$) and postnatal overfeeding enhances the sensibility to insulin ($p<0.0001$), making the LP-PO group the most affected. All groups demonstrated reductions in pancreatic islet size compared to control group ($p<0.05$). **Conclusions:** Even with a normal weight phenotype, the postnatal overfeed offspring of mothers who received the low-protein diet presented morphological disorders in the endocrine pancreas with glucose intolerance and high insulin sensitivity.

Key-words: DOHaD; low-protein maternal diet; early postnatal overfeeding; metabolic programming; glucose homeostasis.

INTRODUCTION

The current prevalence of noncommunicable diseases, such as diabetes and obesity, has a major impact on population health and overloads public health systems worldwide. The DOHaD concept (Developmental Origins of Health and Disease) assists in understanding the current scenario as epidemiological and experimental evidence converges to connect the early nutritional environment with metabolic changes in adulthood [1]. Perinatal and early childhood malnutrition is considered a major global public health problem, mainly in developing countries, where many newborns and lactating women are exposed to hunger and malnutrition that lead to several health risks such as brain damage and insulin resistance, which may lead to early death and other complications, not completely understood [2].

Lactation can be characterized as a period of early life neural plasticity, as in this phase, in rodents, the maturation and development of the central nervous systems (CNS) and endocrine organs occur, mainly during the first weeks after birth [3]. In humans, after birth, occurs substantial structural and functional development in the brain that almost triples in weight till 1 year of age [4], also a positive effect of breast-feeding on cognitive function is extensively reported [5].

Literature has shown that exposure to undernutrition during suckling period can affect neural development leading to metabolic dysfunctions related to energy expenditure and pancreatic beta-cell function in adulthood [6,7]. In this context, the provision of a protein-restricted diet for lactating mothers during the first two thirds of lactation is an alternative experimental model to study early undernutrition with lasting metabolic outcomes [8].

Undernutrition is not alone as a concern regarding early life nutritional stress and metabolism predisposition to later life metabolic disorders, as obesity rates have increased in woman of fertile age and children [9], fetal overnutrition is also associated with later risk of obesity and metabolic disturbances, likely through different pathways [10]. Also, overnutrition during suckling period is associated with late hyperphagia, body weight gain, fat accumulation and basal hyperinsulinemia [11]. In complement, childhood obesity can adversely affect nearly every organ system and often causes serious consequences, including hypertension, dyslipidemia, insulin resistance, type 2 diabetes mellitus (T2DM), fatty liver disease and psychosocial complications [12].

Rodents' postnatally overfed are a well-established experimental model for the study of rapid weight gain from overnutrition and its metabolic consequences, which has proven effective, in later life, in inducing overweight, hyperinsulinemia and metabolic syndrome [13]. Reducing litter size have been shown to develop litters with increased fat deposition, that preserve the obese phenotype for the long-term, accompanied by permanent hyperphagia, hyperinsulinemia, hyperleptinemia, diabetes and cardiovascular disturbances [11].

There are several studies clarifying the lasting metabolic consequences of overnutrition or undernutrition and caloric intake imbalance in early life. Although there is a paucity in studies about bad quality diet with no caloric deficit, even with epidemiological data showing a significant association between food insecurity without hunger and metabolic disorders during childhood [14,15].

Food insecurity, somewhat milder in caloric imbalance, and obesity has been correlated with specific population groups such as immigrants in developed countries [16], and low-income families in emergent countries [17-19]. The high percentage of overweight and obesity found in this population reinforces studies that demonstrate there is as nutritional transition happening in low-income families, of undernutrition diet to excessive consumption of bad quality processed foods, with high caloric value and low cost [17]. This transition is reinforced by the socioeconomic disparities in food quality driven by the elevated costs of healthier diets [20].

A combined maternal low-protein diet with postnatal overfeeding during the suckling phase seems to better fit an early nutritional environment of concomitant bad quality and high quantity. Previously, our group showed that this experimental model developed an impaired glucose homeostasis in young adult's offspring [21]. Bearing in mind the existent data, we hypothesize that a poor-quality diet offered in large quantity during the lactation period can deeply alter the pancreatic beta-cell function in later life even without expressing the obesity phenotype.

MATERIALS AND METHODS

All experimental procedures described below were developed according to the rules of the Ethics Committee for Animal Use and Experimentation of Maringá State University (CEUA Nº 3723280918).

Animals and diet

Adult 70-80 day old Wistar rats were obtained from the central animal facility of the State University of Maringá and housed and maintained in the animal facility of the Department of Biotechnology, Genetics and Cell Biology, of this same institution, under standard light conditions (12-h dark/12-h light cycle) and controlled temperature (23±2° C), where they were kept for 5 days for adaptation. After this period, the animals were induced to mate. The ratio of 1 male to 3 females was adopted, so that different lineages were obtained between the analyzed animals.

The breeding period was followed by pregnant females' isolation until natural delivery, which was considered the postnatal-day 0 (PN0). On PN0, the dams were randomly assigned into two groups, the first group was supplied with a control balanced diet (normal protein—NP—23.3% of protein), while the second group was supplied with a low-protein (LP—4.5% of protein) isocaloric diet (Table 1). The other components of the normal and low-protein diets, such as the mixtures of vitamins and salts, followed the recommendations of the AIN-93 for laboratory rodents [22,23]. The LP group of dams was maintained on this diet for the first 2 weeks (PN14) of the lactation period, and then switched to standard chow in the last week of lactation [24]. At PN2, litter sizes were adjusted to either nine pups for normal litters (NL) or three pups for postnatal-overfeeding (PO) groups. Therefore, the experiments consisted of four groups: normal protein diet – normal litter (NP-NL), normal protein diet – postnatal overfeeding (NP-PO), low protein diet – normal litter (LP-NL) and low-protein diet – postnatal overfeeding (LP-PO). The total number of dams and litters used in this study were nine dams for NP-NL and LP-NL; eight dams for the NP-PO and seven dams for the LP-PO groups. Only male pups were used and cross-fostering (between same diet dams) was made to complete the number of male pups for the normal litters on PN2 when needed.

Food Intake

The dams' food intake was monitored and estimated during the lactation period (PN0-PN21). The four groups' litters were weaned on PN21 by placing in 3 animals per cage and maintaining them on commercial chow (Nuvital®, Curitiba/PR, Brazil). Spillage was not measured and an estimate of food intake (Fi) per rat per day was calculated by determining the difference between the amount of chow remaining (Df) and the total amount of food that was previously placed in the cage (Di). The food consumption was assessed weekly. Food intake (Fi) values were calculated as the

difference between the amount of food remaining (final diet, Df) and the total food available (initial diet, Di), divided by the number of days and the number of rats in the cages: (Fi (g) = Df-Di/7/1 for the lactation period; and, Fi (g) = Df-Di/7/3 for the rats after weaning). The area under the curve (AUC) for the food consumption versus time was calculated for the entire observation period: PN0-PN21 for the dams and PN21–PN90 for the offspring.

Biometric Measurements

To assess the effects of the LP diet and litter size manipulation during the suckling phase, maternal food intake was estimated and body weight monitored during the lactation phase; data are presented as the AUC values for the period PN0 to PN21. Also, the dams uterine, mesenteric and retroperitoneal fat pads were dissected and weighed.

In addition, the body weight of the litters was also assessed at weaning (PN21). At PN90, after an overnight fast, a batch of animals from all groups (n=24 from 9 litters for NP-NL; n=11 from 4 litters for NP-PO, n=19 from 5 litters for LP-NL and n=9 from 4 litters for LP-PO group), which had not undergone surgery, were weighed and euthanized via quick decapitation. Whole pancreas and fat from the periepididimal, retroperitoneal and mesenteric pads were dissected and weighed, the value was correlated with the body weight (bw) of each rat and was calculated as g/100 kg bw.

Dams' milk nutritional profile

After weaning at PN21, the mothers were anesthetized with a thiopental and lidocaine solution (45 and 10 mg/kg bw) and received an injection (2.5 IU/kg bw) of oxytocin (Oxytocin®, Chemical Union, Embu, Sao Paulo, Brazil) to collect milk samples via milking. Milk samples were diluted (1:20 v/v) in saline (0.9% NaCl) and used for subsequent measurement of total protein parameters, which were evaluated by enzymatic colorimetry by commercial kits (Gold Analyzes ® Belo Horizonte, Minas Gerais, Brazil) according to the manufacturer's instructions.

Fasting glycemia and intravenous Glucose Tolerance Test (ivGTT)

On PN90, a batch of animals from all groups (n=12 for NP-NL; n=11 for NP-PO, n=10 for LP-NL and n=7 for LP-PO group) were anesthetized using a ketamine and xylazine (75 and 15 mg/kg of body weight) solution, prior the surgical implantation of a silicone cannula into the right jugular vein, attached to the dorsal region of the neck. The

animals were given a day for recovery from surgery. At PN91, after an overnight fast, the animals received a glucose infusion (1g/kg of body weight) through the cannula, followed by blood samples collected via cannula. The first sample was collected immediately before the glucose infusion (time 0'), and was used for fasting glucose assessment. Glucose infusion was then followed by blood samples collection at 5, 15, 30 and 45 minutes after infusion. The samples were centrifuged and the plasma collected. Blood glucose concentration was determined using the glucose oxidase method [16] with a commercial kit (Gold Analisa®, Belo Horizonte-Brazil).

Intraperitoneal Insulin Tolerance Test (ipITT) and Glucose Decay Constant (KITT)

On PN90, a batch of animals from all groups (n=10 for NP-NL; n=9 for NP-PO, n=5 for LP-NL and n=9 for LP-PO group) were used in the ipITT. After 6 hours fasting, the animals received an intraperitoneal insulin infusion (1U/kg BW), followed by blood samples collected via cutting the tail tip of the animal. The first sample was collected immediately before de insulin infusion (time 0'), then followed by blood samples collection at 15, 30, 45 and 60 minutes after infusion. The samples were immediately analyzed for glycemia by blood glucose monitoring system (Accu-Chek®). The KITT method [25] was applied to quantify the rate at which glucose decays in the bloodstream over the duration of the test.

Histological analyses of the endocrine pancreas and liver

Pancreas and liver samples from litters at PN90 were fixed in 10% buffered formalin, dehydrated, embedded in histological paraffin and sectioned (5μm) in non-seriated cuts (n=4 for animals from 4 different litters per group). Tissue sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (HE). Morphometric analyses were performed using a camera (DM500 plus ICC50 HD®, Leica Microsystems) attached to a light microscope, analyses were performed using 40 digital images (×400 magnification). Islet area analysis was obtained using ICY® software (Institute Pasteur and France-BioImaging, Paris, France). Liver steatosis analysis were obtained using Image Pro Plus v.6® software (Media Cybernetics, Silver Spring, MD, United States).

Statistical Analysis

Results are given as mean ± S.E.M. The data were analyzed by two-way ANOVA (source of variation factors: maternal diet and litter size) followed by Tukey's multiple

comparisons post-test. Mean differences were considered statistically significant at $p<0.05$. Data analyses were performed using GraphPad Prism v7.00 (GraphPad Software Inc., San Diego, CA - USA).

RESULTS

Dams' biometric parameters

Table 2 shows both the LP diet and litter size reduction negatively impact maternal food intake (LP diet $p<0.0001$; Litter Size $p<0.0001$) and body weight (bw) gain during the suckling phase (LP diet $p<0.0001$). The significant factors interaction ($p<0.01$) point that these effects were greater in the LP groups. LP-NL group showed decreased fat pads – retroperitoneal (LP diet $p<0.05$) and uterine (LP diet $p<0.05$) - at weaning. At PN 21 there is no significant difference between the groups concerning total proteins in dams' milk, however postnatal overfeeding appears to decrease total milk proteins according to post-test (litter size $p<0.05$).

Litters' biometric parameters

As the body weight at weaning (PN21) shows, the maternal LP diet during lactation reduced the litters' body weight ($p<0.0001$), while reduction in the litter size increased body weight ($p<0.0001$) (Table 3). Also, during the weaning, the group LP-PO showed a body weight catch-up after diet switch to NP (PN14) (Figure 1).

Body weight on PN90 was reduced by the low-protein diet ($p<0.0001$) and was increased by postnatal overfeeding ($p<0.0001$), the post-test shows no significant difference between the control (NP-NL) and the LP-PO rats' body weight (Table 3). As indicated by the AUC for the food intake after weaning, the maternal LP diet significantly reduced the offspring's food consumption ($p<0.001$), while the postnatal overfeeding increased the litters' food intake ($p<0.001$), but no significant interaction was observed (Table 3).

Periepididymal fat accumulation on PN90 was reduced by the LP diet ($p<0.01$) and increased by postnatal overfeeding ($p<0.05$), the biometric characteristics of the NP-NL control group and the LP-PO rats were not significantly different on adult life. Retroperitoneal and mesenteric fat pads at PN90 were reduced in LP-NL group, post-test showed significant variation in LP diet groups (Table 3).

IvGTT, ipITT and KITT

Fasting glycemia on PN90 was not significantly affected by the maternal LP diet, postnatal overfeeding had a significant effect ($p<0.05$). Only the NP-PO animals showed reduced glycemia, reflecting a significant interaction between maternal diet and litter size ($p<0.05$) (Table 3). The ivGTT demonstrated that litters from dams fed with LP diet are more intolerant to glucose ($p<0.01$), post-test showed LP-PO group as the most intolerant to glucose (Figure 2). IpITT showed through KITT analysis that Maternal LP diet animals are more sensible to insulin ($p<0.0001$) and postnatal overfeeding enhances the sensibility to insulin ($p<0.0001$), reflecting a significant interaction between maternal diet and litter size ($p<0.0001$) (Figure 3B).

Histological analyses of the endocrine pancreas and liver

Figure 4A and 4B shows histological and morphological analyses of the litter's endocrine pancreas at PN90. All groups demonstrated reductions in pancreatic islet size compared to control, NP-NL, group ($p<0.05$). Post-test showed that maternal diet ($p<0.01$) and litter size ($p<0.05$) impairs the size of the pancreatic islets, although there is no significant interaction between both factors. Figure 4C displays histological images from the litter's liver with spots of steatosis at PN90, the two-way ANOVA analysis of the percentage of steatosis in the tissue is discriminated in Figure 4D. Groups NP-PO and LP-PO demonstrated significant higher percentage of steatosis compared to the control group, post-test showed that maternal diet ($p<0.05$) and litter size ($p<0.001$) changes the proportion of steatosis suffered by the liver.

DISCUSSION

Recalling that lactation is a very sensitive phase of development [3], and nutritional stress may affect physiology for long-term [6,7]. Our results demonstrate an indistinct profile of the postnatal overfeed offspring of mothers who received the low-protein diet. They are underweight animals during the entire suckling period, even with an expressive catch-up in the last third of lactation. In adulthood (PN90) they regain their weight and have no significant difference compared to the control group (NP-NL). Despite their normalized body weight, these are animals that have morphological

disorders in the endocrine pancreas with glucose intolerance, high insulin sensitivity and liver steatosis.

The results of body weight, food intake and fat pads weight (Table 3) reinforce the pattern of the experimental models already described in the literature [11,8,21], where the maternal low-protein diet induces the offspring to a low body weight, low fat pads weight and hypophagia till later life. On the other hand, at the same age (PN90), early postnatal overfeeding during lactation leads to overweight and hyperphagia. Now the combination of LP diet and postnatal overfed seems to not affect the final body weight, fat pad weight or food intake, LP-PO even has a mild a reduction of body weight at weaning compared to LP-NL. Which suggests that the combination of factors truly creates a different nutritional environment from other experimental models.

The dams' body weight and food intake (Table 2) also corroborates to the standardization of the model LP-PO, since the low-protein diet significantly reduces the weight of mothers during lactation, demonstrating a deficiency in their diet that may end up being transmitted to the nutritional profile of milk. Despite the amount of total milk proteins showing no significant difference at PN21, studies shows [26] that even in broader models (pregnancy and lactation) of maternal protein undernutrition, in the late period of lactation, milk shows a recovery of its nutritional quality similar to control.

About the consequences of early malnutrition in the function of the endocrine pancreas, as established in the literature [6,7,11,21], all experimental models demonstrated changes in glucose homeostasis (Figure 2), insulin secretion (Figure 3) and pancreatic islet size (Figure 4) at PN90 compared to control (NP-NL). Also, the liver is affected showing a significantly higher percentage of steatosis at PN90 in the groups NP-PO and LP-PO when compared to the control (Figure 4). Interestingly, the LP-PO group proves to be the most dysfunctional in all parameters even with a non-overweight phenotype. Epidemiological data shows that undetected type 2 diabetes is more prevalent among those men whose metabolic syndrome was not associated with obesity [27]. In addition, metabolically unhealthy normal weight individuals more frequently have insulin resistance, nonalcoholic fatty liver disease (NAFLD) and visceral obesity [28].

The weight catch-up presented by the LP-PO litters during breastfeeding is an interesting result as a possible mechanism for the metabolic changes obtained by the group during adulthood. Plagemann [1] showed that animals that went through protein restriction during fetal life followed by neonatal catch-up growth do not have abnormal body weight during adulthood, however acquired impaired glucose tolerance,

hyperinsulinemia and a significantly increased insulin/glucose-ratio, indicating insulin resistance. Also, rats born with low birth weight showed a higher risk of developing diabetogenic disorders at one year of age only if they were additionally exposed to neonatal overnutrition [29].

Our study demonstrates classic results of metabolic dysfunction in experimental models of severe malnutrition during perinatal life, but in a model with mild malnutrition, focused in bad quality diet instead caloric deficit diet, during a short period of lactation. Mimicking in a more realistic way the nutritional environment found by many children nowadays [16,17]. Considering all factors, we have consistent results to affirm that this early nutritional environment profile increases insulin sensitivity and perhaps consequently decreases pancreatic islet mass. Although more results are needed, we suggest that our experimental model (LP-PO) is suitable for the study of malnutrition during lactation due to a poor-quality diet available in larger offer.

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Compliance with ethical standards

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Disclosure of potential conflict of interest

The authors declare that they have not any potential conflict of interest to disclosure, including financial, personal or other relationships with other people or organizations.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

REFERENCES

1. Plagemann, A., Harder, T., Schellong, K., Schulz, S., Stupin, J.H.: Early postnatal life as a critical time window for determination of long-term metabolic health. Best practice & research. Clinical endocrinology & metabolism **26**(5), 641-653 (2012). doi:10.1016/j.beem.2012.03.008
2. Abebe, Z., Zelalem Anlay, D., Biadgo, B., Kebede, A., Melku, T., Enawgaw, B., Melku, M.: High Prevalence of Undernutrition among Children in Gondar Town, Northwest Ethiopia: A Community-Based Cross-Sectional Study. International journal of pediatrics **2017**, 5367070 (2017). doi:10.1155/2017/5367070
3. Lesage, J., Sebaai, N., Leonhardt, M., Dutriez-Casteloot, I., Breton, C., Deloof, S., Vieau, D.: Perinatal maternal undernutrition programs the offspring hypothalamo-pituitary-adrenal (HPA) axis. Stress **9**(4), 183-198 (2006). doi:10.1080/10253890601056192
4. Innis, S.M.: Impact of maternal diet on human milk composition and neurological development of infants. Am J Clin Nutr **99**(3), 734S-741S (2014). doi:10.3945/ajcn.113.072595
5. Evenhouse, E., Reilly, S.: Improved estimates of the benefits of breastfeeding using sibling comparisons to reduce selection bias. Health Serv Res **40**(6 Pt 1), 1781-1802 (2005). doi:10.1111/j.1475-6773.2005.00453.x
6. de Moura, E.G., Passos, M.C.: Neonatal programming of body weight regulation and energetic metabolism. Biosci Rep **25**(3-4), 251-269 (2005). doi:10.1007/s10540-005-2888-3
7. Morimoto, S., Calzada, L., Sosa, T.C., Reyes-Castro, L.A., Rodriguez-Gonzalez, G.L., Morales, A., Nathanielsz, P.W., Zambrano, E.: Emergence of ageing-related changes in insulin secretion by pancreatic islets of male rat offspring of mothers fed a low-protein diet. Br J Nutr **107**(11), 1562-1565 (2012). doi:10.1017/S0007114511004855
8. Moura, A.S., Carpinelli, A.R., Barbosa, F.B., Gravena, C., Mathias, P.C.: Undernutrition during early lactation as an alternative model to study the onset of diabetes mellitus type II. Research communications in molecular pathology and pharmacology **92**(1), 73-84 (1996).
9. Masters, R.K., Reither, E.N., Powers, D.A., Yang, Y.C., Burger, A.E., Link, B.G.: The impact of obesity on US mortality levels: the importance of age and cohort factors in population estimates. American journal of public health **103**(10), 1895-1901 (2013). doi:10.2105/AJPH.2013.301379
10. Gingras, V., Hivert, M.F., Oken, E.: Early-Life Exposures and Risk of Diabetes Mellitus and Obesity. Curr Diab Rep **18**(10), 89 (2018). doi:10.1007/s11892-018-1050-0
11. Plagemann, A., Roepke, K., Harder, T., Brunn, M., Harder, A., Wittrock-Staar, M., Ziska, T., Schellong, K., Rodekamp, E., Melchior, K., Dudenhausen, J.W.: Epigenetic malprogramming of the insulin receptor promoter due to developmental overfeeding. Journal of perinatal medicine **38**(4), 393-400 (2010). doi:10.1515/JPM.2010.051
12. Gungor, N.K.: Overweight and obesity in children and adolescents. J Clin Res Pediatr Endocrinol **6**(3), 129-143 (2014). doi:10.4274/Jcrpe.1471
13. Boullu-Ciocca, S., Dutour, A., Guillaume, V., Achard, V., Oliver, C., Grino, M.: Postnatal diet-induced obesity in rats upregulates systemic and adipose tissue glucocorticoid metabolism during development and in adulthood: its relationship with the metabolic syndrome. Diabetes **54**(1), 197-203 (2005). doi:54/1/197 [pii]
14. Thompson, A.L., Bentley, M.E.: The critical period of infant feeding for the development of early disparities in obesity. Social science & medicine **97**, 288-296 (2013). doi:10.1016/j.socscimed.2012.12.007
15. Metallinos-Katsaras, E., Must, A., Gorman, K.: A longitudinal study of food insecurity on obesity in preschool children. Journal of the Academy of Nutrition and Dietetics **112**(12), 1949-1958 (2012). doi:10.1016/j.jand.2012.08.031

16. Dondi, A., Piccinno, V., Morigi, F., Sureshkumar, S., Gori, D., Lanari, M.: Food Insecurity and Major Diet-Related Morbidities in Migrating Children: A Systematic Review. Nutrients **12**(2) (2020). doi:10.3390/nu12020379
17. Fernanda, W., Bianca Inês, E., Luciano, L.: Insegurança Alimentar em Famílias do Programa Bolsa Família. Segurança Alimentar e Nutricional **25**(2) (2018). doi:10.20396/san.v25i2.8649841
18. Monteiro, F., Schmidt, S.T., Costa, I.B.d., Almeida, C.C.B., Matuda, N.d.S.: Bolsa Família: insegurança alimentar e nutricional de crianças menores de cinco anos %J Ciência & Saúde Coletiva. **19**, 1347-1358 (2014).
19. Souza, N.N.d., Dias, M.d.M.e., Sperandio, N., Franceschini, S.d.C.C., Priore, S.E.: Perfil socioeconômico e insegurança alimentar e nutricional de famílias beneficiárias do Programa Bolsa Família no município de Viçosa, Estado de Minas Gerais, Brasil, em 2011: um estudo epidemiológico transversal %J Epidemiologia e Serviços de Saúde. **21**, 655-662 (2012).
20. Darmon, N., Drewnowski, A.: Contribution of food prices and diet cost to socioeconomic disparities in diet quality and health: a systematic review and analysis. Nutr Rev **73**(10), 643-660 (2015). doi:10.1093/nutrit/nuv027
21. Almeida, D.L., Simoes, F.S., Saavedra, L.P.J., Praxedes Moraes, A.M., Matiusso, C.C.I., Malta, A., Palma-Rigo, K., Mathias, P.C.F.: Maternal low-protein diet during lactation combined with early overfeeding impair male offspring's long-term glucose homeostasis. Endocrine **63**(1), 62-69 (2019). doi:10.1007/s12020-018-1719-9
22. Malta, A., de Moura, E.G., Ribeiro, T.A., Tofolo, L.P., Abdennebi-Najar, L., Vieau, D., Barella, L.F., de Freitas Mathias, P.C., Lisboa, P.C., de Oliveira, J.C.: Protein-energy malnutrition at mid-adulthood does not imprint long-term metabolic consequences in male rats. European journal of nutrition **55**(4), 1423-1433 (2016). doi:10.1007/s00394-015-0960-8
23. Reeves, P.G., Nielsen, F.H., Fahey, G.C., Jr.: AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. The Journal of nutrition **123**(11), 1939-1951 (1993). doi:10.1093/jn/123.11.1939
24. Barbosa, F.B., Medina, A.R., Balbo, S.L., de Freitas Mathias, P.C.: Low protein diets administered to lactating rats affect in a time-dependent manner the development of young. Research communications in molecular pathology and pharmacology **106**(1-2), 63-76 (1999).
25. Bonora, E., Moghetti, P., Zancanaro, C., Cigolini, M., Querena, M., Cacciatori, V., Corgnati, A., Muggeo, M.: Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. J Clin Endocrinol Metab **68**(2), 374-378 (1989). doi:10.1210/jcem-68-2-374
26. Bautista, C.J., Bautista, R.J., Montano, S., Reyes-Castro, L.A., Rodriguez-Pena, O.N., Ibanez, C.A., Nathanielsz, P.W., Zambrano, E.: Effects of maternal protein restriction during pregnancy and lactation on milk composition and offspring development. The British journal of nutrition **122**(2), 141-151 (2019). doi:10.1017/S0007114519001120
27. Pajunen, P., Kotronen, A., Korpi-Hyovalti, E., Keinanen-Kiukaanniemi, S., Oksa, H., Niskanen, L., Saaristo, T., Saltevo, J.T., Sundvall, J., Vanhala, M., Uusitupa, M., Peltonen, M.: Metabolically healthy and unhealthy obesity phenotypes in the general population: the FIN-D2D Survey. BMC Public Health **11**, 754 (2011). doi:10.1186/1471-2458-11-754
28. Stefan, N., Schick, F., Haring, H.U.: Causes, Characteristics, and Consequences of Metabolically Unhealthy Normal Weight in Humans. Cell Metab **26**(2), 292-300 (2017). doi:10.1016/j.cmet.2017.07.008
29. Neitzke, U., Harder, T., Plagemann, A.: Intrauterine growth restriction and developmental programming of the metabolic syndrome: a critical appraisal. Microcirculation **18**(4), 304-311 (2011). doi:10.1111/j.1549-8719.2011.00089.x

TABLE AND FIGURE LEGENDS

Table 1. Composition of the control and low protein diets. [a] The mineral salts and vitamins mixture that was used in the manufactured diet followed the AIN-93 recommendation. The dietary component values are presented as g kg^{-1} of diet and the energy in kJ kg^{-1}.

Table 2. The effects of low-protein diet and litter size reduction in the dams estimated food intake (AUC) and body weight evolution (AUC) during the lactation period (PN0-PN21); uterine, ovarian, retroperitoneal and mesenteric fat accumulation after weaning; total protein on dams' milk at PN21. The values are presented as the mean ± SEM. The n of dams is: NP-NL=9, NP-PO=9, LP-NL=8 and LP-PO=7. The source of variation factors was F_1: Maternal diet; F_2: Litter size (postnatal early overfeeding); I: interaction between factors F_1 and F_2, *p value (p<0.05)* indicates significant effect and *ns* indicates not significant effect. Letters (A, B, C and D) indicates the post-test results when the difference among groups were significant ($p<0.05$).

Table 3. The effects of maternal low-protein diet and litter size reduction in the litters body weight at weaning period (PN21); estimated food intake from PN21 to PN90; body weight and periepididymal, retroperitoneal, mesenteric fat accumulation at PN90; litters fasting glycemia at PN90. The values are presented as the mean ± SEM. The source of variation factors was F_1: Maternal diet; F_2: Litter size (postnatal early overfeeding); I: interaction between factors F_1 and F_2, *p value (p<0.05)* indicates significant effect and *ns* indicates not significant effect. Letters (A, B, C and D) indicates the post-test results when the difference among groups were significant ($p<0.05$).

Figure 1. Evolution of litters body weight till PN21. Data are presented as the mean ± SEM.

Figure 2A. Blood glucose levels during the ivGTT at PN90. Data are presented as the mean ± SEM. **2B.** Area under the curve for glycemia in ivGTT: source of variations-maternal diet and litter size; post-test differences among groups are presented as the level of significance (when $p<0.05$) inside the figure. * indicates significant difference ($p<0.05$) when compared to control group (NP-NL).

Figure 3A. Blood glucose levels during the ipITT at PN90. Data are presented as the mean ± SEM. **3B.** KITT analysis trough ipITT test data. Maternal diet and Litter size (postnatal early overfeeding) were considered as the source of variation factors, p value ($p<0.05$) indicates significant effect and ns indicates not significant effect. * indicates significant difference ($p<0.05$) when compared to control group (NP-NL).

Figure 4A. Offspring endocrine pancreas morphology at PN90. Representative photomicrography (×400 magnification, scale bars = 50μm) shows pancreatic sections stained with hematoxylin and eosin (HE). **4B.** Quantitative analyses of islet area. Data are presented as the mean ± SEM. Maternal diet and Litter size (postnatal early overfeeding) were considered as the source of variation factors, p value ($p<0.05$) indicates significant effect and ns indicates not significant effect. **4C**. Histological images from the litter's liver at PN90. Representative photomicrography (×400 magnification, scale bars = 50μm) shows liver sections stained with hematoxylin and eosin (HE), arrows disclose steatosis

spots in the tissue. **4D.** Quantitative analyses of liver steatosis. Data are presented as the mean ± SEM. Maternal diet and Litter size (postnatal early overfeeding) were considered as the source of variation factors, p value ($p<0.05$) indicates significant effect and ns indicates not significant effect. * indicates significant difference ($p<0.05$) when compared to control group (NP-NL).

TABLES

Table 1. Composition of the control and low protein diets

Diet components	**Control Diet**		**Low-protein Diet**	
	g kg^{-1}	kJ kg^{-1}	g kg^{-1}	kJ kg^{-1}
Cornstarch	527.5	8.828	642.5	10.753
Casein (88% protein)	233.3	3.905	45.5	0.761
Sucrose	127.2	2.129	200	3.347
Soybean oil	48	1.807	48	1.807
Mix of minerals[a]	32.0	-	32.0	-
Mix of vitamins[a]	16.0	-	16.0	-
Fish Oil	16.0	0.602	16.0	0.602
Total	1000.0	17.272	1000.0	17.272

Table 2. The effects of low-protein diet and litter size reduction in the dams

Parameters							
	NP-NL ^{A}	**NP-PO** ^{B}	**LP-NL** ^{C}	**LP-PO** ^{D}	**Factors**		
					F_1	**F_2**	**I**
AUC-Food intake (PN0-PN21)	1167 ± 25 ^{BCD}	748 ± 15 ^{ACD}	420± 22 ^{AB}	385 ± 18 ^{AB}	p< 0.0001	p< 0.0001	p< 0.0001
AUC-body weight (PN0-PN21)	6011 ± 84 ^{CD}	5912 ± 55 ^{CD}	4391 ± 25 ^{ABD}	4732 ± 61^{ABC}	p< 0.0001	ns	p<0.01
Uterine fat pad (g/100g of bw)	0.72 ± 0.07	0.82 ± 0.03	0.47 ± 0.02	0.79 ± 0.12	ns	p<0.05	ns
Ovarian fat pad (g/100g of bw)	0.73 ± 0.11	0.77 ± 0.05	0.40 ± 0.01	0.79 ± 0.12	ns	p<0.05	ns
Retroperitoneal fat pad (g/100g of bw)	0.74 ± 0.09	1.05 ± 0.12	0.34 ± 0.03 ^{B}	0.60 ± 0.08 ^{B}	p<0.01	p< 0.05	ns
Mesenteric fat pad (g/100g of bw)	0.63 ± 0.05	0.58 ± 0.05	0.40 ± 0.09	0.61 ± 0.06	ns	ns	ns
Milk total proteins (g/dL)	24.4 ± 1.29	22.3 ± 2.20	27.1 ± 1.68	21.0 ± 1.98	ns	p<0.05	ns

Table 3. The effects of maternal low-protein diet and litter size reduction in the offspring.

Parameters							
	NP-NL [A]	**NP-PO** [B]	**LP-NL** [C]	**LP-PO** [D]	**Factors**		
					F_1	**F_2**	**I**
Body weight PN21 (g)	45.9 ± 1.31 [BCD]	61.7 ± 1.42 [ACD]	20.9 ± 0.72 [ABD]	38.9 ± 1.14 [ABC]	p< 0.0001	p< 0.0001	ns
Final body weight (g)	360.9 ± 8.04 [BCD]	408.5 ± 8.15 [ACD]	305.8 ± 3.62 [ABD]	370.9 ± 8.15 [BC]	p< 0.0001	p< 0.0001	ns
AUC food intake (PN21-PN90)	1593 ± 65 [C]	1664 ± 55 [C]	1284 ± 58 [ACD]	1569 ± 43 [C]	p<0.01	p<0.01	ns
Periepididymal fat pad (g/100g of bw)	0.96 ± 0.056	1.17 ± 0.110 [C]	0.79 ± 0.013 [B]	0.92 ± 0.097	p<0.05	p<0.05	ns
Retroperitoneal fat pad (g/100g of bw)	1.11 ± 0.057 [C]	1.19 ± 0.116 [C]	0.74 ± 0.055 [AB]	0.96 ± 0.083	p<0.01	ns	ns
Mesenteric fat pad (g/100g of bw)	0.46 ± 0.023	0.52 ± 0.062 [C]	0.34 ± 0.034 [B]	0.42 ± 0.019	p<0.01	ns	ns
Fasting glycemia at PN90 (mg/dL)	96 ± 3.28 [B]	81 ± 1.91 [A]	90 ± 3.51	88 ± 2.54	ns	p<0.05	p<0.05

FIGURES

Figure 1.

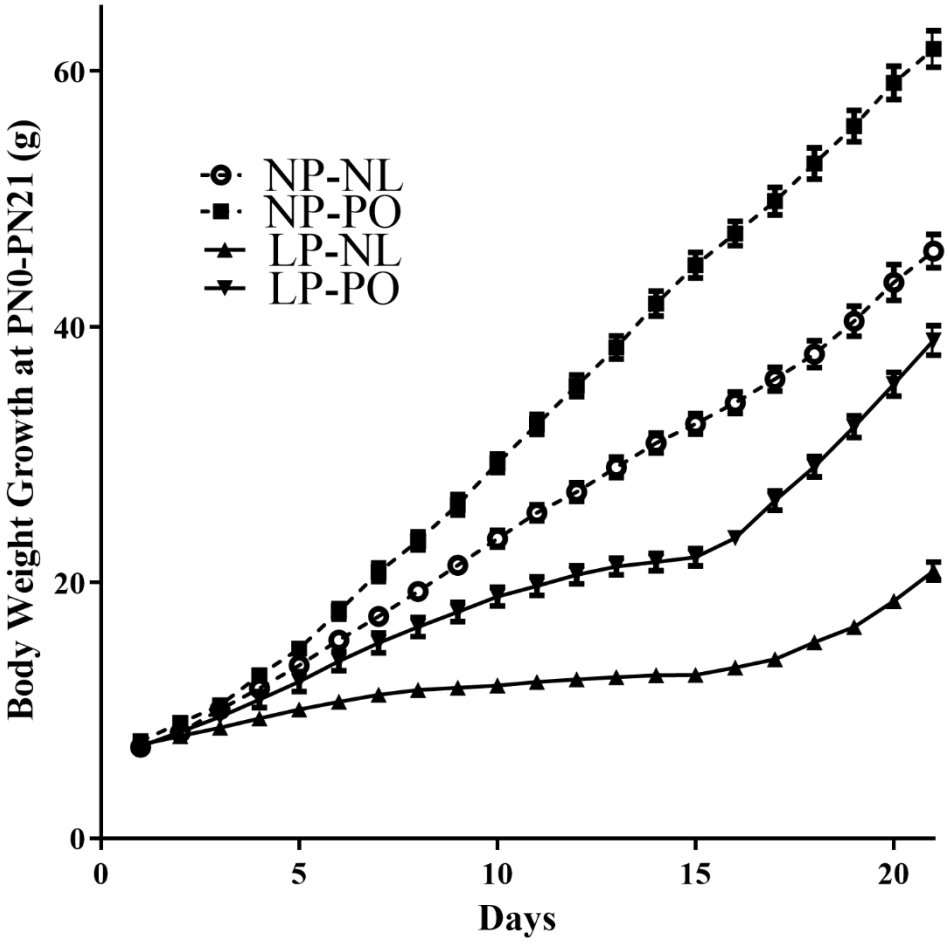


Figure 2.

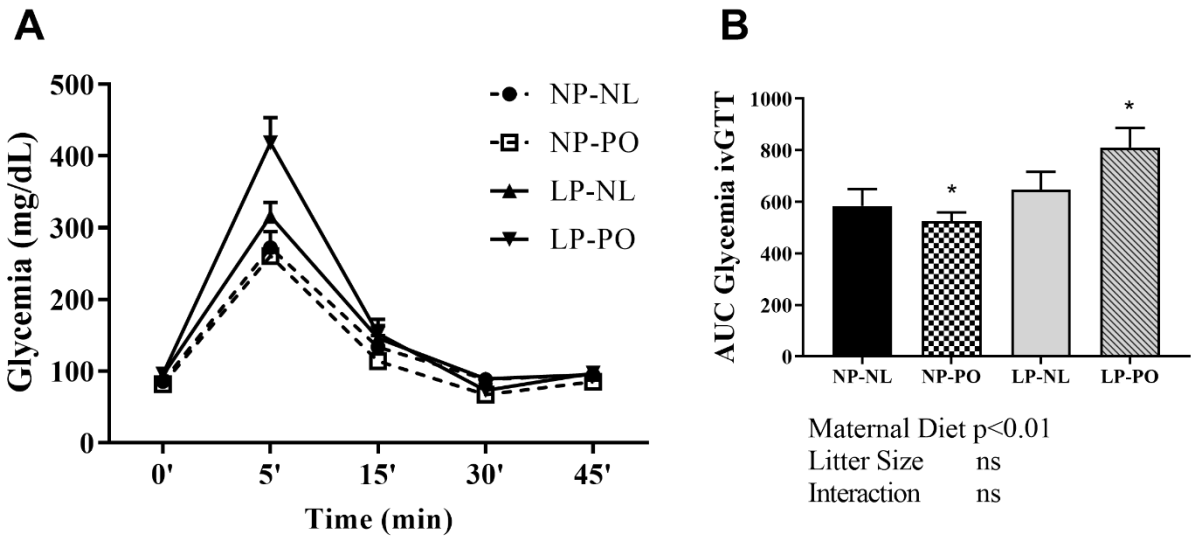


Figure 3.

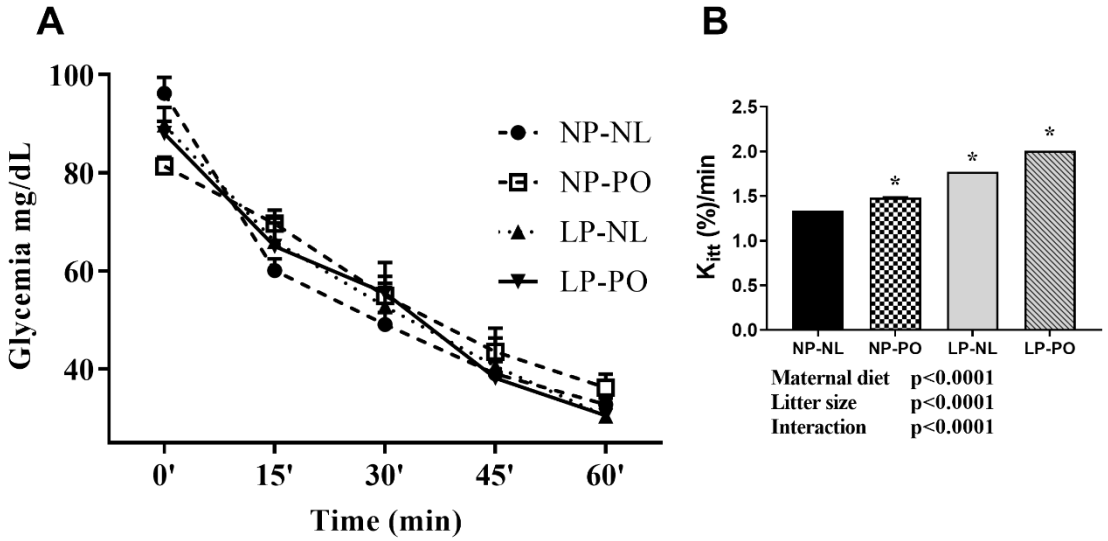
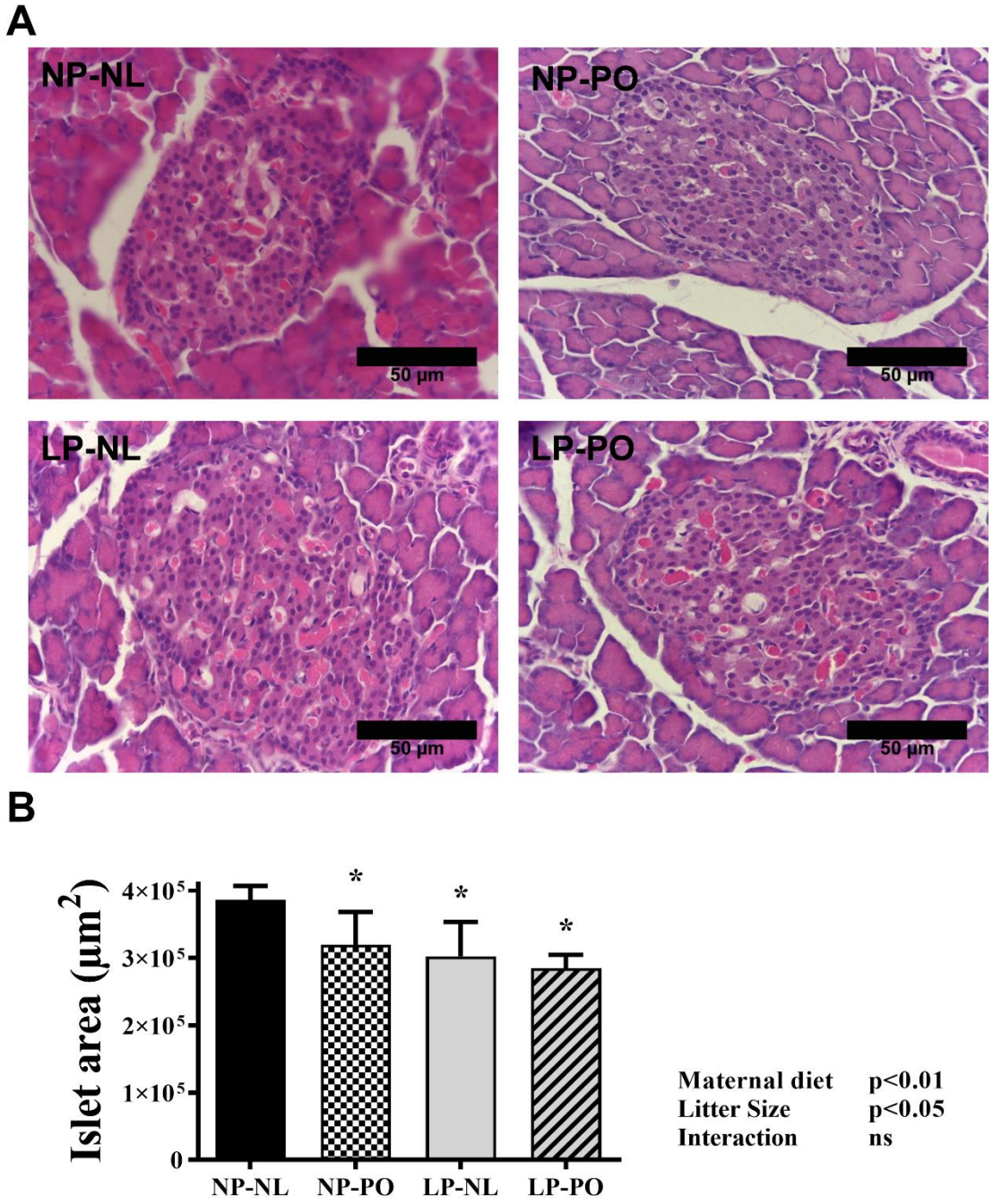
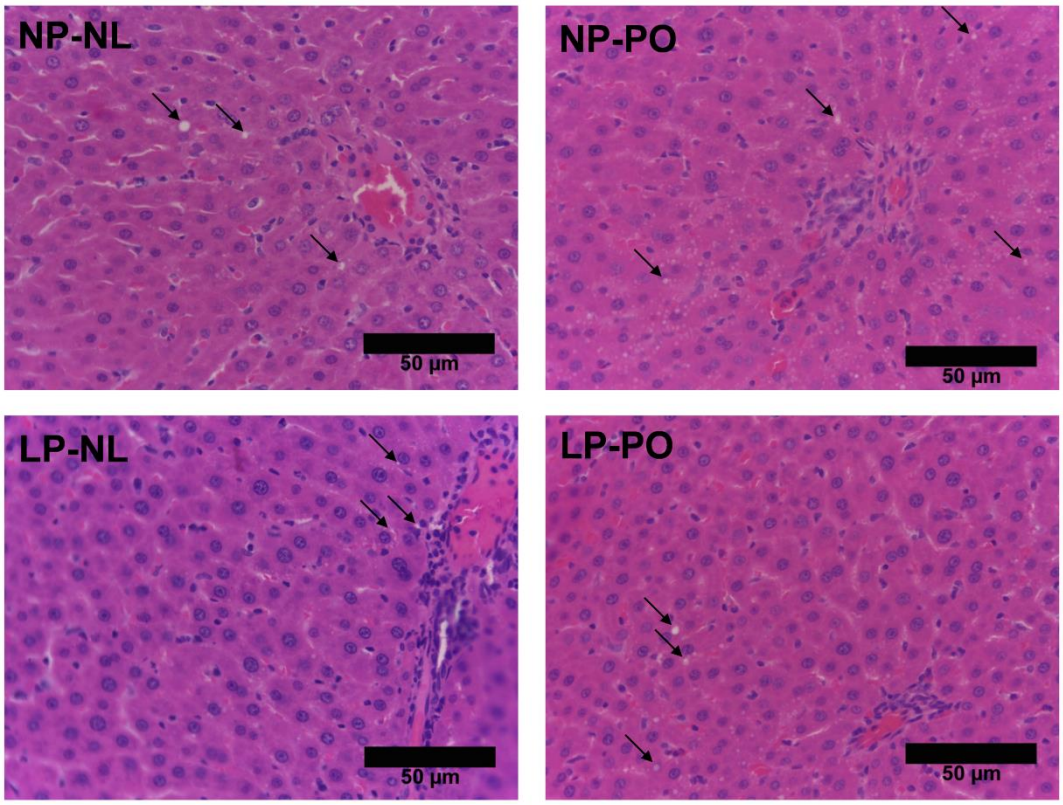


Figure 4.


A
NP-NL
50 µm
NP-PO
50 µm
LP-NL
50 µm
LP-PO
50 µm
B
Islet area (µm2)
4×10^5
3×10^5
2×10^5
1×10^5
0
*
*
*
NP-NL
NP-PO
LP-NL
LP-PO
Maternal diet p<0.01
Litter Size p<0.05
Interaction ns

C



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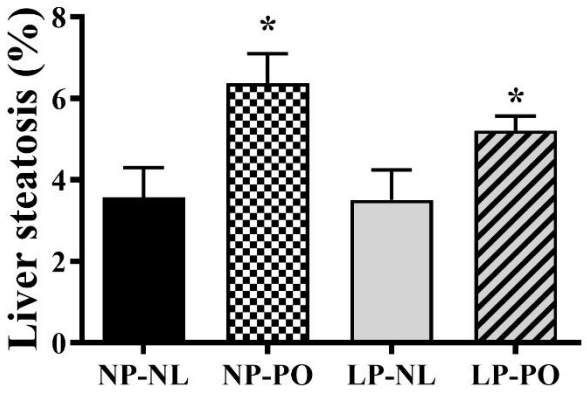

Maternal diet	p<0.05
Litter Size	p<0.0001
Interaction	ns