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Avaliação da função reprodutiva ovariana de ratas adultas expostas à hiperglicemia materna intraútero e submetidas à dieta hiperlipídica após o desmame

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Doutora em Tocoginecologia.

Orientadora: Profa. Dra. Débora Cristina Damasceno
Coorientadores: Prof. Dr. Gustavo Tadeu Volpato
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Todos esses que aí estão
Atravancando meu caminho,
Eles passarão...
Eu passarinho!

Mário Quintana

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Aos meus pais, Sélio e Cristiane, por todo incentivo, apoio e, principalmente, por todo amor que sempre me deram.

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Aos animais utilizados no experimento pois, sem eles, esse trabalho não seria possível.

Biografia da aluna

Graduada em Biomedicina pela Universidade Federal de Mato Grosso (UFMT), Campus universitário do Araguaia no ano de 2016, foi bolsista voluntária de Iniciação Científica durante os 4 anos do curso, onde desenvolveu o projeto intitulado "*Efeitos abortivos e teratogênicos do tratamento com Phyllanthus niruri (quebra pedra) na prenhez de ratas"* sob a orientação do Prof. Dr. Gustavo Tadeu Volpato, que foi publicado na revista *Journal of Ethnofharmacology* (fator de impacto = 4,36) no ano de 2020. Durante sua Graduação, participou ativamente do movimento estudantil, como membro do Diretório Central dos Estudantes e do Centro Acadêmico de Biomedicina, onde fez parte da comissão organizadora do I Congresso de Biomedicina do Araguaia no ano de 2014. É mestra em Imunologia e Parasitologia Básicas e Aplicadas pelo programa de mestrado PPGIP - UFMT em 2018, tendo desenvolvido fisicamente parte do projeto de pesquisa intitulado *"Avaliação do perfil glicêmico e imunológico de ratas expostas ao diabete moderado intraútero e submetidas à dieta hiperlipídica no período pós-natal"* na Faculdade de Medicina de Botucatu (FMB)/UNESP, sob orientação do Prof. Dr. Gustavo Tadeu Volpato e coorientação da Profa. Dra. Débora Cristina Damasceno. Este estudo foi publicado na revista *Biology of Reproduction* (fator de impacto = 4,28) no ano de 2022. No ano de 2018, ingressou no Programa de Pós-graduação em Tocoginecologia (Nível: Doutorado) pela FMB/UNESP para desenvolver o projeto intitulado *"Avaliação da função reprodutiva ovariana de ratas adultas expostas à hiperglicemia materna intraútero e submetidas à dieta hiperlipídica após o desmame"* sob a orientação da Profa. Dra. Débora Cristina Damasceno. Este projeto resultou em 6 manuscritos, sendo 3 de autoria da aula e 3 como coautoria. Como coautora, participou assiduamente da realização prática dos experimentos e discussão dos resultados, no entanto, outras alunas integrantes do grupo de pesquisa foram autoras principais, visto que o projeto também se inseria em suas teses. Faz parte como membro do Grupo de Pesquisa do CNPq "Estresse oxidativo e Anomalias congênitas" liderado pela Profa. Débora C. Damasceno. Durante o desenvolvimento de seu Doutorado, realizou projetos de pesquisa voltados para o entendimento dos mecanismos envolvidos na disfunção diabética e mal nutrição em fêmeas com idade reprodutiva, com o objetivo de promover e prevenir doenças relacionadas ao binômio diabete e gestação. Nesta etapa, auxiliou outros alunos de Mestrado e de Iniciação Científica na redação de seus projetos de pesquisa e no desenvolvimento técnico também e redigiu diversos artigos para publicação, sendo que publicou nove que estão indexados no PUBMED (2 deles relacionados à sua tese de Doutorado). Estes artigos foram publicados em importantes revistas internacionais de grande relevância para a área.

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Considerações Gerais

A maioria dos animais vivíparos, incluindo humanos, tem placenta e útero que protegem o feto do ambiente externo, fornecendo condição ideal para o desenvolvimento fetal (Arima e Fukuoka, 2020). Entretanto, mudanças ambientais ocorridas durante as janelas críticas do desenvolvimento (período intrauterino e neonatal) podem alterar permanentemente as estruturas e funções de órgãos específicos (Langley-Evans & McMullen 2010), aumentando a suscetibilidade de desenvolvimento de doenças na vida adulta. A teoria das doenças do adulto de origem fetal (FOAD - *fetal origin adult diseases*), proposta por Barker, baseia-se na premissa da plasticidade do desenvolvimento, durante o qual eventos intrauterinos específicos podem produzir diferentes fenótipos levando à programação fetal (Barker, 2007; Calkins e Devaskar, 2011).

Posteriormente, o conceito do FOAD avançou para o conceito das Origens Desenvolvimentistas da Saúde e da Doença (DOHaD – *Developmental origins of health and diseases*), ampliando os períodos críticos e se mostrando um modelo adequado para observações epidemiológicas que ligam o ambiente intrauterino e fases iniciais da vida, à saúde na vida adulta (Michels, 2018). Esse conceito delineia a capacidade do feto de se adaptar ao ambiente intrauterino ao custo de modificar as perspectivas de saúde a longo prazo, podendo causar comprometimentos futuros e risco de doenças na vida adulta (Gillman *et al*., 2007). Esses períodos abrangem desde a pré-concepção (maturação dos oócitos e desenvolvimento dos espermatozoides), período embriofetal e neonatal se estendendo aos primeiros anos de vida. Estas fases estão relacionadas ao conceito, mundialmente, disseminado pela campanha "Primeiros 1000 dias" *("First 1000 Days"* - https://thousanddays.org), a qual está inserida na temática do DOHaD e tem como objetivo uma conscientização sobre a importância da nutrição nos primeiros 1000 dias de vida (Suzuki *et al.,* 2018).

Estudos epidemiológicos e experimentais demonstram que a obesidade e o diabete materno podem favorecer o desenvolvimento de doenças nos descendentes (Tsoulis *et al*., 2016; Marciniak *et al*., 2017) e, se somada a fatores ambientais, como a disponibilidade de dietas ricas em lipídios e diminuição da atividade física, contribuem significativamente para o aumento da prevalência de sobrepeso, obesidade, *Diabetes mellitus* do tipo 2 (DM2), *Diabetes mellitus* gestacional (DMG), doenças cardiovasculares, renais e problemas reprodutivos na vida adulta dos descendentes (Clement *et al*., 1998; Hattersley, 1998; Shepherd, 1999; Rinaudo & Wang, 2012, Tsoulis *et al*., 2016). Alterações epigenéticas estão entre os mecanismos moleculares que explicam como o ambiente intrauterino inadequado pode ser prejudicial anos após a exposição, levando ao aparecimento de doenças na idade adulta (Bouchard *et al*., 2012).

Diabete e obesidade são importantes problemas de saúde pública. O diabete atinge cerca de 451 milhões de pessoas no mundo e a previsão para 2045 é que 628 milhões de pessoas entre 20-79 anos sejam diabéticas, além de 587 milhões de pessoas com intolerância à glicose (Cho *et al*., 2018). Por sua vez, a prevalência de obesidade aumentou tanto que, atualmente, mais de um terço da população mundial é classificada com sobrepeso ou obesidade (Chooi *et al*., 2019).

Mulheres portadoras do DM2 e/ou sobrepeso/obesidade apresentam problemas reprodutivos, menor reserva ovariana em relação a mulheres saudáveis, fertilidade reduzida e maiores taxas de perdas embrionárias após a implantação embrionária (Platt *et al*., 2002; Ramalho-Santos *et al*., 2008; Talmor *et al*., 2014). Entretanto, os mecanismos pelos quais isso ocorre ainda precisam ser melhor elucidados e é de grande interesse para os pesquisadores (Nayki *et al*., 2016). A infertilidade, particularmente relacionada a distúrbios de ovulação e bloqueio tubário, está significativamente associada a DM2 e obesidade. Mesmo controlando os fatores de risco relacionados ao estilo de vida e à saúde, não foi possível descartar a possibilidade de uma disfunção metabólica entre infertilidade e diabete, ou seja, existe um "solo comum". Como exemplo desta relação, podemos citar a Síndrome do Ovário Policístico (SOP), que é um distúrbio metabólico caracterizado por disfunção ovulatória, resistência à insulina e hiperinsulinemia. Mulheres com SOP têm risco elevado de diabete e obesidade (Huang, 2012; Celik *et al*., 2014). No entanto, a causalidade ou a relação entre ovulação irregular ou anovulação e resistência à insulina na SOP não é clara (Tobias *et al*., 2015).

Para entender a influência do diabete no processo reprodutivo, modelos animais são amplamente utilizados (Kleinert *et al*., 2018). A estreptozotocina (STZ) é um produto químico utilizado em animais de laboratório para induzir e estudar modelos de diabete e suas complicações (Sinzato *et al*., 2021). É uma droga citotóxica que causa destruição das células beta (β)-pancreáticas e, consequentemente, síntese deficiente de insulina, levando à hiperglicemia (Shafrir, 2010). Pode ser utilizada em diferentes doses e vias de administração, linhagem e idade dos animais para atingir diferentes níveis de glicose no sangue, reproduzindo a glicemia encontrada no DM2 e DMG. A indução do diabete pode ser realizada no período neonatal (Taqui *et al*., 2020; Soares *et al.*, 2021), durante a gravidez (Merzouk *et al*., 2002; Caluwaerts *et al*., 2003; Lopez-Soldado & Herrera, 2003; Soulimane *et al*., 2005) ou em ambos os períodos (Saito *et al*., 2010). Nesses modelos, os animais apresentam intolerância à glicose e estado de estresse oxidativo na idade adulta (Bueno *et al.*, 2020; Macedo *et al.*, 2021).

A obesidade induzida por dietas ricas em lipídeos, chamadas *High-fat diets* (Dieta Hiperlipídica - DHL) tem considerável valor translacional com a obesidade humana (Nilsson

et al., 2012) e, consequentemente, são amplamente utilizadas para estudar os efeitos da programação fetal da obesidade em modelos animais (Kleinert *et al*., 2018). Animais alimentados com DHL apresentam intolerância à glicose, hiperglicemia, dislipidemima, resistência à insulina e são mais suscetíveis a desenvolver distúrbios metabólicos (Glavas *et al*., 2010; Sanchez-Garrido *et al*., 2015; Kayser *et al*., 2015). Além disso, as fêmeas apresentam comprometimento no desenvolvimento ovariano e distúrbios reprodutivos (Akamine *et al*., 2010; Skaznik-Wikiel *et al*., 2016; Wu *et al*., 2019).

Ratas que foram alimentadas com dieta hiperlipídica por 120 dias após o desmame apresentaram morfologia ovariana anormal (Akamine *et al*., 2010). Houve diminuição nos níveis dos hormônios ovarianos (estrógeno e progesterona), aumento de apoptose (Hussain *et al*., 2016; Patel e Shah, 2018) e prejuízos na via de sinalização da insulina, com diminuição da expressão de receptores de insulina nas células ovarianas (Nteeba *et al*., 2013; Wang *et al*., 2017). Além disso, essas alterações foram encontradas na geração seguinte, indicando que o prejuízo para a função reprodutiva pode se perpetuar entre os descendentes (Tsoulis *et al*., 2016).

Frente a estes estudos, compreender como diabete e obesidade impacta a função reprodutiva é importante, pois as gerações futuras serão afetadas. Nosso grupo de pesquisa tem se dedicado a investigar os efeitos do diabete e suas repercussões no organismo materno e fetal. Nós estudamos as repercussões do diabete sobre a prole feminina adulta a fim de entender se o fenótipo diabetôgenico é transmitido e como o organismo é afetado. Para isso, foram avaliadas filhas de ratas diabéticas aos 120 dias de vida. Foi possível observar hiperglicemia, intolerância à glicose, hiperlipidemia e disfunção das células β-pancreáticas (Paula *et al*., 2022) nestas fêmeas. Além disso, associamos o diabete intrauterino ao consumo pós-desmame de DHL para mimetizar uma condição muito frequente na população com o consumo de dietas ricas em gordura, o que tem sido cada vez mais elevado, principalmente entre as mulheres. As ratas expostas ao ambiente intrauterino diabético e que consumiram DHL desde o desmame até os 120 dias de vida tiveram comprometimento da função reprodutiva com diminuição do número de implantações, fetos vivos, peso da ninhada e aumento do percentual de perdas antes da implantação embrionária em relação ao grupo diabético que não consumiu a dieta (Sinzato *et al*., 2022).

A partir destes estudos, visando compreender melhor as repercussões do diabete e da dieta hiperlipídica na função reprodutiva das fêmeas e investigar os mecanismos pelos quais isso ocorre, os objetivos dessa tese de Doutorado foram:

- *Entender como a pesquisa envolvendo ovário e DHL vêm sendo conduzida e o que seus resultados nos mostram, bem como quais as lacunas de conhecimento que ainda precisam ser preenchidas por meio da realização de revisão sistemática e meta-análise (Capítulo 1).*
- *Investigar como a associação entre diabete e DHL podem prejudicar os ovários visando apontar os possíveis mecanismos pelos quais isso ocorre (Capítulo 2).*
- *Investigar as repercussões das alterações metabólicas reprodutivas sobre a prenhez de filhas de ratas diabéticas em comparação com ratas não prenhes (Capítulo 3).*

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CAPÍTULO I

Este capítulo da tese de Doutorado é uma revisão sistemática e meta-análise que foi publicada na revista *Nutrition Reviews* (Fator de Impacto = 7,11) no ano de 2022.

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Special Article

Intergenerational high-fat diet impairs ovarian follicular development in rodents: a systematic review and meta-analysis

Verônyca G. Paula, Giovana Vesentini, Yuri K. Sinzato, Rafaianne Q. Moraes-Souza, Gustavo T. Volpato, and Débora C. Damasceno

Context: *Excessive consumption of high-fat diets has increased in the population over time and is harmful to female fertility.* ***Objective:*** *To investigate and discuss the effects of a high-fat diet on ovarian follicles in rodents.* ***Data source:*** *A systematic literature search of PubMed, EMBASE, Web of Science, and SCOPUS was carried out.* ***Data Extraction:*** *Study characteristics, including study design, population, intervention, outcome, and risk of bias were analyzed.* ***Data Analysis:*** *Twenty-two articles were included in a systematic review. Given the availability of studies, a quantitative meta-analysis included 12 studies that were performed for outcomes. There was a decrease in primordial follicles in female rodents that received a high-fat diet compared with the standard diet group. The offspring of mothers exposed to a high-fat diet showed an increased number of cystic follicles and a decreased number of secondary follicles and antral follicles, compared with the control diet group. Therefore, these high-fat diet–induced follicular alterations might impair the fertility of dams and their female newborns.* ***Conclusion:*** *The consumption of a high-fat diet causes damage to ovarian follicular development, and this commitment will persist in the next generation.* ***Systematic Review Registration:*** *PROSPERO registration no. CRD42019133865.*

INTRODUCTION

The prevalence of overweight and obesity in women of reproductive age has increased over the past 30 years, with 38.3% of women over 20 years classified as obese.[1] Also, consumption of diets rich in fats and/or carbohydrates with high-calorie rates (ie, high-fat diets—HFDs) has also increased and is linked to metabolic syndrome and is associated with comorbidities, including reproductive dysfunction and infertility.[2,3] Problems in the reproductive system may occur at different stages, including ovarian follicular recruitment, oocyte development and quality, fertilization, and embryo implantation.[4] Among the most prevalent reproductive diseases, polycystic ovarian syndrome (PCOS) affects 6%–20% of women of reproductive age.[5] This syndrome is characterized mostly by anovulation, hyperandrogenism, and the presence of cysts in the ovaries.[6,7]

The ovary is the central organ in studies on fertility because the ovarian reserve concerns 2 crucial elements for reproduction: the size of primordial follicle stock and the quality of oocytes, and decline in fertility is

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Key words: corpora lutea, cysts, maternal diet, mouse, ovary, rat

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related to follicle depletion and reduced capacity of ovaries to produce competent oocytes.[8,9] Ovarian development in rodents is similar to that in humans, with development time being a determining factor.[10] At birth, the rat and mouse ovary consist of cords and oogonia.[10] Primordial follicles are formed on the first 3 days after birth, and the first pool of follicles develop into antral follicles within the next 3 weeks.[11–15] The fully developed secondary follicles are found on day 7 of life. Minimal ovarian cell apoptosis, responsible for selecting which follicles will survive and develop, can be found until day 18 of life, when early antral follicles are apparent.[16,17]

The use of laboratory animals is essential for gaining understanding of the mechanisms related to diseases and for developing methodologies for their prevention, diagnosis, and treatment. Standards for animal care have been set to ensure animal research is conducted ethically.[18] Rodents present a rapid process from birth to death, compared with other developed mammals, which makes them particularly useful for research.[19] Over 95% of animal studies are performed in mice, and such studies are essential for a better understanding of the complex processes of disease.[19]

Environmental, behavioral, and genetic predisposition factors contribute to the development of obesity, which may occur due to an energy imbalance between high caloric food intake and low energy expenditure.[20] Understanding how obesity and HFD exposure impact the reproduction of females and their offspring is very important, especially considering the need for preventive measures to minimize adverse effects.

There is evidence that certain environmental factors early in life influence the expression of the individual genetic load, determining a peculiar health–disease pattern. Clinical and experimental studies show a strong association between environmental problems that occur during fetal life or in the early stages of extrauterine life and the emergence of chronic diseases throughout life.[21] These findings point to new causal relationships influencing early metabolic adjustments and determining outcomes throughout life. This review article aims to address these ideas, currently grouped in a new branch of scientific knowledge called "Developmental origins of health and disease (DOHaD)."[21] The concept of DOHaD comes from the original idea created by Barker as "Fetal origins of adult disease (FOAD)."[22] However, the results of several studies have found that the process of health and disease in adulthood comes not only from early fetal life events but also from the entire developmental period, childhood, and adolescence.[23] Therefore, insults during perinatal life can program the individual for positive or negative changes in health pattern at later stages of life.[21]

Although there are studies that demonstrate the effects of HFD exposure on rodent ovaries, the results are still conflicting with respect to how a HFD affects the development and survival of ovarian follicles and how the effect is transmitted between generations. Additionally, there are currently no existing systematic reviews or meta-analyses on the effects of a HFD on ovarian follicles. Thus, a systematic review and meta-analysis were performed with animal models (rodents) exposed to a HFD during post-weaning, during pregnancy and/or lactation, or during postnatal life to investigate and discuss the effects of HFD exposure on ovarian follicular development.

METHODS

The guidelines were followed as outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (for the PRISMA checklist see Table S1 in the Supporting Information online).[24] The protocol was registered in the PROSPERO database under the ID number CRD42019133865 (https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019133865). Studies measuring ovarian development in rodents that consumed a HFD and a standard diet were reviewed. The PubMed (Public/Publisher MEDLINE), EMBASE (Excerpta Medica Database), Web of Science, and SCOPUS databases were searched from inception to July 7, 2020, using the following key word combinations: (mouse OR rat) AND (high-fat diet) AND (follicular phase OR polycystic ovary syndrome) (see Table S2 in the Supporting Information online). Potential additional studies that were not found with the initial electronic search were then manually searched via reviews, forward and backward searches, and author-based searches. The records were retrieved from each database and imported, and duplicates were removed using Endnote Version X7.7.1. The records identified by the literature searches were examined by two independent reviewers (V.G.P. and Y.K.S.) according to the title, abstract, and full text for potentially relevant studies in Rayyan software.[25] When information was unavailable and when there was any doubt about making a valid judgment of the eligibility criteria, the reference was included for full-text screening. Disagreements were resolved by discussion with a third reviewer (D.D.).

Eligibility criteria

Original animal studies were eligible for the systematic review using the PICOS (population, intervention, comparison, outcome, and study design) model, according to the inclusion and exclusion criteria shown in Table 1. Although intervention is the standardized term for

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Table 1 **PICOS criteria for inclusion and exclusion of studies**

Parameter	Inclusion criterion	Exclusion criterion
Population	Female rats or mice and/or their offspring of all ages	Human; in vitro; genetically modified animals; animals other than rats and mice
Intervention	Studies that compared: exposure to a high-fat diet in females; exposure in dams during preconception, pregnancy, and/or lactation. No limitations were applied regarding the duration and period of exposure or fat content.	Studies that combined high-fat diet with additional disease (eg, diabetes, high blood pressure), intervention (eg, surgery, drugs, stress, and exercise); any other type of diet that was not HFD or standard diet (eg, cafeteria diets, junk food diet, supplements)
Comparator	Standard diet	
Outcomes	Number and size of follicles, follicular stage, corpora lutea, and cysts	
Study design	Original animal studies	Reviews; observational studies; conference abstracts; editorials and opinion pieces

Abbreviation: HFD, high-fat diet.

systematic reviews according to PICOS criteria, the HFD herein is referred to as an insult, because the outcomes are the result of a negative insult. For a study to be eligible, screened articles should meet the following inclusion criteria: in vivo studies in female rats or mice exposed to a HFD; dams fed a HFD before and/or during the whole or any part of pregnancy and/or lactation, and their offspring were subjected to control or a HFD after weaning. To establish a broad search, studies with a HFD that had a higher fat content than the standard diet were included. For comparison, a standard diet was considered as a control group. To be considered for this review, outcomes of research on either rats subjected to a HFD or their offspring had to include: number or size of follicles, all follicular stages (classified as primordial, primary, secondary, antral, and atretic), and number of corpora lutea or cysts. There was no limitation on the year of publication or rodent species, thus rats and mice were both considered while conducting this review.

Data collection and extraction

Two reviewers (V.G.P. and G.V.) extracted data using a spreadsheet standardized extraction from the selected full texts, with discrepancies decided after discussion. If a consensus was not reached, a third reviewer (D.C.D.) was consulted. Descriptive data about animal strain, general characteristics of studies, the HFD and the standard diet characteristics (eg, fat content and period of exposure), and age were collected. For each included study, the outcome values were collected as means or counts, standard deviation (SD) or standard error (SE), and the number of animals. If the results were given only in graphs, the WebPlotDigitizer 4.2 (https://apps.automeris.io/wpd/) was used to extract the values. If data about the studies were insufficient, the authors were e-mailed to obtain more information (2 replies were received).

Risk of bias assessment

The SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) risk of bias tool was used to assess the methodological quality.[26] This tool is based on the Cochrane Collaboration risk of bias tool to address key aspects of bias in animal intervention studies related to selection, performance, detection, attrition, and reporting.[27] Two reviewers (V.G.P. and G.V.) independently assessed the risk of bias of each included study, and disagreements were resolved by consensus. The items were classified as having low, unclear, or high risk of bias. The risk of bias score for each of the articles was defined as a percentage (0%–100%), which determined whether it was considered in the low, unclear, or high risk category.[26] Studies were not excluded based on the risk of bias, because there was insufficient contrast between studies to allow a sensitivity analysis based on the risk of bias.

Data analysis

Meta-analysis was conducted if more than 2 studies on an outcome were available, using Review Manager 5.3 software.[28] Studies were excluded when not all outcome data was available or could be obtained (ie, mean, SD, SEM, and the number of animals). The results were grouped for common results after the first ovulation (ie, between 32 and 35 days of life),[10] and subgrouped as follows: (1) adult females that consumed a HFD (HFD), (2) females exposed to a HFD during pregnancy and/or lactation (F0HFD) and offspring that consumed a standard diet after weaning (F1 SD), and (3) females exposed to a HFD during pregnancy and/or lactation (F0 HFD) and offspring that consumed a HFD after weaning (F1 HFD). The individual and pooled effect sizes of a HFD on ovarian development for continuous variables were calculated using the standardized mean difference (SMD) along with a 95% confidence interval

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(CI), aligned to a point in the same direction. The SMD was chosen because different studies may quantify the same outcome in different ways.[29] In the case of 1 reference that investigated different time points for different groups of animals, the groups were analyzed as independent experiments. If a study contained more than one experimental group of animals with similar methods of insult that were compared with the same control group, the experimental groups were then pooled using the recommendations in the Cochrane Handbook.[29] The random effect models were used in all analyses due to expected high heterogeneity. Statistical heterogeneity was quantified using the I^2 test and was defined according to the I^2 range as follows: less than 40% low, 30%–60% moderate, 50%–90% substantial, and greater than 75% considerable heterogeneity.[29] Statistically significant P values were set to less than 0.05. To explore possible causes of heterogeneity and to assess the influence of the length of exposure of the HFD on follicle development, subgroup analyses were performed. This analysis was planned for the groups of adult female rats that consumed a HFD (HFD) using the following duration of insult: up to 90 days, 90–120 days, and more than 120 days of exposure to a HFD. Furthermore, the subgroup of mothers exposed to aHFD (F0 HFD/F1 SD or F0 HFD/F1 HFD) included the following periods: (1) mother exposed to a HFD during pre-pregnancy, pregnancy, and lactation, and (2) mother exposed to a HFD during pregnancy and lactation. However, there was not sufficient data to perform a quantitative meta-analysis for mothers exposed to HFD during lactation or the pre-pregnancy period only. Publication bias assessment (using the funnel plot method) was planned if at least 10 studies could be included in the same outcome.

RESULTS

Description of the data set

The results of the literature search are presented in Fig. 1. The initial electronic search in 4 databases resulted in a total of 7776 references. After removing 1192 duplicates, 6584 were evaluated for title and

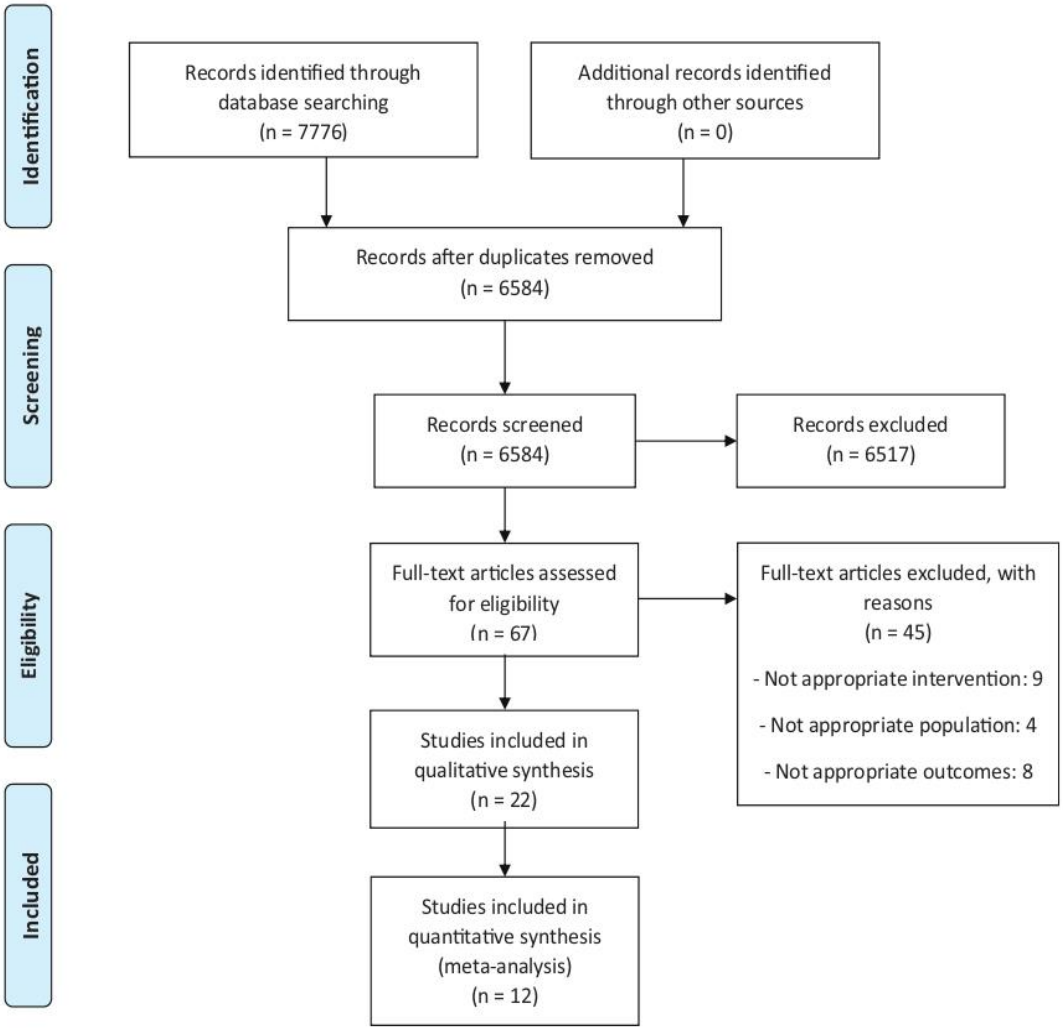


Figure 1 **Flow diagram of search and selection process of articles based on PRISMA recommendations**

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abstracts, and then 6517 articles were excluded. The remaining 67 publications were conducted for evaluation of the full text, and after excluding 45 articles that did not meet the inclusion criteria, the final data set contained 22 studies (Fig. 1).[30–51]

Study characteristics

The characteristics of the studies are presented in Table S3 in the Supporting Information online. The articles included were published between 2010 and 2020, with 13.6% ($n=3$) published in 2014, 18% ($n=4$) published in 2019, and 18% ($n=4$) published in 2020. In this systematic review, 12 articles (54.5% $n=12$) used rat models, and among them[30,31,37,38,40–43,46,47,49,51] the Sprague–Dawley strain was the most used (66.6% $n=8$). Data from 10 articles using mouse models (45.4% $n=10$) showed that 8 articles (80%) used the C57BL6/5 strain.[32,33,35,36,44,45,48,50] The HFDs differed in their fat content and composition. Five articles reported the source of fat used: 4 used lard,[40,43,45,47] and 1 used soybean oil and coconut oil.[43] The fat content in the HFD varied between 10% and 60%. Most studies (54.54% $n=12$) used a HFD with between 58% and 60% of fat[30,31,34–36,38,39,44,45,48,49,51]: 5 (22.7%) studies used a HFD with between 10% and 30% of fat,[32,37,40,47,50] and 5 (22.7%) studies used a HFD with between 40% and 52% of fat.[33,41–43,46] The energy values were reported by 15 studies,[30,32,34–38,43,45–51] and for the HFD the values ranged from 4.38 kcal/g (19 kJ/g reported by the authors) to 5.80 kcal/g.[47,50]

The exposed period of the diets in adult females (HFD) varied from 63 days to 224 days.[34,45] In studies in which mothers consumed HFD (F0 HFD), the duration of the insult ranged from 42 days (pregnancy and lactation) to 98 days (since post-weaning, including the pre-mating period, pregnancy, and lactation).[42,46] The consumption period of the offspring (F0 HFD/F1 HFD) ranged from 59 days to 160 days.[38,39]

Of the 22 articles included, 8 articles performed the insult with the HFD on the mother and/or the newborns, and the repercussions on the follicles were evaluated in the offspring.[31–33,38,39,42,46,51] The other 14 articles performed the insult with the HFD in rats that did not undergo the mating period.[30,34–37,40,41,43–45,47–50]

Among the outcomes considered in the eligibility criteria, 4 studies evaluated the total number of follicles,[37,43,47,50] and only 1 study evaluated the size of the follicles.[43] Eleven studies evaluated the HFD repercussions on the primordial follicles,[31–36,40,45–47,50] 12 studies evaluated the primary and secondary follicles,[31,34–36,40–42,45–47,50,51] 12 studies evaluated the antral follicles,[31,33,35,36,38,40,42,45–47,50,51] and 9 studies analyzed the presence of atretic follicles.[31,34,39,41,42,46,47,50,51] Regarding cell types, only 1 study investigated granulosa cells and teak cells.[30] The corpora lutea count was carried out in 14 studies.[31,32,34–40,43,44,47,48,50] The presence of cystic follicles was assessed in 4 studies.[31,46,47,50] Akamine et al[30] and Zheng et al[49] showed descriptive morphological results for follicular analysis and therefore were not included in Table S3 in the Supporting Information online.

The quantitative analysis included 12 studies that assessed the effects of a HFD on the number of primordial follicles, primary follicles, secondary follicles, antral follicles, atretic follicles, cysts, and corpora lutea. The outcomes were stratified into 3 subgroups: females that received a HFD[33–38,41,45,46]; offspring of mothers that received a HFD before/during pregnancy/lactation and received a standard diet until adulthood[31–33,38,42,46]; offspring of mothers that received a HFD before/during pregnancy/lactation and received a HFD until adulthood.[32,33,38,46] The Patel et al[43] study met the eligibility criteria and outcome data was available; however, this study was not included in the meta-analysis for 2 reasons: (1) insult: the authors submitted the rats to a progressive percentage of fat, which was increased weekly; (2) outcome measures: the study did not present a clear methodological description for follicle classification. Such characteristics were not found in any other included study, and for that reason, substantial heterogeneity was found, which explains the exclusion of this article from the meta-analysis.

Effects of consumption of a HFD on follicular development

The meta-analysis included 10 studies[32–38,41,45,46] and showed that adult females fed with a HFD (HFD) had a lower number of primordial follicles (SMD −0.90, 95% CI −1.72 to −0.07, $P=0.03$; $=76\%$) (Fig. 2A)[31–36,45,46] when compared with the rodents exposed to a standard diet. There was no effect of HFD consumption on the number of primary follicles (SMD −0.28, 95% CI −0.90 to 0.34, $P=0.37$; $I^2=58\%$) (Fig. 2B)[31,34–36,41,42,45,46], secondary follicles (SMD 0.54, 95% CI −0.19 to 1.26, $P=0.15$; $I^2=55\%$) (Fig. 3A)[31,34–36,41,42,46], antral follicles (SMD 0.11, 95% CI −0.45 to 0.67, $P=0.70$; $I^2=60\%$) (Fig. 3B)[31,33–36,38,42,45], atretic follicles (SMD 3.79, 95% CI 1.18 to 8.76, $P=0.14$; $I^2=91\%$) (Fig. 4A)[34,42,46], and corpora lutea (SMD 0.67, 95% CI −0.34 to 1.67, $P=0.19$; $I^2=71\%$) when compared with a standard diet (Fig. 4B)[31,32,34–38].

In the subgroup analysis, stratified according to the period of exposure, for adult females that consumed a HFD (HFD) for up to 90 days, there was a decrease in primordial follicles (SMD −1.55; 95% CI −2.71 to −0.39; $P=0.009$; $I^2=81\%$), compared with rodents

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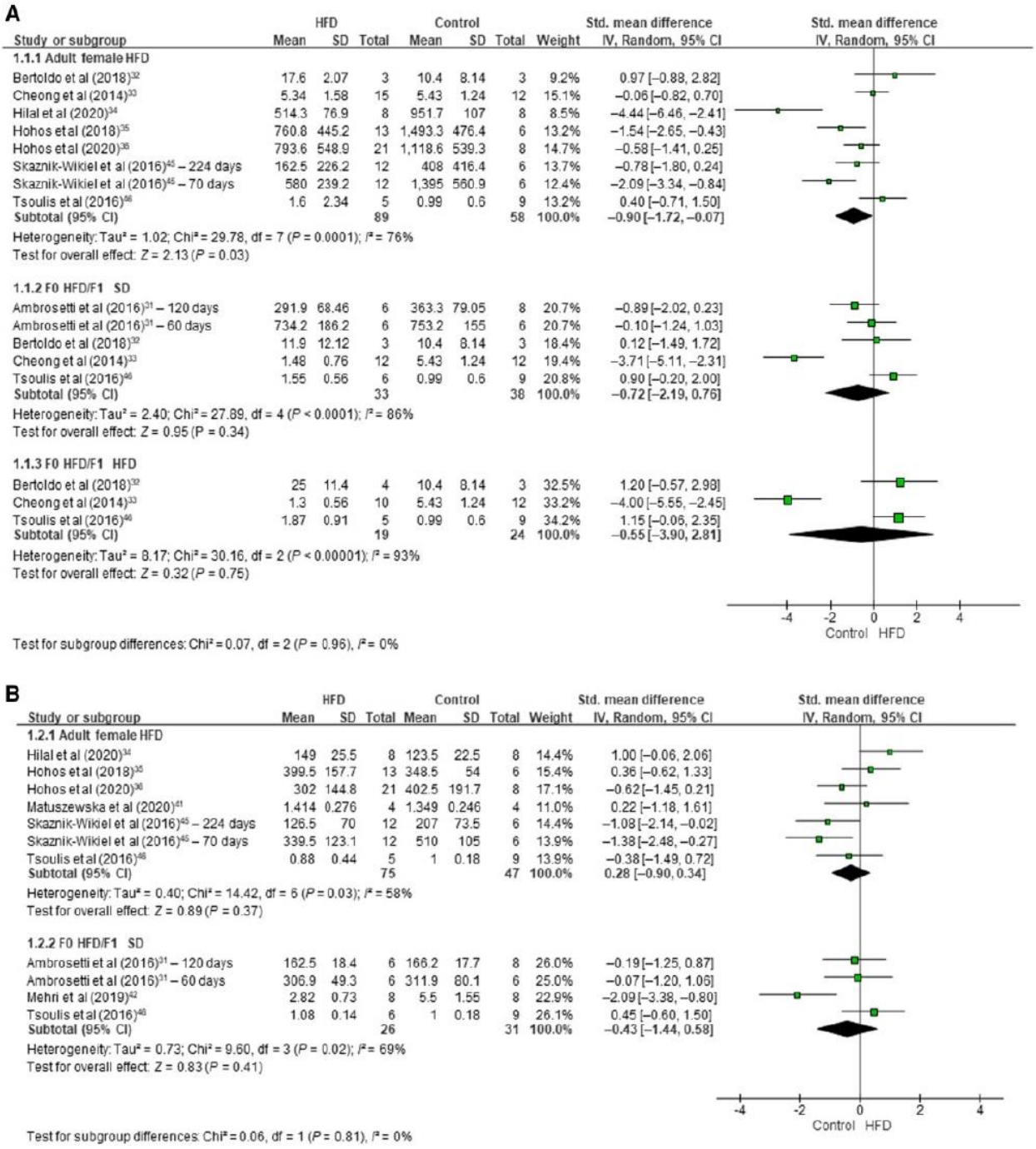


Figure 2 **(A) Meta-analysis of primordial follicles of the adult female that was fed HFD (HFD) (1.1.1), offspring of mothers that received HFD and received standard diet until adulthood (F0 HFD/F1 SD) (1.1.2), and offspring of mothers that received HFD and received HFD until adulthood (F0 HFD/F1 HFD) (1.1.3) compared with controls.** (B) Meta-analysis of primary follicles of the adult female that was fed HFD (HFD) (1.2.1), offspring of mothers that received HFD and received standard diet until adulthood (F0 HFD/F1 SD) (1.2.2) compared with controls; 95% CI, 95% confidence interval; IV, inverse variance

exposed to a standard diet. For females that consumed a HFD for more than 120 days, there was a decrease in primary follicles (SMD −0.88; 95% CI −1.57 to −0.18; $P = 0.01$; $I^2 = 0\%$), compared with rodents exposed to a standard diet (Table S4 in the Supporting Information online).

Effects of maternal and offspring consumption of a HFD on follicular development of offspring

The meta-analysis included 6 studies[31–33,38,42,46] and showed that offspring (F1 SD) of mothers exposed to a HFD (F0 HFD) presented an increase in the number of

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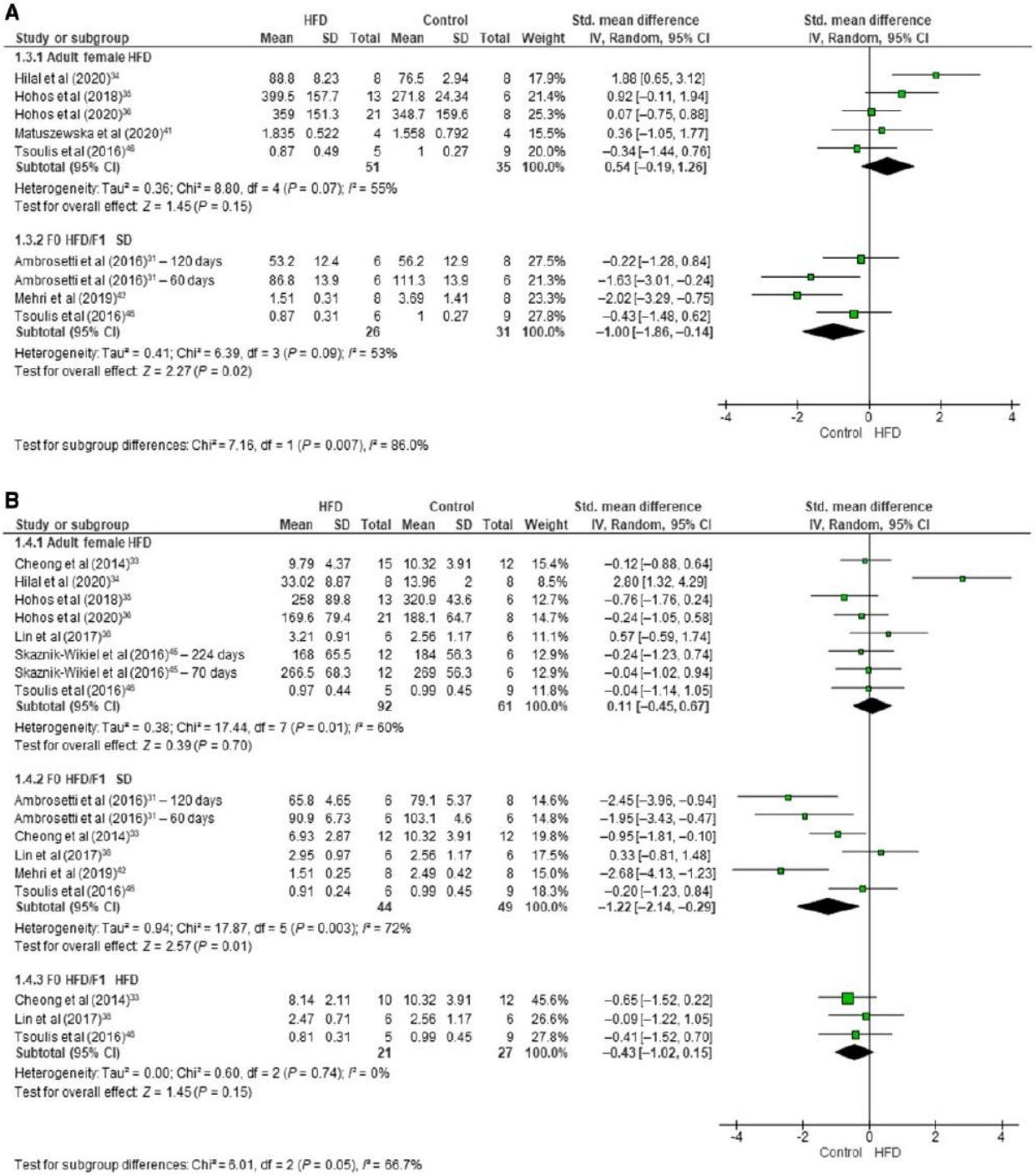


Figure 3 **(A) Meta-analysis of secondary follicles of the adult female that was fed HFD (HFD) (1.3.1), and offspring of mothers that received HFD and received standard diet until adulthood (F0 HFD/F1 SD) (1.3.2) compared with controls.** (B) Meta-analysis of antral follicles of the adult female that was fed HFD (HFD) (1.4.1), offspring of mothers that received HFD and received standard diet until adulthood (F0 HFD/F1 SD) (1.4.2), and offspring of mothers that received HFD and received HFD until adulthood (F0 HFD/F1 HFD) (1.4.3) compared with controls; 95% CI, 95% confidence interval; IV, inverse variance

cystic follicles (SMD 1.30; 95% CI 0.18 to 2.43; $P = 0.02$; $I^2 = 54\%$) (Fig. 4C) and a decrease in the number of secondary follicles (SMD −1.00; 95% CI −1.86 to 0.14; $P = 0.02$; $I^2 = 53\%$) (Fig. 3A) and antral follicles (SMD −1.22; 95% CI −2.14 to −0.29; $P = 0.01$; $I^2 = 72\%$) compared with offspring of mothers exposed to a standard diet (Fig. 4B). There was no effect on offspring (F1 SD) of mothers that consumed a HFD (F0 HFD) in terms of primordial follicle number (SMD −0.72; 95% CI −2.19 to 0.76; $P = 0.34$; $I^2 = 86\%$) (Fig. 2A), primary

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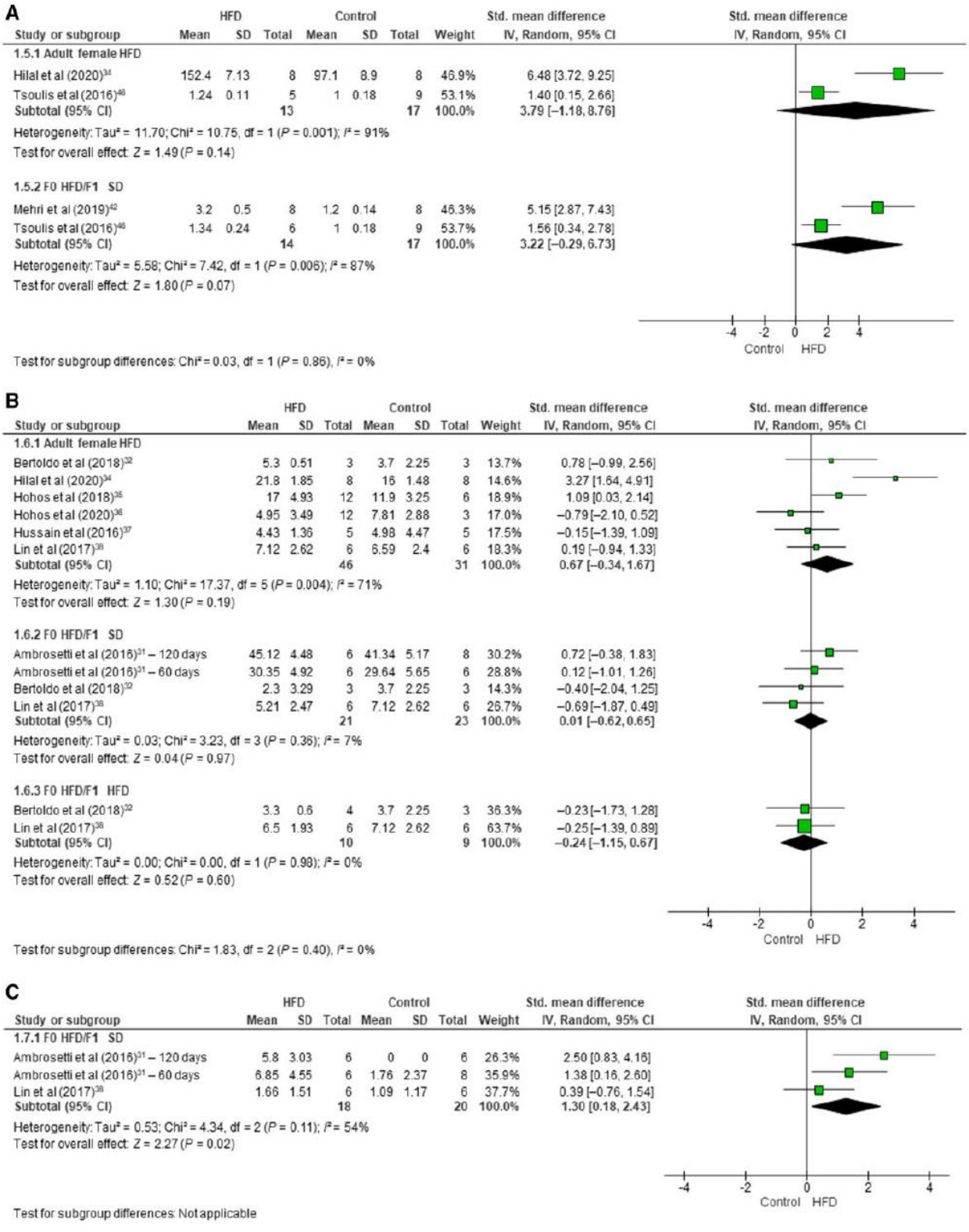


Figure 4 **(A) Meta-analysis of atretic follicles of the adult female that was fed HFD (HFD) (1.5.1), and offspring of mothers that received HFD and received standard diet until adulthood (F0 HFD/F1 SD) (1.5.2) compared with controls**. (B) Meta-analysis of corpora lutea of the adult female that was fed HFD (HFD) (1.6.1), offspring of mothers that received HFD and received standard diet until adulthood (F0 HFD/F1 SD) (1.6.2), and offspring of mothers that received HFD and received HFD until adulthood (F0 HFD/F1 HFD) (1.6.3) compared with controls. (C) Meta-analysis of cystic follicles of offspring of mothers that received HFD and received standard diet until adulthood (F0 HFD/F1 SD) (1.7.1) compared with controls; 95% CI, 95% confidence interval; IV, inverse variance

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follicles (SMD −0.43; 95% CI −1.44 to 0.58; $P = 0.83$; $I^2 = 69\%$) (Fig. 2B), atretic follicles (SMD 3.22; 95% CI −0.29 to 6.73; $P = 0.07$; $I^2 = 87\%$) (Fig. 4A) or corpora lutea (SMD 0.01; 95% CI −0.62 to 0.65; $P = 0.97$; $I^2 = 7\%$) (Fig. 4B). When mothers and their offspring consumed a HFD (F0 HFD/F1 HFD), the number of primordial follicles (SMD −0.55; 95% CI −3.90 to 2.81; $P = 075$; $I^2 = 93\%$) (Fig. 3A), antral follicles (SMD −0.43; 95% CI −1.02 to 0.15; $P = 0.15$; $I^2 = 0\%$) (Fig. 4B), and corpora lutea (SMD −0.24; 95% CI −1.15 to 0.67; $P = 0.60$; $I^2 = 0\%$) (Fig. 4B) showed no difference compared with offspring of mothers exposed to a standard diet.

The results of subgroup analysis according to the period of exposure reflect the results of the primary analysis, that offspring of mothers exposed to HFD during pre-pregnancy, pregnancy, and lactation (F0 HFD/F1 SD) showed a decreased number of secondary (SMD −1.23; 95% CI −2.38 to −0.09; $P = 0.03$; $I^2 = 61\%$), antral (SMD −1.45; 95% CI −2.53 to −0.38; $P = 0.008$; $I^2 = 73\%$), atretic (SMD 5.15; 95% CI 2.87 to 7.43; $P < 0.00001$; $I^2 =$ not applicable) (Table S4 in the Supporting Information online) and an increased number of cystic follicles (SMD 1.30; 95% CI 0.18 to 2.43; $P = 0.02$; $I^2 = 54\%$), when compared with offspring of mothers exposed to a standard diet (Table S4 in the Supporting Information online).

All the other subgroup analysis was stratified according to the period of exposure; however, the offspring of the mothers that consumed a HFD during pre-pregnancy, pregnancy, and lactation (F0 HFD/F1 SD) (primordial and primary follicle) and the offspring of the mothers that consumed a HFD during pregnancy and lactation (primordial, primary, secondary, antral, and atretic follicle) (F0 HFD/F1 HFD) did not show any significant differences (Table S4 in the Supporting Information online).

Analysis of bias

Information on the sequence of group allocation was absent in 8 studies (36.36% high risk, 63.63% unclear risk), and all studies reported baseline characteristics (100% low risk). Information on the methods used for animal allocation concealment sequence (100% unclear risk), evidence of animals randomly housed (100% unclear risk), and evidence from caregivers and/or researchers blinded by the insult each animal received during experiments (100% unclear risk) were absent in all studies. No information about the random evaluation of the results was reported (100% unclear risk). The assessor's blinded evidence about the results was found in 6 studies (18.18% low risk and 81.81% unclear risk). Information on incomplete data was unclear in most studies (77.27% unclear risk, 22.72% low risk). There was a high risk of selective outcome reporting in 2 studies (9.09% high risk, 90.90% low risk), and there was a high risk of another source of bias in 1 study (4.54% high risk, 22.72% unclear risk, 72.72% low risk) (Figs S1 and S2 in the Supporting Information online).

DISCUSSION

Main findings

The present review aimed to summarize the existing literature and to analyze the impact of a HFD compared with a standard diet on the development of ovarian follicles in adult females who consumed a HFD (HFD) and also on the offspring (F1 SD/F1 HFD) of mothers who consumed a HFD (F0 HFD) during pregnancy and/or lactation. After considering the eligibility criteria, 22 original papers were included. The meta-analysis found evidence that HFD feeding seems to be associated with a detrimental effect on the development of ovarian follicles in rats and their offspring. In the adult females fed a HFD (HFD) compared with those fed a standard diet, the number of primordial follicles was lower. Maternal exposure to a HFD induced greater cystic follicular number and lower antral and secondary follicular number in offspring that consumed a standard diet (F0 HFD/F1 SD), showing an intergenerational effect that occurs by passing the effect from mother to the first-generation offspring.[52]

Implications

Few studies on humans have been conducted with a focus on fat intake in relation to reproductive health.[53] Therefore, the use of rodents as a model for studying HFD-induced changes has been of great importance in understanding the mechanisms by which obesity and overweight lead to impaired fertility in women.[4] In addition, this model has been considered a good candidate for studies on the mechanisms of PCOS, since it exhibits similar changes to those found in clinical studies, such as insulin resistance, hyperandrogenism, and anovulation, with morphological changes in follicles being quite frequent.[43,45,54]

The role of obesity and overweight in the development of reproductive problems involves the fact that white adipose tissue, mainly peri-ovarian adipose tissue in rodents, plays an important role in the secretion and release of reproductive hormones and folliculogenesis.[55–57] Normal white adipose tissue levels are critical to maintaining the integrity of the hypothalamic–pituitary–gonadal axis, so excess adiposity can be

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detrimental to the reproduction and normal development of ovarian follicles.[47]

Ovarian decline is related to loss of follicles and reduced ability to produce competent oocytes, and primordial follicles are the basis of the ovarian reserve.[9] Primordial follicles are subjected to initial gonadotropin-independent recruitment in the growing pool of follicles, while most follicles are destined to suffer atresia.[58] After puberty, at 32 days in mice, and 35 days in rats,[10] a small proportion of growing antral follicles may respond to gonadotropins and undergo cyclical recruitment. These processes of recruitment of primordial and atretic follicles will continue until the eventual depletion of the ovarian reserve; therefore, the reproductive life span of the female is critically dependent on the size of the primordial follicular pool.[59] Exposure to a HFD in adult female rats (HFD) caused a decrease in the number of primordial follicles, and analysis of subgroups by period of exposure showed that the loss occurs in the first 90 days of exposure to a HFD.

The dysregulation of immune cells and/or altered inflammatory signaling can have profound effects on the ovarian reserve.[60] The negative effects of a HFD, as a result of obesity or overweight, on primordial follicles can be mediated by the local increase in inflammation, as indicated by the increase in macrophage infiltration in the ovaries.[45] It is suggested that macrophages can regulate cell proliferation, apoptosis in the ovary, inflammation, and steroidogenesis through the secretion of cytokines.[61] Increased macrophage infiltration, as well as increased expression of inflammatory markers (such as TNF-α and IL-6) in the ovaries or peri-ovarian adipose tissue may increase the expression of pro-apoptotic markers.[60] Female rats fed with a HFD often show increased expression of FoXO3a, which is considered a key regulator of the development of the initial follicles (primordial and primary) involved in the upregulation of the gene encoding BCL2-like 11 (*BIM*), a member of the B-cell CLL/lymphoma 2 (*BCL2*) family, increasing the expression of the pro-apoptotic proteins Bax, Bim, and Bad.[62–64] In the study by Cheong et al,[33] the observation of a marked reduction in the number of primordial follicles in the ovaries of offspring of rats fed with a HFD during pregnancy but which consumed either a standard diet or a HFD in postnatal life were a consequence of apoptosis induced by increased levels of FoXO3a.

High levels of inflammatory cytokines are also associated with the generation of reactive oxygen species that can have harmful consequences for fertility.[65] Exposure to a HFD results in an increase in fatty acids, and the consumption of superoxide dismutase (SOD) during the oxidation of fatty acids leads to accumulation of the free radical peroxide malondialdehyde (MDA) in the ovaries.[66] In addition, it has been found that the gene expression of markers related to oxidative stress was increased in the ovaries of rats that consumed a HFD and also in the ovaries of their offspring.[67] The occurrence of oxidative stress is related to apoptotic pathways and FOXO3a, thus causing a cycle between inflammation, apoptosis, and oxidative stress that results in the loss of primordial follicles (Fig. 5).[45,68,69]

Although the increased number of atretic follicles was not observed in quantitative analysis, a qualitative analysis of the studies included in this review shows that this is an important mechanism by which a HFD leads to a depletion of the follicle pool.[31,33,34,47] Increased follicular activation is another mechanism by which a HFD causes a decrease in the number of primordial follicles, which promotes the rapid maturation of follicles and consequently accelerates losses,[58] as demonstrated by Wang et al[47] and Zhou et al.[50] Therefore, exposure to a HFD is likely to induce premature aging of the ovaries in rodent females, leading to infertility.[59]

Despite the data on overall effects that showed no difference in corpora lutea number between groups, changes in the number of corpora lutea are frequently found in female rats fed with a HFD. Qualitative analysis showed divergent results concerning the corpora lutea number. While some studies showed an increase,[47,50] some no changes,[31,35,37,38,40] and others a decrease in the corpora lutea number, this might be explained by differences in the methodology of follicle counting between studies,[43,44,48,53] small sample sizes, and differences in the standard diet and the HFD between studies. The increase in the number of corpora lutea can be directly linked to the increase in follicular activation and consequently to the rapid maturation of follicles. This is caused by the excess of fat in the diet,[47] and will not result in a good reproductive performance, since the early follicular development results in deficient oocytes.[46,70,71]

Excessive weight gain and maternal obesity are risk factors for maternal and fetal comorbidities,[72] and it is known that these effects are transmitted to the succeeding generations, regardless of the lifestyle and genetic factors of the offspring, resulting in the multigenerational perpetuation of metabolic disorders.[73,74] Rats fed with a HFD when compared with rats fed a standard diet were found to have an abnormal estrous cycle. This can impair mating,[51,75] delay maturation of oocytes, and reduce the size of oocytes,[76] causing higher percentages of embryofetal deaths during pregnancy,[77] and impacting the reproductive function of the offspring,[33,46] as seen in the present study. Consumption of a HFD by rodent females during pregnancy and/or lactation increases body weight and also the deposition

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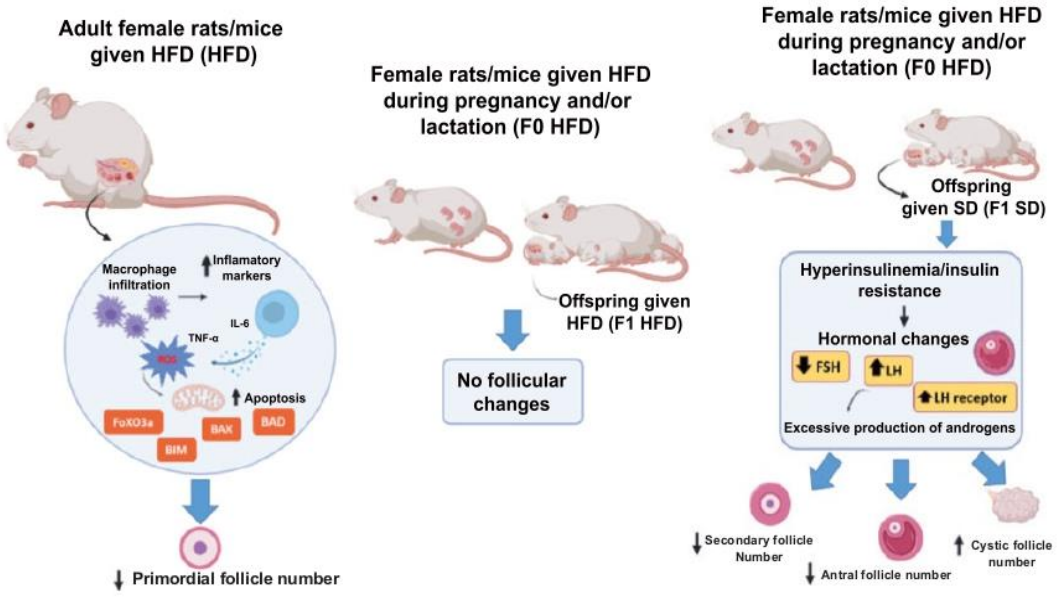


Figure 5 **Mechanisms associated with follicular changes caused by insult with HFD**. *Abbreviations and symbols*: HFD, high-fat diet; FoxO3a, Forkhead box O (FoxO) 3a; BAX, Bcl-2–associated X protein; BIM, activator subtype of BH3-only proteins; BAD, Bcl-2 antagonist of cell death; IL-6, interleukin 6, TNF-α, tumor necrosis factor α; LH, luteinizing hormone; FHS, follicle-stimulating hormone; ↓, decreased; ↑, increased

of white adipose tissue in the offspring long-term[78]; in addition, fetuses of mothers who consumed a HFD during pregnancy showed greater expression of pro-inflammatory markers in their adipose tissue.[79,80] The role of adiposity and weight gain in the relationship between maternal HFD consumption and follicular changes in offspring was reported in the study by Tsoulis et al,[46] in which the increase in peritoneal fat and leptin levels of mothers who were fed a HFD showed a negative correlation with the survival of oocytes in fetuses, suggesting that increased maternal–fetal inflammation caused by the consumption of a HFD by mothers induces apoptosis in the developing fetal ovary. However, the mechanisms by which maternal consumption of a HFD impairs embryo development still need to be better elucidated.[81]

It is known that hyperglycemia and insulin resistance may play a role in the perpetuation of metabolic dysfunction in the offspring. Hyperglycemia and insulin resistance caused by maternal consumption of a HFD causes an increase in granulosa cell apoptosis in preovulatory follicles, a decrease in the size of oocytes, a reduction in the survival of blastocysts, and a decrease in expression of the IGF-IR receptor in 4-day embryos.[76,82] IGF-IR is critical for normal insulin signaling and glucose transport in the pre-implantation blastocyst.[83] Maternal insulin resistance resulting from a HFD is the factor determinant for the decrease in expression of IGF-IR.[76] Another important factor recently discovery is the decrease of the Stella protein in oocytes of mice fed with a HFD, which causes high-level DNA damage in the pro-nucleus of the oocyte, and later causes a delay in embryonic development.[81] It is important to highlight that Stella is a well-known maternal factor, and its expression begins during the specification of the primordial germ cells[84]; it is expressed again exclusively in the oocytes and persists in the embryos pre-implantation.[85] These findings might help explain the effect of fetal programming caused by a HFD, although gaps remain to be filled.

The meta-analysis also showed that offspring of mothers exposed to a HFD during pregnancy and/or lactation had a decreased number of secondary follicles and antral follicles, and an increased number of cystic follicles. These changes indicate that maternal metabolic changes may impact the ovarian follicular development of the next generation, even after exclusive consumption of a standard diet by the offspring.

The presence of cysts is associated with changes in hormones such as insulin, which plays an important role in this pathway.[86] Insulin resistance and/or hyperinsulinemia is often found in rats that have consumed a HFD, as well as in their offspring,[30,31,46,87] and this can predispose the rats to androgen overproduction,[49,88] decreased follicle-stimulating hormone secretion,[88,89] and increased luteinizing hormone secretion, as well as increased affinity of luteinizing hormone receptors in granulosa cells.[88] The overproduction of luteinizing hormone relative to follicle-stimulating hormone promotes the cellular production of androgens in the theca

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cell, which interferes with the aromatization of granulosa cells to estrogen and impairs follicle maturation and ovulation (Fig. 5).[90,91] The excessive production of androgens affects the hypothalamic–pituitary axis, causing the vicious cycle of overproduction of androgens that characterizes PCOS pathogenesis.[92] Polycystic ovaries, hyperandrogenism, and oligo and/or anovulation are characteristics of PCOS, an endocrine disorder with numerous metabolic and reproductive consequences.[93] These follicular developmental changes in offspring are the result of fetal programming caused by maternal exposure to a HFD and can shorten the reproductive life of the females, resulting in premature ovarian failure due to the depletion of the ovarian reserve; in addition, they can impair the pregnancy process.

The meta-analysis showed no difference in the number of atretic follicles. However, as the period of exposure to a HFD increased, the analysis showed that there was an increase in atretic follicles in adult female rats (HFD) and in the offspring of mothers fed with a HFD (F0 HFD/F1 SD). However, this is the individual effect-size and not a pooled analysis, since only 1 study was included in the subdivision per exposure period. There are some articles in the literature reporting that exposure to a HFD leads to increased atretic follicles.[34,46,47,50] We note that, of the studies selected for the meta-analysis, most did not contain information about atretic follicles, and this may have influenced the result. In general, subdivision by exposure period did not yield a different result when compared with the primary analysis, neither lower heterogeneity. This shows that, generally, the effect of exposure to a HFD was independent of the period of exposure.

The fact that the diets differed considerably in terms of fat percentage, composition, and energy content, and that these were not always reported clearly, opens up a debate about how representative these values are for translational studies. According to the literature, humans consume approximately 30% of their daily calories from fat, ie, less than the 40%–60% of calories being obtained from fat used in many rodent studies on consumption of a HFD.[94,95] Thus, the translational value of results obtained using a HFD with almost 80% of calories from fat, may be questionable.[94] It is currently believed that rodents fed with a HFD better mimic the human state of obesity and its effects than most genetically modified models, and that the HFD may be the best choice for testing possible therapeutic measures.[96] It is essential to note that researchers who wish to work with these models take into account the strains, how the HFD will be offered, how the animals will be kept in housing, and choose percentages of fat that will be used so that the results have a greater translational effect.[94]

Limitations and strengths

The studies included in this review have some important limitations. The percentage of fat used in diets differed between studies, and lack of information about diet composition was common. It is known that small differences between diets, such as the source of fat used and the proportion of minerals and fatty acids, can significantly affect metabolic parameters (eg, insulin resistance),[97,98] an important hormone for follicular development.[86] Five included studies reported the source of fat used in the HFD, and most studies showed energy values, which did not vary much between studies. The percentages of fat, however, were very different. There were also differences in the characteristics of the standard diets, which might affect the results. These differences suggest that there has not been a consensus in research involving the HFD and its effect on fertility, and this lack of standardization makes the reproducibility of the experiments challenging.

Furthermore, the period of exposure for the adult female rats (HFD) and the pregnant rats (F0 HFD) to the HFD was very different. Although the period of exposure was not specified in the eligibility criteria for this review, it is known to be a key factor for data interpretation.[99] The dietary durations were detailed in the study by Akamine et al,[30] in which females exposed for 180 days to a HFD exhibited abnormal ovarian morphology compared with those fed for 120 days.

The studies also showed heterogeneity concerning the included species and the applied methodologies for follicle counting. Overall, the follicle classifications were uniform, except for a single study that considered primary and secondary follicles as a single unit for statistical analysis,[44] and 2 studies presented no clear methodology for follicular classification.[43,49] However, the methods used to perform the histological sections analysis, as well as the follicle counting for each section, were quite different between studies. Methodological differences in the morphological analysis might confound the data,[53] eg, the fact that some studies counted follicles in each ovarian section, and others counted only a representative section. Although the evaluation of the cell types that form the follicles was the aim of this review, only Akamine et al[30] analyzed these cells. Therefore, it was not possible to carry out an analysis of the effects of a HFD on follicular cells in this review. Another important limitation was the lack of numerical outcome data that prevented the inclusion of several studies in the meta-analysis and decreased the power of the calculation of the overall effect size of the outcomes.

This review performed a methodological evaluation using an appropriate tool applied to animal studies. Among the good principles for ensuring low risk of bias

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in an experimental study are sequence generation, allocation concealment, and blinding.[100,101] Although some studies reported on randomization, none informed the reader of the method of randomization in the experimental groups. Concerning random housing and blinding of caregivers and/or investigators, none of the included studies reported such information. However, in studies with experimental diets, blinding of personnel is challenging, given the fact that the diets generally have visual characteristics that easily allow their distinction. Although some studies reported the blinding of outcome assessors, most of them did not mention blinding. Blinding is particularly important when there is a subjective element in the recording of observations, eg, the analysis of histological preparations.[101] The presentation of a clear methodology is essential for understanding the results, for critical analysis of the study, and for minimizing the bias of the results.[101] Due to insufficient information, as demonstrated by the analysis of the risk of bias, most of the studies were rated as unclear risk of bias. Thus, it was not possible to carry out an analysis of publication bias or sensitivity analysis. To increase the usefulness and reliability of animal studies, the UK's National Center for Refinement and Reduction of Animals in Research has developed the ARRIVE guidelines (Animal Research: In Vivo Experiment Reports),[102] which has been used by several investigators.

Despite the limitations, this review stands out for being the first to present a systematic review with meta-analysis on the effects of a HFD on the development of ovarian follicles in rodents. The search was comprehensive, including several databases and a large number of keywords, which made it possible to increase the number of analyzed studies. The fact that the current review analyzed the exposure of adult females and mothers and/or their offspring to a HFD made it possible to analyze the intergenerational effect of HFD consumption. Also, the increasing use of this model for the study of PCOS provides important information about the mechanisms of the disease, although caution is required in the translational interpretation of studies.

CONCLUSION

This systematic review and meta-analysis study of female rodents that consumed a HFD compared with a standard diet provides evidence that HFD exposure leads to impaired follicular development, caused mainly by a loss of primordial follicles and an increased number of cystic follicles. However, it is difficult to draw definitive conclusions about the impact of a HFD on ovarian follicles at the moment due to important variations in the methodological conditions and the reporting of results. More studies are needed, and the researchers must strive to ensure that the methodology described guarantees the reliability and reproducibility of the studies. As demonstrated by the 90-day HFD exposure of mothers in the period of pregnancy and/or lactation, it was possible to observe changes in the follicles, and thereby to explore possible mechanisms involved in the follicular changes—possible targets for the study of therapeutic measures. A HFD including approximately 40%–50% fat could be considered the most appropriate for obtaining results with translational value.

These findings are important because consumption of a diet rich in fats is increasingly a part of the Western lifestyle. This analysis indicates that preventive measures aimed at improving the nutritional conditions of women and raising awareness about the role of nutrition in the healthy development of the ovary are of importance.

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Author contributions. V.G.P., G.V., and D.C.D designed the study; V.G.P., Y.K.S, and G.V. conducted the literature search; V.G.P. and G.V. assessed eligibility and risk of bias and extracted data from studies; V.G.P. wrote the manuscript; V.G.P. and G.V. had primary responsibility for the final content; and D.C.D., Y.K.S, R.Q.M.S, and G.T.V. provided other contributions (including revising the paper critically for important intellectual content). All authors read and approved the final manuscript.

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Declaration of interest. The authors have no relevant interests to declare.

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SUPPORTING INFORMATION

The following Supporting Information is available through the online version of this article at the publisher's website.

Figure S1 **Risk of bias score for each item in animal studies, as assessed using the SYRCLE tool**

Figure S2 **Risk of bias summary for each item in animal studies, as assessed using the SYRCLE tool**

Table S1 **PRISMA checklist**

Table S2 **Search strategy**

Table S3 **Influence of a high-fat diet (HFD) and maternal HFD during pregnancy or follicle development**

Table S4 **Summary of findings of subgroup meta-regression according to high-fat diet (HFD) exposure period**

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CAPÍTULO II

Este manuscrito será posteriormente formatado para submissão à revista *Metabolism – Clinical and Experimental* (Fator de Impacto = 8,694).

AVALIAÇÃO DA FUNÇÃO MITOCONDRIAL, ESTRESSE OXIDATIVO E APOPTOSE NOS OVÁRIOS DE RATAS ADULTAS EXPOSTAS À HIPERGLICEMIA MATERNA E SUBMETIDAS À DIETA HIPERLIPÍDICA APÓS O DESMAME

Mitochondrial function, oxidative stress and apoptosis in ovaries of adult rats exposed to maternal hyperglycemia and submitted to high-fat diet after weaning

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RESUMO

Diabetes e obesidade podem prejudicar a função reprodutiva tanto em mulheres quanto em animais de laboratório e há necessidade de estudar se essas alterações persistem na próxima geração. Neste estudo, avaliamos a influência da hiperglicemia materna e do consumo pós-desmame de uma dieta hiperlipídica na função mitocondrial, estresse oxidativo e apoptose nas células ovarianas da prole adulta de ratas diabéticas. Ratas fêmeas receberam tampão citrato (Controle – C) ou *Streptozotocin* (droga citotóxica de células beta-pancreáticas para induzir diabetes – D) no dia 5 pós-natal. Na vida adulta, foram acasaladas para obtenção da prole feminina (F) que foi distribuída nos grupos experimentais de acordo com a dieta a ser utilizada, dieta padrão (DP) ou hiperlipídica (DHL), desde o desmame até a idade adulta. FC/DP e FC/DHL representam filhas de mães controle e que receberam DP ou DHL, respectivamente; FD/DP e FD/DHL representam filhas de mães diabéticas e receberam DP ou DHL, respectivamente. Na idade adulta, foi realizado o Teste Oral de Tolerância à Glicose (TOTG) e AUC e, em seguida, as ratas foram anestesiadas e eutanasiadas. Amostras de sangue foram coletadas para determinar as concentrações hormonais. Os ovários foram coletados e separados para análises. Os grupos FC/DHL, FD/DP e FD/DHL apresentaram maior AUC aos 120 min do TOTG. As concentrações de progesterona foram menores nos grupos FC/DHL, FD/DP e FD/DHL. Houve disfunção mitocondrial representada pela menor expressão de MFF e MFN2 nos grupos FD/DP e FD/DHL, assim como prejuízos na proliferação e no processo de apoptose nos ovários. A contagem de folículos primordiais, em crescimento e antrais foi menor nos grupos FD/DP e FD/DHL. Menor intensidade de marcação para SOD e maior intensidade de marcação para MDA foram encontradas nas células lúteas e estromais nos grupos FC/DHL, FD/DP e FD/DHL. Portanto, concluímos que a programação fetal comprometeu a secreção hormonal, função mitocondrial, reserva ovariana e estresse oxidativo dos ovários das filhas de diabéticas, expostas ou não à dieta hiperlipídica pós-desmame. Já, a associação de diabetes materno e dieta hiperlipídica foi responsável por aumentar a intolerância à glicose e a ocorrência de diabetes nas descendentes.

Palavras-chave: Ovário, Mitocôndria, Dieta hiperlipídica, Diabete, Ratas.

1. Introdução

O processo reprodutivo da fêmea depende da função coordenada de eventos que vão desde a diferenciação e liberação do oócito maduro para fertilização até a propagação bem-sucedida da espécie (McGee & Hsueh, 2000). O ovário é o órgão central nos estudos de fertilidade, pois a reserva ovariana contém dois elementos essenciais para a reprodução: o estoque dos folículos primordiais e qualidade dos oócitos (te Velde & Pearson, 2002). Na espécie humana, a formação da reserva ovariana ocorre na vida embrionária e a mulher nasce com um *pool* de folículos primordiais formados (Sánchez & Smitz, 2012). Já em roedores, o desenvolvimento folicular ocorre na vida pós-natal, sendo que os folículos primordiais só começam a se desenvolver a partir do 3º dia de nascimento (Rajah *et al*. 1992; McGee & Hsueh, 2000; Zambrano *et al.*, 2014). O desenvolvimento folicular é controlado pelo processo de apoptose e, durante cada ciclo reprodutivo, os folículos sofrem apoptose como uma forma de selecioná-los no ovário (Hussein 2005; Nayki *et al*., 2016).

A função ovariana depende da ação combinada da função mitocondrial e dos hormônios envolvidos no processo reprodutivo. Nas mitocôndrias das células da granulosa, ocorre produção de energia e nelas há um ambiente propício para a síntese de lipídios e hormônios (17 β-estradiol e progesterona) para manter o desenvolvimento folicular e oocitário no ovário (Morohaku *et al*., 2013; Chowdhury *et al*., 2016; Liu *et al*., 2018). As proteínas MFF (fator de fissão mitocondrial) e MFN2 (mitofusina 2) são fundamentais para a manutenção da dinâmica e função mitocondrial controlando a fissão, essencial para o crescimento e divisão das células para preenchê-las com um número adequado de mitocôndrias, bem como a fusão das mitocôndrias, que permite uma interconexão dos conteúdos mitocondriais e a geração de extensas redes de novas mitocôndrias (Shukla & Mukherjee, 2020). O papel dessas proteínas para o desenvolvimento ovariano ainda não está totalmente claro, mas MFF é visto como regulador da proliferação e maturação das células da granulosa e diminuição da sua expressão está relacionada ao aumento do processo apoptótico (Long *et al*., 2013). A MFN2 é um grande alvo de estudo envolvendo a função reprodutiva, sendo que sua baixa expressão pode levar a estresse oxidativo e prejudicar a foliculogênese (Chen *et al*., 2015). Além disso, baixas expressões de MFF e MFN2 estão relacionadas a problemas na embriogênese. Já foi demonstrado que camundongos *knockout* para MFN2 morreram no útero no meio da prenhez devido a uma deficiência placentária (Chen *et al*., 2010; Youle & Bliek, 2012). Na presença de uma disfunção mitocondrial, pode haver a produção anormal de hormônios ovarianos bem

como o aumento de espécies reativas de oxigênio (ROS), afetando a função das células da granulosa e o desenvolvimento de oócitos (May-Panloup *et al*., 2016; Zhao *et al*., 2019).

Mulheres em idade reprodutiva podem apresentar diabetes, obesidade e complicações decorrentes destes problemas de saúde (Wu *et al*.,2017), o que leva a prejuízos no desenvolvimento folicular, especialmente na presença de uma hiperglicemia, podendo causar infertilidade (Chang *et al*., 2005; Cretu *et al*., 2020). A hiperglicemia está associada à disfunção mitocondrial, estresse oxidativo e diminuição da reserva ovariana (Erbas *et al*., 2014; Nayki *et al*., 2016). Há evidência de alterações na imunoexpressão de marcadores de morte celular por apoptose, como diminuição de Bcl2 e aumento de Bax, FAS e de caspase-3 nos ovários de camundongos diabéticos, com consequente aumento de apoptose e redução no número de folículos (Nayki *et al.*, 2016; Fraunhoffer *et al*., 2018). Além disso, a hiperglicemia pode alterar a cascata de sinalização de insulina, hormônio que tem papel na esteroidogênese ovariana e no desenvolvimento folicular, atráves da sua ligação com receptores que estão difundidos nas células da teca e da granulosa. A partir dessa ligação, há deflagração das vias intracelulares que inclui as vias de sinalização da fosfatidilinositol-3-cinase (PI3K) e das proteínas quinases ativadas por mitógenos (MAPK) (Kido *et al*., 2001). PI3K tem sido explorada por participar diretamente da proliferação celular no ovário (Johnson *et al*., 2001; Carvalho *et al*., 2003; Lima *et al*., 2006; Li *et al*., 2017), além de ativar a via da MAPK, incluindo as reguladas por sinais extracelulares quinases (ERKs) e p38 quinases. ERK1/2 e p38 participam efetivamente da esterodoigênese ovariana (Manna & Stocco, 2011). Essas proteínas também envolvidas na ativação de mecanismos apoptóticos nas células da granulosa e ovócito (Seidman *et al.*, 2001; Lu e Xu, 2006; Lan *et al*., 2015; Liu *et al*.,2019).

Assim, alterações nos fatores de transcrição envolvidos no quadro de resistência à insulina induzidas pelo diabete e, sendo parte da cascata de sinalização insulínica, podem comprometer o desenvolvimento folicular saudável nos ovários e interferir no processo reprodutivo (Butte, 2000; Després & Lemieux, 2006; Gambineri *et al*., 2006). Estes estudos são bem descritos em animais diabéticos e mostram aumento de apoptose nos folículos ovarianos, bem como atraso na maturação dos oócitos em camundongos (Chang *et al.*, 2005), diminuição do *pool* de folículos com presença de fibrose (Erbas *et al*., 2014) e aumento de degeneração folicular com aumento da expressão de caspase-3 em ratas (Nayki *et al.*, 2016).

Estas repercussões podem ocorrer por influências intrauterinas e também devido ao ambiente pós-natal, dentre eles o consumo de uma dieta inadequada que também interfere na programação fetal, prejudicando o processo reprodutivo dos descendentes (Sullivan *et al*.,

2012; Desai *et al*., 2013). Estudos têm avaliado a influência da dieta pós-natal nas funções reprodutivas. Ratas alimentadas com dieta hiperlipídica por 120 dias após o desmame apresentaram resistência à insulina, hiperinsulinemia e morfologia ovariana alterada (Akamine *et al.*,2010). Houve diminuição nos níveis dos hormônios ovarianos (estrógeno e progesterona), aumento de apoptose nas células da granulosa (Hussain *et al*., 2016; Patel & Shah 2018) e prejuízos na via de sinalização da insulina, com diminuição da expressão de receptores de insulina nas células ovarianas (Nteeba *et al*., 2013; Wang *et al*.,2017).

Embora existam estudos sobre as repercussões do diabete e/ou de uma dieta com excesso de gordura nos ovários e os mecanismos apoptóticos envolvidos nesses animais, não há qualquer investigação sobre as alterações em descendentes provenientes de um ambiente intrauterino inadequado, causado pelo diabete materno, e que consomem uma dieta hiperlipídica após o desmame. O efeito na maioria dos órgãos de roedores é semelhante aos efeitos em seres humanos e os achados de modelos animais fornecem uma ferramenta valiosa para estudar e compreender melhor os mecanismos envolvidos na programação metabólica e fetal (Williamns *et al.*, 2014).

Diante dessas evidências e, considerando que o ambiente intrauterino hiperglicêmico ou a resistência à insulina podem prejudicar a função reprodutiva tanto em mulheres como em modelos experimentais e a impossibilidade de obter amostras de ovários de mulheres em idade reprodutiva, há necessidade de estudar se as alterações em modelos animais persistem na próxima geração. Nossa hipótese é que descendentes de ratas que se desenvolvem em um ambiente intrauterino diabético e submetidas à dieta hiperlipídica na vida pós-natal (do desmame à vida adulta), apresentarão função mitoncondrial alterada e estresse oxidativo, interferindo no desenvolvimento folícular e levando a modificações na estrutura e na função ovariana. Estes achados poderão justificar a menor fertilidade e a reprodução prejudicada na vida adulta destes animais.

2. Materiais e método

Animais

Foram utilizados ratos adultos machos e fêmeas (150-250 g), da linhagem *Sprague-Dawley* que acasalaram para fornecer descendentes fêmeas para indução de diabete. Os animais foram mantidos no Biotério do Laboratório de Pesquisa Experimental de Ginecologia e Obstetrícia da Faculdade de Medicina de Botucatu-Unesp sob condições controladas de

temperatura (22 ± 2ºC), umidade (50 ± 10%) e ciclo claro/escuro (12 h) em gaiolas de polipropileno forradas com maravalha. Como forma de enriquecimento ambiental, bolas de papel foram utilizadas nas gaiolas (Simpson & Kelly, 2011). Água filtrada e ração comum foram oferecidas *ad libitum*. O Comitê de Ética no Uso de Animais de nossa Instituição aprovou todos os métodos adotados neste estudo (Protocolos CEUA: Número 1218-2017 – projeto maior/guarda-chuva e Número: 1334/2019). Este trabalho foi financiado pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – Processo Número: 2016/25207-5).

Desenho experimental

O desenho experimental está resumido na Figura 1.

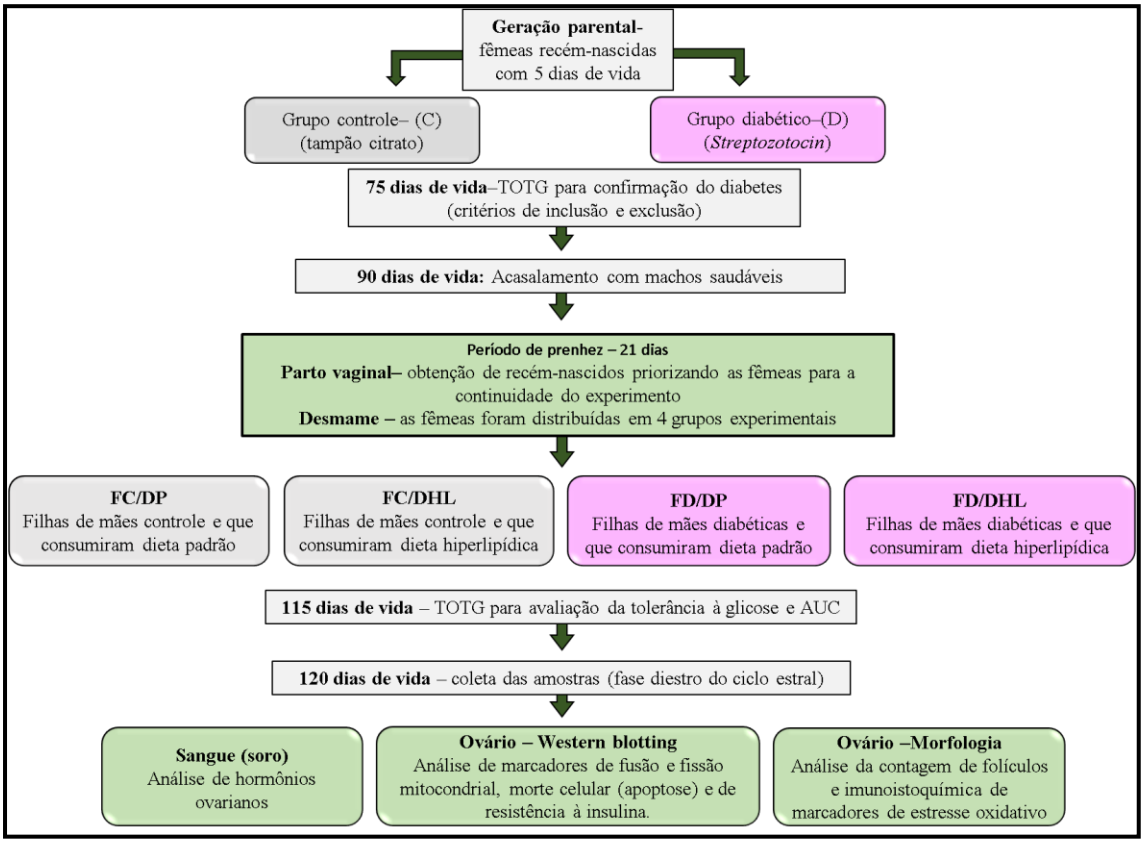


Figura 1. Desenho experimental

Indução do diabete

Para a indução do diabete nas ratas fêmeas, foi utilizada a droga beta-citotóxica *Streptozotocin* (Sigma Aldrich®, MA, EUA - dose de 70 mg/kg, via subcutânea) no dia pós-natal 5 (DPN). Para composição do grupo não diabético (controle), as ratas fêmeas receberam o volume equivalente de veículo (tampão citrato - 0,01 M, pH 4,5) conforme descrito anteriormente (Damasceno *et al*., 2011). Os níveis de glicemia no teste oral de tolerância à glicose (TOTG) foram classificados de acordo com a *American Diabetes Association* (ADA) (ADA, 2021) e Sinzato *et al*. (2021). As ratas foram consideradas diabéticas quando aos 75 dias de vida apresentaram glicemia ≥ 200 mg/dL em pelo menos um momento durante o TOTG. No grupo controle, foram incluídas apenas ratas com glicemia < 140 mg/dL pelo menos em três momentos durante o TOTG. Ratas fêmeas que não apresentaram as características acima, foram anestesiadas, passaram por eutanásia e foram excluídas deste estudo.

Período de acasalamento, prenhez e lactação

Na idade adulta, as fêmeas diabéticas (D) e controle (C) acasalaram durante a noite com ratos machos normoglicêmicos não diabéticos. Na manhã seguinte, a prenhez foi confirmada quando os espermatozoides foram encontrados no esfregaço vaginal, e este foi designado como dia 0 de prenhez. Ratas fêmeas que não acasalaram após 15 dias consecutivos foram consideradas inférteis e excluídas deste estudo (Moraes-Souza *et al*., 2017). A prole foi obtida através de parto vaginal e, após o nascimento, as mães foram mantidas com pelo menos oito filhotes por ninhada, sendo 4 machos e 4 fêmeas, até o desmame (DPN 22). Quando a ninhada tinha até oito filhotes, se ambos os sexos estivessem presentes, não houve manipulação. Quando as ninhadas excederam 8 filhotes, machos e fêmeas foram selecionados de forma randomizada. Se a ninhada fosse inferior a oito filhotes por mãe, essa mãe e sua ninhada foram excluídas de nosso experimento, mas mantidas em nosso biotério para outro estudo.

Grupos experimentais e dietas

Após o desmame, as filhas de mães diabéticas (FD) e controle (FC) foram pseudo-randomizadas por sorteio respeitando no máximo quatro fêmeas por mãe, sendo utilizadas apenas duas irmãs/mãe/grupo. Posteriormente, os grupos FD e FC foram divididos em dois outros grupos de acordo com a dieta com a qual foram alimentadas, seja uma dieta padrão (DP - com teor de Kcal: 28,54% proteína, 62,65% carboidrato, 8,7% gordura, Purina®, Brasil) ou dieta hiperlipídica (DHL- com teor de Kcal: 23,43% proteína, 46,63% carboidrato, 30%

gordura, utilizando banha de porco como fonte de gordura). A comparação bromatológica adicional entre as dietas é apresentada no Anexo1.

As ratas foram distribuídas em quatro grupos experimentais: FC/DP - filhas de mães controle e alimentadas com dieta padrão após o desmame; FC/DHL - filhas de mães controle e alimentadas com dieta hiperlipídica após o desmame; FD/DP - filhas de mães diabéticas e alimentadas com dieta padrão após o desmame; FD/DHL - filhas de mães diabéticas e alimentadas com dieta hiperlipídica após o desmame. A DHL foi feita artesanalmente em nossa instituição, adequadamente suplementada com vitaminas e minerais e mantida sob refrigeração até o momento do uso. Como as dietas apresentaram características visuais que permitiam facilmente a sua distinção, não foi possível o alojamento aleatório dos animais e o cegamento dos cuidadores e/ou investigadores.

Teste oral de tolerância à glicose (TOTG) e Área sob a curva (AUC)

No DPN 115, o TOTG foi realizado conforme já descrito anteriormente (Tai, 1994; Gallego *et al*., 2019) para avaliação da tolerância à glicose, e a resposta total de glicose foi avaliada calculando a área sob a curva (AUC) usando o método trapezoidal (Tai, 1994) levando em consideração a quantidade de glicose circulando em 120 min do TOTG.

Eutanásia dos animais, coleta de sangue e ovários

A partir dos 120 dias de vida, as ratas foram analisadas quanto à fase do ciclo estral, no período da manhã no biotério. As ratas que se encontravam em diestro (fase do ciclo que sofre menos interferências hormonais) foram anestesiadas com tiopental sódico (Thiopentax®) - dose de 120 mg/kg via intraperitoneal) no laboratório e decapitadas. As amostras de sangue foram coletadas em tubos de ensaio sem anticoagulante, incubadas em gelo por 30 min e, depois, centrifugadas a 1.575 *x g* por 10 min a 4°C. O soro foi coletado e armazenado a -80°C até posterior análise dos parâmetros hormonais. Posteriormente, as ratas foram submetidas à laparotomia para retirada dos ovários.

Avaliação do perfil hormonal

A partir daqui todos os procedimentos foram realizados com o investigador cego para as análises. As concentrações séricas de 17 β-estradiol (#501890*)* e progesterona *(*#582601) *(Cayman Chemical*®, Michigan, USA) foram avaliados por ELISA segundo as recomendações do *kit*.

Imunodetecção de proteínas por Western blotting

Um ovário de cada rata foi retirado e imediatamente congelado em nitrogênio líquido e armazenados em freezer a -80°C. As amostras foram homogeneizadas em extração com RIPA *Lysis Buffer* (Millipore®, EUA) adicionado de inibidor de protease e fosfatase (Sigma Chemical Company®, EUA). Posteriormente, foram centrifugados a 1699 *x g* a 4°C por 20 min para remover o material insolúvel. A determinação da concentração proteica foi realizada pelo método de Bradford (Bradford, 1976), e posteriormente foi realizada a quantificação de proteínas nas amostras. As amostras foram normalizadas para alíquotas contendo 70 μg de proteínas totais, e as amostras (n=6 animais por grupo) foram separadas em gel de poliacrilamida (DPS-PAGE). O peso molecular relativo das bandas foi determinado de acordo com o padrão de peso molecular marcado com cores, Kaleidoscope (Bio-Rad®, USA), que foi colocado junto na corrida eletroforética. Após a eletroforese, as proteínas foram transferidas para membrana de nitrocelulose (Millipore®, EUA). As ligações a proteínas não específicas foram bloqueadas pela incubação da membrana com 5% de leite desnatado diluído em tampão TBS-T por 1h em temperatura ambiente. As membranas foram incubadas por 16 horas a 4°C com anticorpos primários: MFF (CellSignaling®, #84580, 1:800), Mitofusina-2 (MFN-2) (CellSignaling®, #9482, 1:800), PI3-Kinase (BD Bioscience®, #610045, 1:1000), MAPK (Erk1/2) (CellSignaling®, #4695, 1:800), Fosfo-p44/42 MAPK (Erk1/2) (CellSignaling®, #4377, 1:800), Bcl-2 (CellSignaling®, #15071, 1:800), Fas (CD-95) (BD Bioscience®, #610197, 1:800), BAX (Elabscience® #13814, 1:800), Caspase-3 clivada (Abcam®, #ab2302, 1:500), PCNA (PC10) (Santa Cruz®, #sc-56, 1:1000) e β-Actina (CellSignaling®, #3700, 1:1000). Após lavagem em TBS-T, as membranas foram incubadas por 90 min com um anticorpo secundário conjugado com HRP e específico para cada anticorpo primário (CellSignaling®, #7074, #7076, 1:10.000). A reação foi desenvolvida por um *kit* de reagentes ECL (Bio-Rad®, EUA) e as imagens das bandas foram capturadas usando uma câmera CCD (sistema G:BOX, Syngene®, Cambridge, Reino Unido). As densidades ópticas integradas (IODs) das bandas de proteína-alvo foram medidos usando o *software ImageJ (National Institutes of Health*, EUA). Posteriormente, os níveis de expressão foram normalizados pelos valores obtidos do controle interno (β-actina) e os resultados expressos em *fold change* com média ± desvio-padrão (DP).

Avaliação histológica dos ovários

O segundo ovário de cada rata foi pesado e fixado em formaldeído a 10% por 24 horas, desidratado em concentrações progressivas de álcool e incluído em parafina. Foram utilizados 5 ovários/grupo, os quais foram seccionados em seções de 8 μm de espessura cada um utilizando um micrótomo rotativo. Para realização da análise histológica convencional e contagem dos folículos ovarianos, cada 10ª seção foi corada com hematoxilina e eosina (H&E) (Kundu *et al*., 2018). As imagens foram capturadas por meio do sistema de imagem computadorizado (Software KS-300, versão 3.0, Zeiss®, alemão), que recebe uma imagem de câmera digital (CCD-IRIS/RGB, Sony®, Japão), acoplada ao microscópio (DMR, Leica®). A contagem foi realizada utilizando o *software Image J*®. Os folículos foram classificados como *primordiais* se apresentassem cada oócito circundado por uma única camada de células da granulosa achatadas; *primários* se apresentassem o oócito circundado por uma única camada de células da granulosa cuboides; *folículos em crescimento* se apresentassem um oócito circundado por, pelo menos, duas camadas de células granulosas cuboides e *folículos antrais* se o oócito contivesse material nuclear para evitar dupla contagem dos tipos de folículos maiores que podem abranger várias seções (Kundu *et al*., 2018). Para garantir que nenhum corpo lúteo fosse contado duas vezes, essa estrutura foi analisada em uma imagem única (objetiva de 4x) e foi seguida em sua totalidade através do ovário e contada apenas uma vez, mesmo que aparecesse na próxima seção a ser contada (Kundu *et al*., 2018).

Avaliação imunoistoquímica (IHQ) do ovário

Para a técnica IHQ, cada 9ª seção foi considerada e as células do corpo lúteo, células estromais e células da granulosa foram as estruturas analisadas. Os anticorpos primários Malondialdeído (anti-MDA, Abcam® #ab6463, 1:500) e Superóxido-dismutase (anti-SOD-1, Abcam® #ab16831, 1:200), foram utilizados. Não houve recuperação antigênica. O bloqueio de peroxidase endógena foi realizado com metanol e peróxido de hidrogênio (na concentração de 20%) por 40 min em temperatura ambiente, e com a utilização do bloqueio pronto para uso (inibidor da peroxidase contendo peróxido de hidrogênio e azida de sódio 15mM - Dako®, CA, EUA), com incubação em estufa a 27ºC por 20 min. Para o bloqueio de proteínas inespecíficas, foram utilizados os bloqueios com leite desnatado na concentração de 10% por 2 horas e o *Protein Block*® (caseína 0,25% em PBS, contendo proteína carreadora e azida sódica 15mM - Dako®) por 30 min em estufa a 27°C. Após a incubação do anticorpo primário, o anticorpo secundário (Histofine®) foi adicionado por 30 min em estufa a 27°C. Para a revelação da

peroxidase, o cromógeno DAB (3,3 diaminobenzidina) foi usado por 3 min à temperatura ambiente. Em seguida, as lâminas foram contracoradas em hematoxilina de Harris e montadas. As imagens foram capturadas por meio do sistema de imagem computadorizado (Software KS-300, versão 3.0, Zeiss®, alemão), que recebe uma imagem de câmera digital (CCD-IRIS/RGB, Sony®, Japão), acoplada ao microscópio (DMR, Leica®). Ambos anticorpos apresentaram imunomarcação citoplasmática e as análises foram realizadas pela intensidade da imunocoloração utilizando o *software Image J*®.

Análise estatística

Todas as análises foram realizadas com o auxílio do especialista em Bioestatística do Escritório de Apoio à Pesquisa (EAP) da Faculdade de Medicina de Botucatu, Unesp. Para calcular o tamanho da amostra (n), foi utilizado um delineamento randomizado, com base em valores obtidos de experimentos prévios, sendo um "n" mínimo de cinco animais/grupo). Para a análise imunoistoquímica, o cálculo foi estimado em cinco fotos/estrutura/animal/grupo. Os dados foram apresentados como média ± desvio padrão. Para AUC e concentrações séricas de progesterona e 17β-estradiol, foi utilizado o Two-way ANOVA seguido do Teste de Comparação Múltipla de Tukey. Para os dados que não apresentassem uma distribuição normal (curva de Gauss), foi realizado ajustes de todos os dados e, em seguida, analisados por testes de distribuição, dentre eles a expressão proteica dos diferentes marcadores e da intensidade de marcação para Superóxido Dismutase 1 (SOD-1) e Malondialdeído (MDA), os quais foram submetidos aos testes de Distribuição Gama seguidos do Teste de Comparações Múltiplas de Wald. Para a análise da contagem de folículos ovarianos e corpo lúteo, os testes de distribuição de Poisson, binomial negativo e teste de Wald foram utilizados. O teste de correlação de Pearson foi utilizado para verificar a existência de correlações entre os dados. Para todas as comparações, foi considerado um limite mínimo de confiança de 95% ($p<0,05$).

3. **Resultados**

Glicemia circulante durante o TOTG para verificar a proporção de ratas com diabete

A Tabela 1 apresenta os dados de área sob a curva (AUC) e porcentagem (%) de ratas diabéticas aos 115 dias de vida (DPN 115). A quantidade de glicose circulante durante os 120 min do TOTG foi maior nos grupos FC/DHL, FD/DP e FD/DHL em comparação ao grupo controle (FC/DP). As filhas de mães diabéticas e que consumiram dieta rica em gordura na vida

pós-natal (FD/DHL) apresentaram maior AUC ainda em relação aos grupos FC/DHL e FD/DP, levando à maior porcentagem de ratas que ficaram diabéticas no grupo FD/DHL (40,00%) em relação aos grupos outros (11,11%).

Tabela 1. Área sob a Curva (AUC) e porcentagem (%) de ratas com diabete (prole feminina) de mães controle (FC) ou mães diabéticas (FD) que consumiram uma dieta padrão (DP) ou hiperlipídica (DHL) desde o desmame até os 120 dias de vida pós-natal.

Grupos	AUC (mg/dL/120min)	% Ratas com diabete
FC/DP	117,30 ± 10,37	0%
FC/DHL	138,68 ± 11,01*	0%
FD/DP	155,97 ± 9,35*	11,11%
FD/DHL	164,70 ± 7,24*$^{\#\$}$	40,00%*$^{\#\$}$

Valores expressos como média ± desvio padrão. n=8 ratas/grupo

**p<0,05 - comparado ao grupo FC/DP; $^{\#}$p<0,05 - comparado com o grupo FC/DHL; $^{\$}$p<0,05 - comparado com o grupo FD/DP (ANOVA seguida do teste de comparações múltiplas de Tukey).*

Avaliação da concentração sérica de hormônios ovarianos e ciclo estral

Aos 120 dias de vida das ratas-filhas, houve diminuição das concentrações séricas de progesterona nos grupos FC/DHL, FD/DP e FD/DHL em comparação com o grupo FC/DP (Figura 2A). No grupo FD/DHL as concentrações de progesterona foram menores em comparação com os grupos FC/DHL e FD/DP. Já, as concentrações séricas de 17β-estradiol não diferiram entre os grupos (Figura 2B). A avaliação do ciclo estral foi realizada em ambos os grupos de filhas de mães diabéticas e o grupo OD/SD permaneceu mais tempo na fase estro (*p=0,0332*).

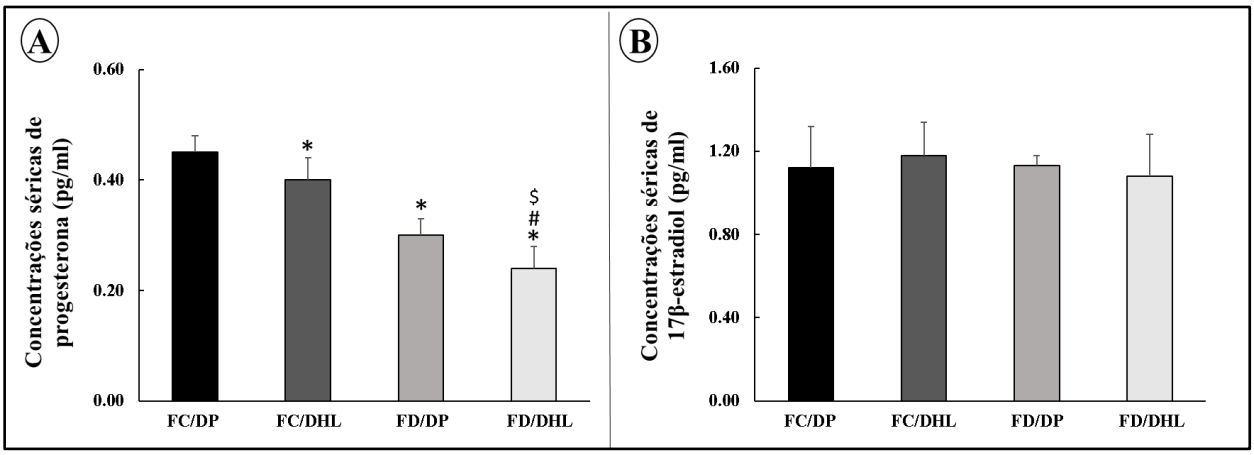


Figura 2. Concentrações séricas de (A) Progesterona e (B) 17 β-estradiol da prole feminina de mães controle (FC) ou mães diabéticas (FD) que consumiram uma dieta padrão (DP) ou hiperlipídica (DHL) deDPe o desmame até os 120 dias de vida pós-natal.

Os valores são expressos como média ± desvio padrão. n=8 ratas/grupo

**p<0,05 - comparado ao grupo FC/DP; $^{\#}$p<0,05 - comparado com o grupo FC/DHL; $^{\$}$p<0,05 - comparado com o grupo FD/DP (ANOVA seguido de teste de comparações múltiplas de Tukey).*

Avaliação de proteínas envolvidas na via de sinalização à insulina

A expressão da proteína PI3K foi maior no grupo FC/DHL em comparação com o grupo FC/DP e FD/DHL (Figura 3A e 3B). Em relação à expressão de MAPK (ERK1/2 –fosforilada e total), não houve alterações entre os grupos (Figuras 3A e 3C).

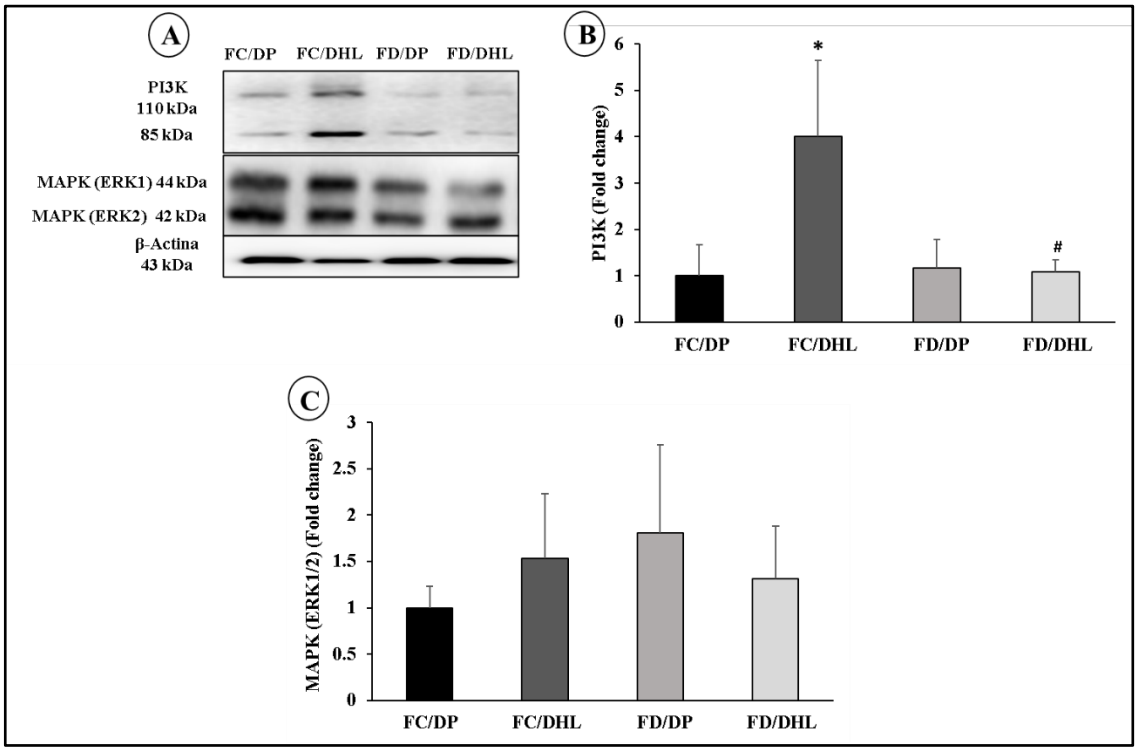


Figura 3. Análise da expressão de proteínas marcadoras de resistência à insulina. (A) Membranas, (B) PI3K, (C) MAPK (ERK1/2) da prole feminina de mães controle (FC) ou mães diabéticas (FD) que consumiram uma dieta padrão (DP) ou rica em gordura (DHL) desde o desmame até os 120 dias de vida pós-natal. Legenda: PI3K - fosfatidilinositol-3-cinase, MAPK (ERK1/2) – Proteína quinase ativada por mitógeno (quinase regulada por sinal extracelular 1/2).

Os valores são expressos como média ± desvio padrão, n=6 ratas/grupo.

**p<0,05 - comparado ao grupo FC/DP; #p<0,05 - comparado com o grupo FC/DHL (Distribuição Gama seguida do teste de comparações múltiplas de Wald).*

Avaliação da função mitocondrial, apoptose e proliferação celular

Houve diminuição da expressão da proteína MFF nos grupos FC/DHL, FD/DP e FD/DHL quando comparadas às expressões das ratas FC/DP (Figura 4A e 4B). A proteína MFN-2 foi menos expressa nos grupos de filhas de ratas diabéticas comparada às do grupo controle (FC/DP). No grupo FD/DHL, a expressão de MFN-2 também foi menor do que nos grupos FC/DH FD/DHL (Figura 4C).

Os marcadores relacionados ao processo de apoptose e proliferação celular também foram alterados. Houve diminuição da expressão de Bcl-2 nos grupos diabéticos (FD/DP e FD/DHL) em comparação ao grupo controle (FC/DP) (Figura 4A e 4D). A expressão de Bax também foi menor no grupo FD/DHL em relação aos demais grupos (Figura 4A e 4E). Os grupos de filhas de ratas diabéticas apresentaram diminuição da expressão de Caspase-3 clivada (Figura 4A e 4F) e do marcador de proliferação celular PCNA em comparação com os grupos FC/DP e FC/DHL (Figura 4A e 4G). Não houve alterações na expressão de FAS entre os grupos (Figura 4A e 4H).

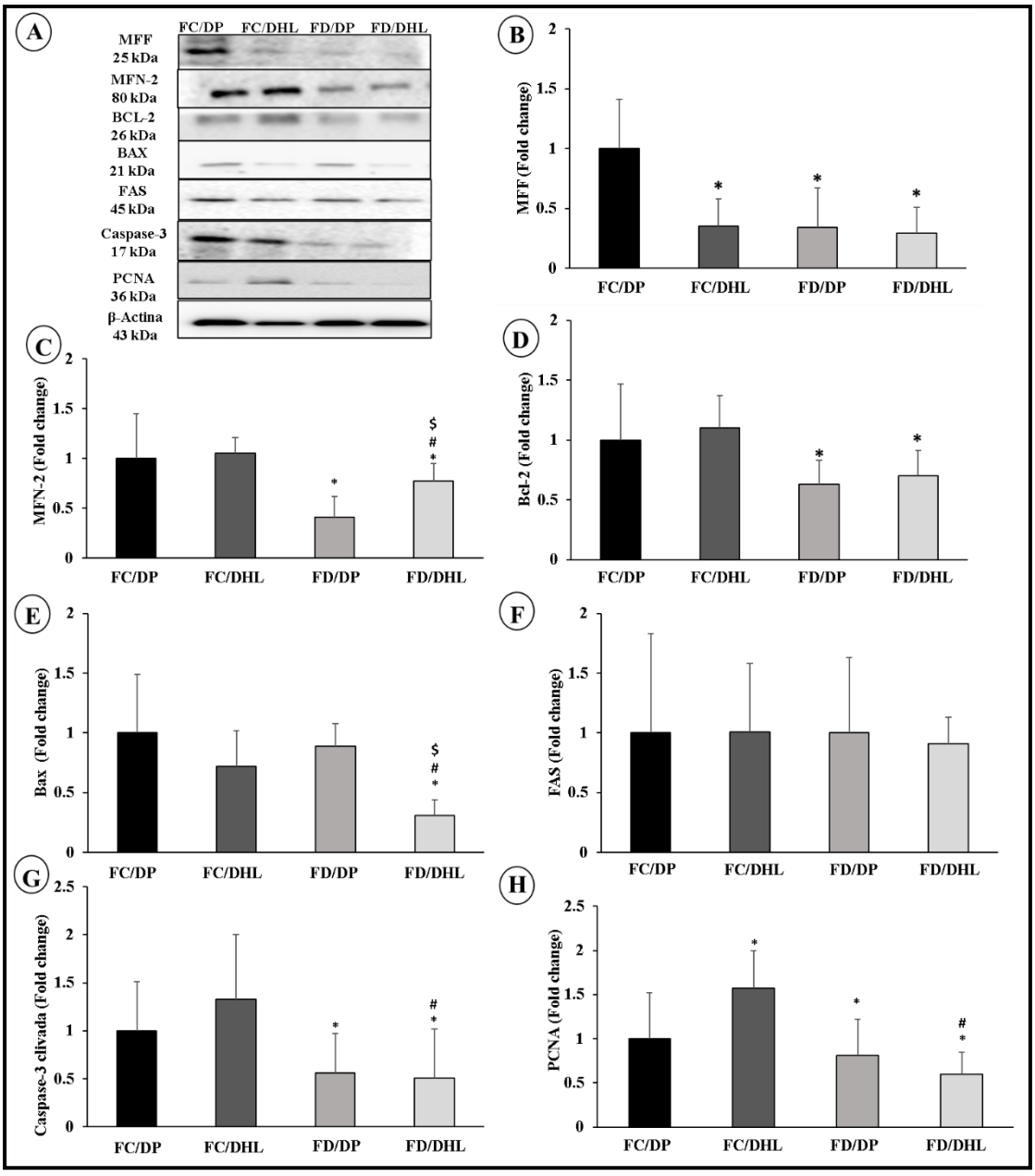


Figura 4. Análise da expressão de proteínas envolvidas na dinâmica mitocondrial e apoptose. (A) Membranas, (B) MFF, (C) MFN-2, (D) Bcl-2, (E) Bax, (F) Caspase-3 clivada, (G) FAS e (H) PCNA de filhas de ratas controle (FC) ou diabéticas (FD) que consumiram uma dieta padrão (DP) ou hiperlipídica (DHL) desde o desmame até os 120 dias de vida pós-natal.

Legenda: MFF – Fator de fissão mitocondrial, MFN-2 – Mitofusina-2, Bcl-2 – linfoma de células B-2, Bax - Proteína X associada a Bcl-2, FAS - Superfamília de receptores do fator de necrose tumoral (TNF) membro 6, PCNA - antígeno nuclear de célula em proliferação**.**

Os valores são expressos como média ± desvio padrão, n=6 ratas/grupo.

**p<0,05 - comparado ao grupo FC/DP; #p<0,05 - comparado com o grupo FC/DHL; $p<0,05 - comparado com o grupo FD/DP (teste de Distribuição Gama e ANOVA seguido de teste de comparações múltiplas de Wald).*

Avaliação da contagem de folículos ovarianos

A tabela 2 apresenta os dados da contagem de folículos ovarianos. Houve diminuição de folículos primordiais, primários e folículos em crescimento nos grupos FD/DP e FD/DHL em comparação com o grupo FC/DP. Os grupos FC/DHL, FD/DP e FD/DHL apresentaram diminuição de folículos antrais quando comparados ao grupo FC/DP, sendo que no grupo FD/DHL também houve diminuição de folículos antrais comparado ao grupo FC/DHL. Não houve alterações na contagem de corpos lúteos.

Tabela 2. Contagem de folículos ovarianos da prole feminina de mães controle (FC) ou mães diabéticas (FD) que consumiram uma dieta padrão (DP) ou hiperlipídica (DHL) desde o desmame até os 120 dias de vida pós-natal.

Variáveis / Grupos		**DP**	**DHL**
Folículo Primordial			
	FC	75,8 ± 11,0	55,8 ± 12,8
	FD	34,6 ± 14,6*	35,6 ± 17,2*
Folículo Primário			
	FC	37,4 ± 12,3	33,0 ± 16,2
	FD	19,8 ± 5,7*	21,6 ± 6,4*
Folículos em Crescimento			
	FC	155,8 ± 25,0	148,6 ± 33,6
	FD	88,4 ± 16,4*	89,0 ± 50,5*
Folículo Antral			
	FC	63,4 ± 7,5	50,6 ± 22,4*
	FD	20,0 ± 7,9*	25,2 ± 12,5*$^{\#}$
Corpo Lúteo			
	FC	8,8 ± 1,8	7,8 ± 1,6
	FD	8,0 ± 1,6	8,2 ± 2,8

Os valores são expressos como média ± desvio padrão. n=5 ratas/grupo

**p<0,05 - comparado ao grupo FC/DP; $^{\#}$p<0,05 - comparado com o grupo FC/DHL (Distribuição de Poisson seguido de teste de Wald – folículos primordial, primário, antral e corpo lúteo; Teste binomial negativo seguido de teste de Wald – folículo em crescimento).*

Avaliação imunoistoquímica do quadro de estresse oxidativo nas células ovarianas

A intensidade de marcação para SOD-1 foi menor nas células do corpo lúteo nos grupos FC/DHL, FD/DP e FD/DHL em comparação ao grupo FC/DP. O grupo FD/DHL também apresentou menor intensidade de marcação para SOD-1 em relação aos demais grupos (FC/DP, FC/DDHL e FD/DP) (Figura 5). Nas células estromais, a intensidade de marcação para SOD-1 também foi menor nos grupos FC/DHL, FD/DP e FD/DHL em comparação ao grupo FC/DP e menor ainda no grupo FD/DHL em relação ao grupo FC/DHL (Figura 6). Nas células da granulosa, houve aumento da intensidade de marcação para SOD-1 no grupo FD/DP em comparação aos grupos FC/DP e FD/DHL (Figura 7).

Não houve alterações na intensidade de marcação para MDA nas células do corpo lúteo entre os grupos (Figura 8). Houve aumento da intensidade de marcação para MDA nas células estromais dos grupos FC/DHL, FD/DP e FD/DHL comparados ao grupo FC/DP (Figura 9). A intensidade de marcação para MDA no grupo FD/DHL foi ainda maior em comparação ao grupo FC/DHL (Figura 9). Foi verificado aumento da intensidade de marcação para MDA nas células da granulosa nos grupos FC/DHL e FD/DHL em relação à das ratas FC/DP (Figura 10).

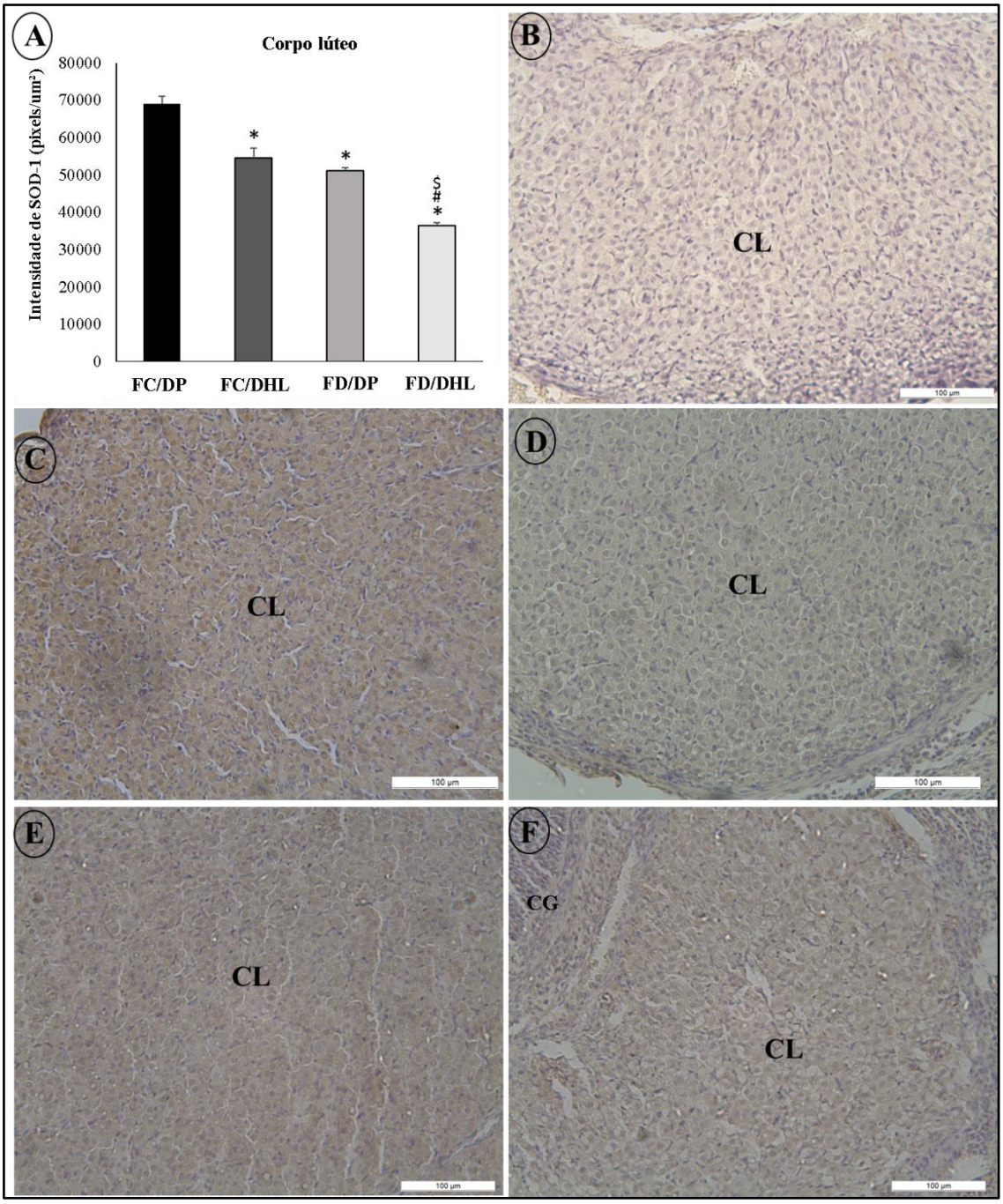


Figura 5. Análise da intensidade de marcação de superóxido dismutase (SOD-1) no corpo lúteo. (B) Controle negativo, (C) Grupo FC/DP, (D) Grupo FC/DHL, (E) Grupo FD/DP, (F) Grupo FD/DHL. Legenda: CL - corpo lúteo; CG - células da granulosa. *Aumento: 20x.*

Os valores são expressos como média ± desvio padrão, n=5 ratas/grupo.

**p<0,05 - comparado ao grupo FC/DP; #p<0,05 - comparado com o grupo FC/DHL; $p<0,05 - comparado com o grupo FD/DP (teste de Distribuição Gama e ANOVA seguido de teste de comparações múltiplas de Wald).*

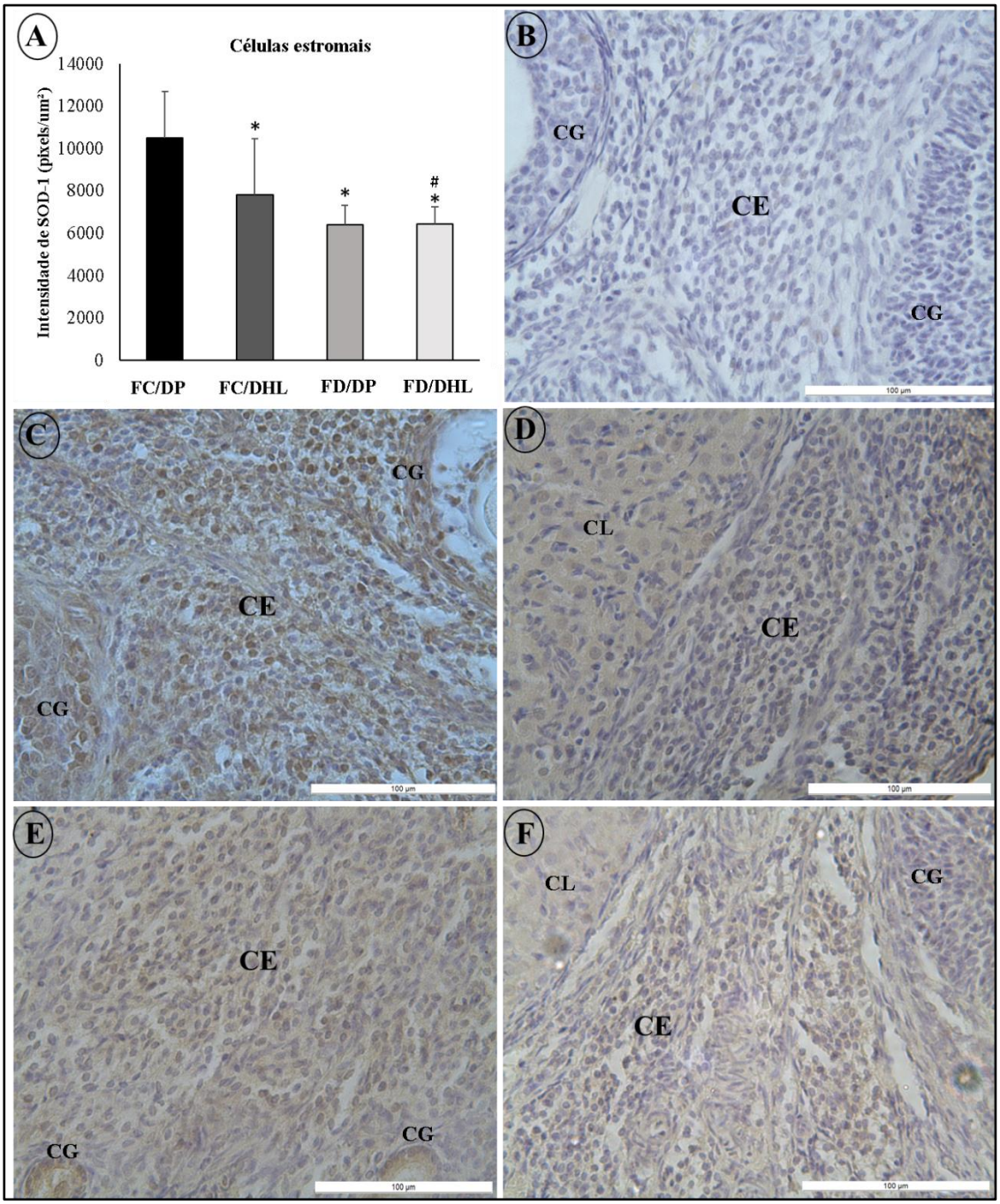


Figura 6. Análise da intensidade de marcação de SOD-1 nas células estromais. (B) Controle negativo, (C) Grupo FC/DP, (D) Grupo FC/DHL, (E) Grupo FD/DP, (F) Grupo FD/DHL.

Legenda: CE - células estromais, CL - corpo lúteo, CG - células da granulosa. *Aumento: 40x.*

Os valores são expressos como média ± desvio padrão, n=5 ratas/grupo.

**p<0,05 - comparado ao grupo FC/DP; #p<0,05 - comparado com o grupo FC/DHL (teste de Distribuição Gama e ANOVA seguido de teste de comparações múltiplas de Wald).*

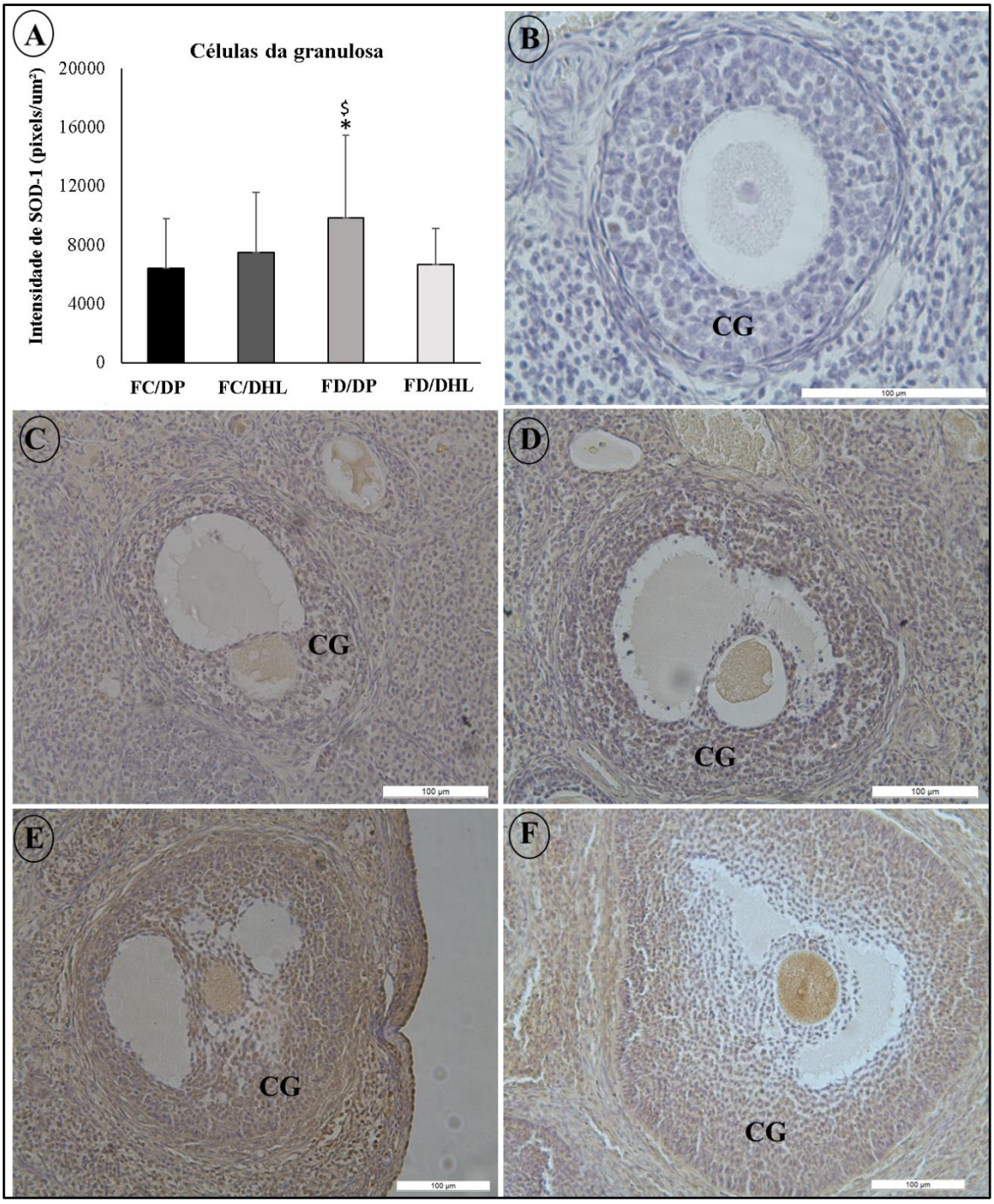


Figura 7. Análise da intensidade de marcação de SOD-1 nas células da granulosa. (B) Controle negativo (ampliação 40x), (C) Grupo FC/DP, (D) Grupo FC/DHL, (E) Grupo FD/DP, (F) Grupo FD/DHL. Legenda: CG - células da granulosa. *Aumento: 20x.*

Os valores são expressos como média ± desvio padrão, n=5 ratas/grupo.

**p<0,05 - comparado ao grupo FC/DP; $^{\#}$p<0,05 - comparado com o grupo FC/DHL; $^{\$}$p<0,05 - comparado com o grupo FD/DP (teste de Distribuição Gama e ANOVA seguido de teste de comparações múltiplas de Wald).*

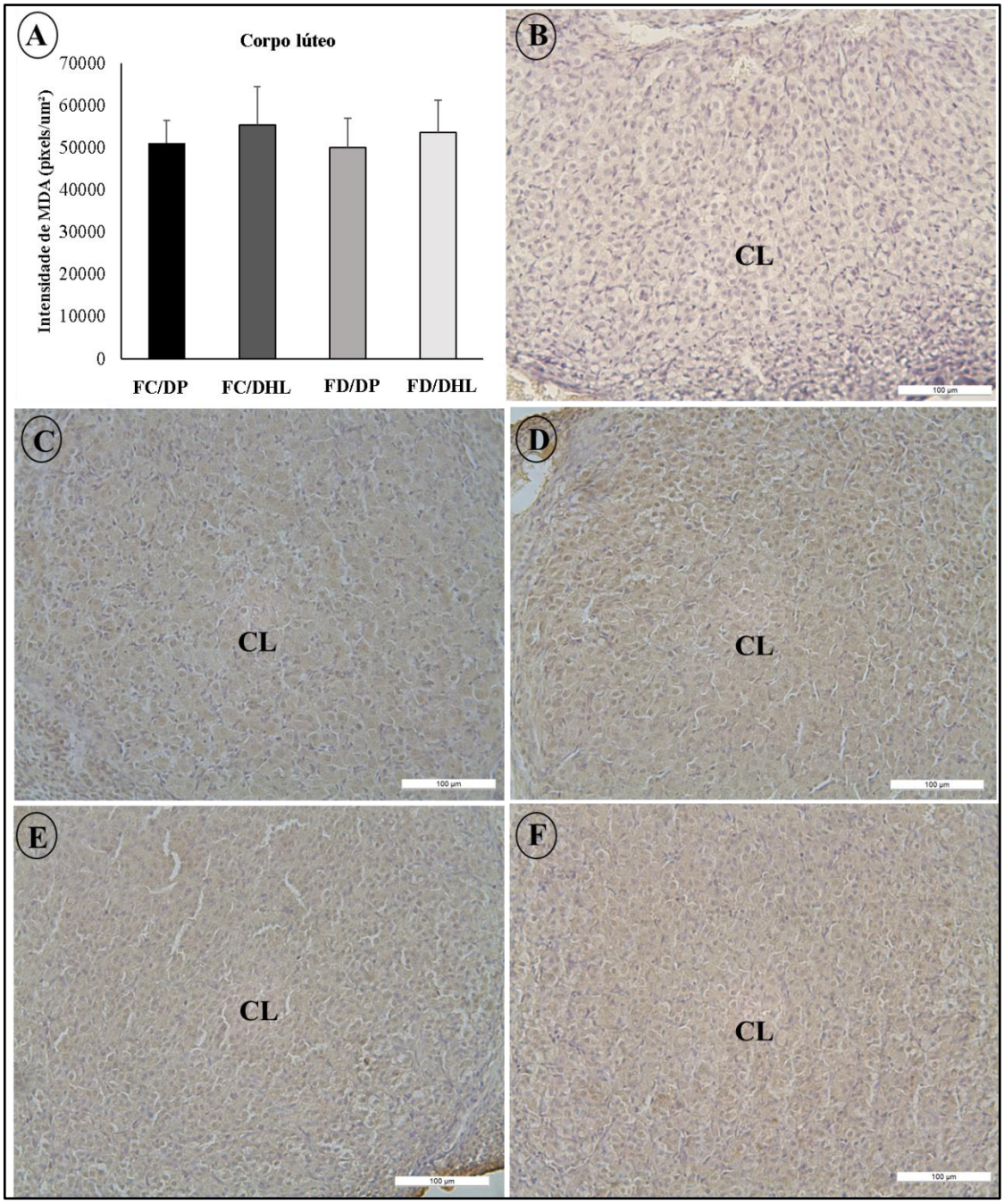


Figura 8. Análise da intensidade de marcação de malondialdeído (MDA) no corpo lúteo. (B) Controle negativo, (C) Grupo FC/DP, (D) Grupo FC/DHL, (E) Grupo FD/DP, (F) Grupo FD/DHL.
Legenda: CL - corpo lúteo. *Aumento: 20x.*
Os valores são expressos como média ± desvio padrão, n=5 ratas/grupo.
p> 0,05 – sem diferença estatisticamente significativa.

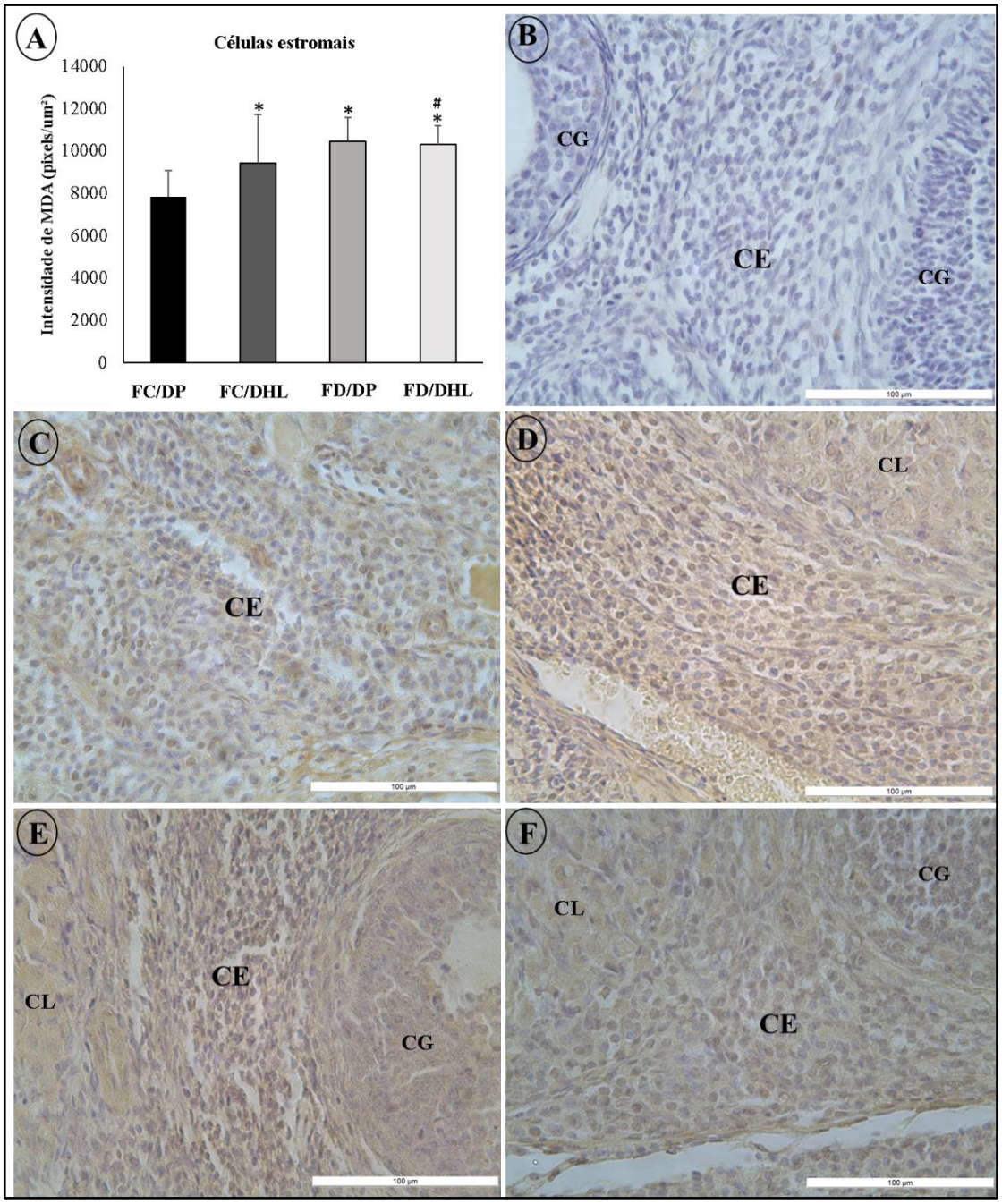


Figura 9. Análise da intensidade de marcação de malondialdeído (MDA) nas células estromais. (B) Controle negativo, (C) Grupo FC/DP, (D) Grupo FC/DHL, (E) Grupo FD/DP, (F) Grupo FD/DHL. Legenda: CE - células estromais; CL - corpo lúteo; CG - células da granulosa. *Aumento: 40x.*

Os valores são expressos como média ± desvio padrão, n=5 ratas/grupo.

**p<0,05 - comparado ao grupo FC/DP; #p<0,05 - comparado com o grupo FC/DHL (teste de Distribuição Gama e ANOVA seguido de teste de comparações múltiplas de Wald).*

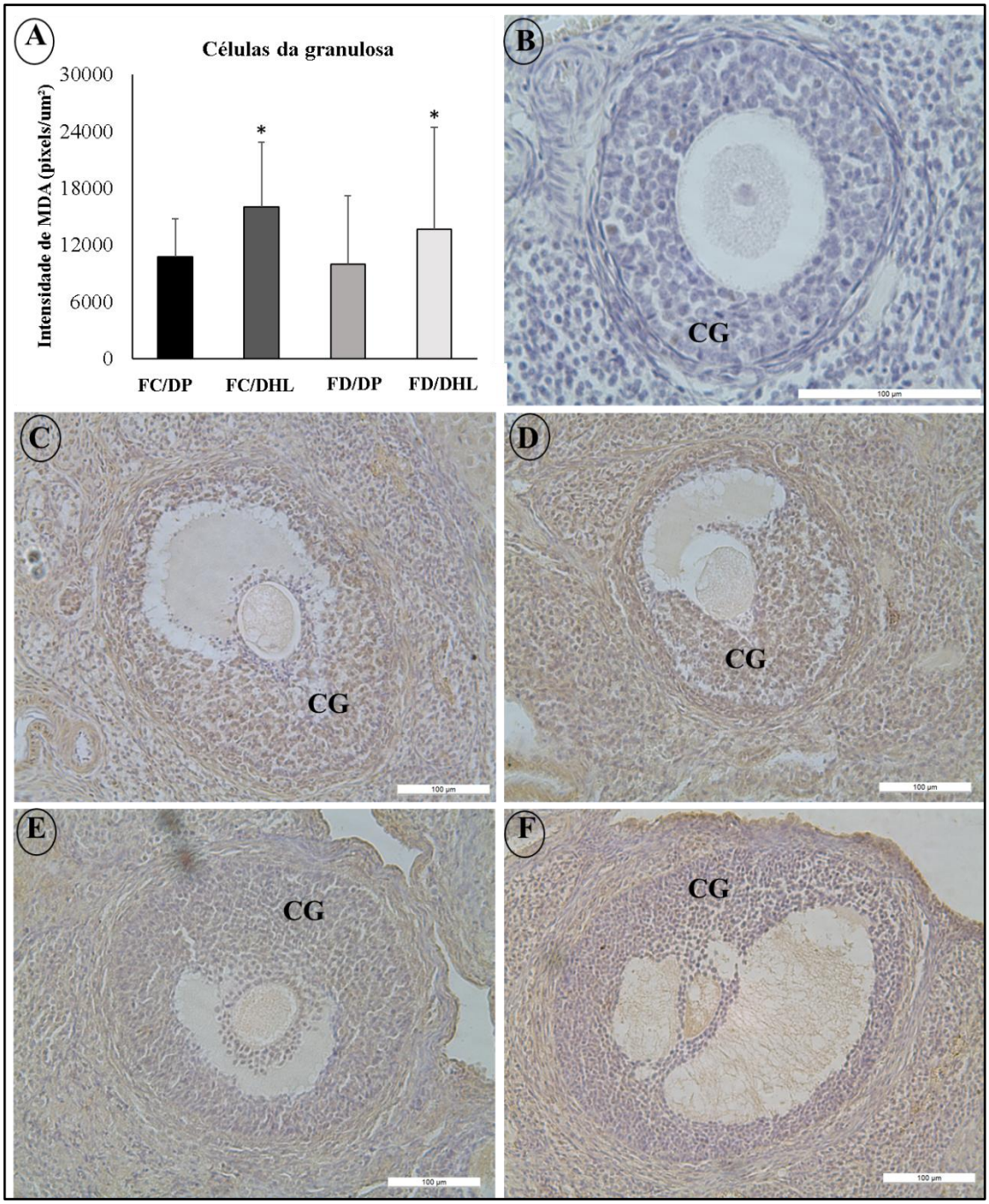


Figura 10. Análise da intensidade de marcação de malondialdeído (MDA) nas células da granulosa. (B) Controle negativo (aumento de 40x), (C) Grupo FC/DP, (D) Grupo FC/DHL, (E) Grupo FD/DP, (F) Grupo FD/DHL.

Legenda: CG - células da granulosa. *Aumento: 20x.*

Os valores são expressos como média ± desvio padrão, n=5 ratas/grupo.

**p<0,05 - comparado ao grupo FC/D (teste de Distribuição Gama e ANOVA seguido de teste de comparações múltiplas de Wald).*

Correlações entre AUC, concentração sérica de progesterona, marcadores de apoptose, função mitocondrial e de estresse oxidativo

As análises de correlação dos dados são apresentadas na figura 11. Em relação a glicemia, houve correlações negativas entre AUC/120min x Progesterona (Figura 11A), AUC/120min x MFF (Figura 11B) e AUC/120min x MFN2 (Figura 11C). As correlações também foram positivas entre MFF x Progesterona (Figura 11D), MFN2 x Progesterona (Figura 11E) e Caspase-3 clivada x Progesterona (Figura 11F). Entre as células ovarianas, no corpo lúteo houve correlação positiva entre MFF x Intensidade de SOD no corpo lúteo (Figura 11G) e correlação negativa entre AUC/120min x Intensidade de SOD no corpo lúteo (Figura 11H). Nas células do estroma ovariano, houve correlação negativa entre Intensidade de SOD nas células estromais x AUC/120min (Figura 11I) e correlações positivas entre MFF x Intensidade de SOD nas células estromais (Figura 11J) e MFN2 x Intensidade de SOD nas células estromais (Figura 11K).

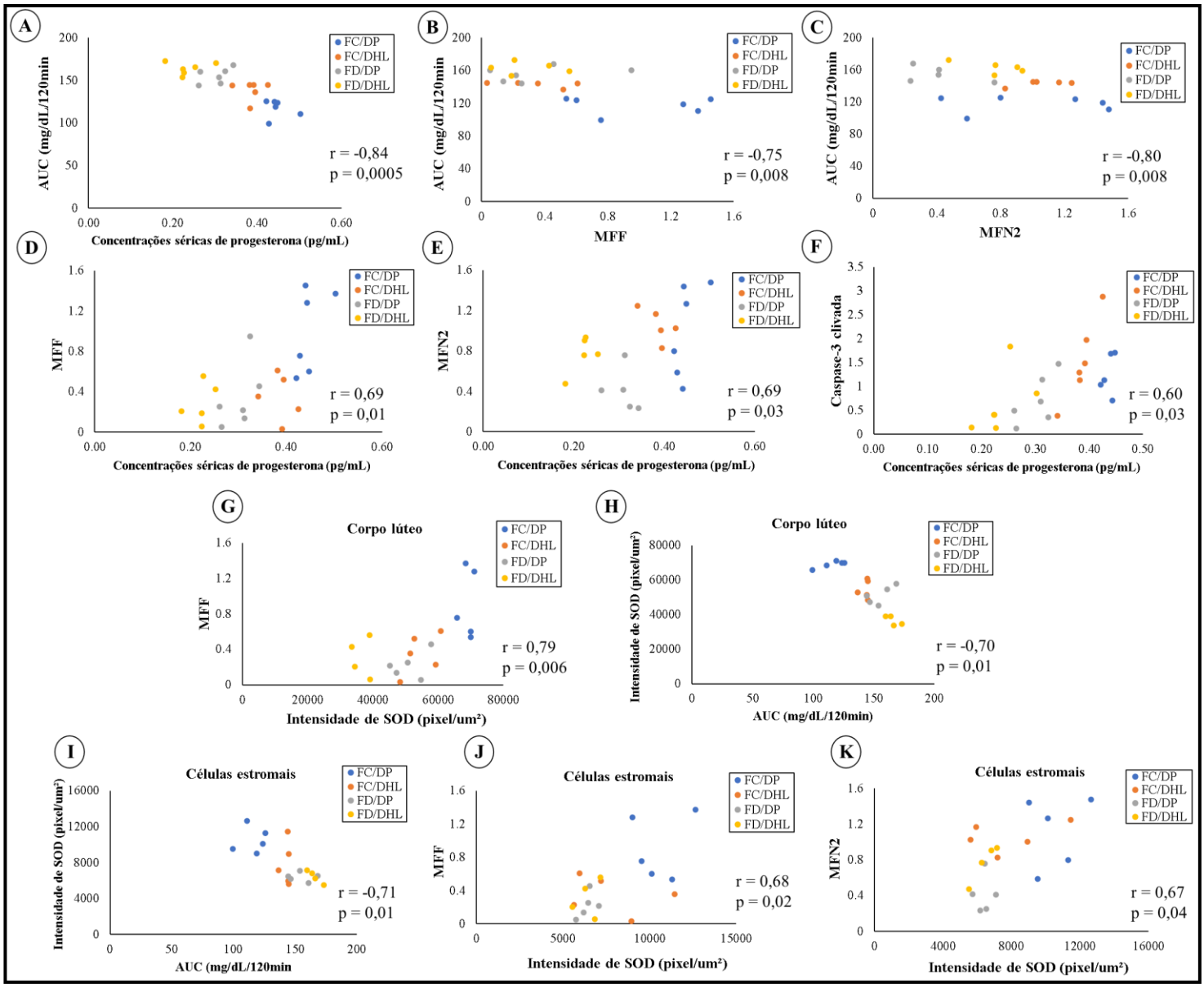


Figura 11. Análise de correlação entre os dados. (A) Correlação entre AUC/120min x Concentrações séricas de progesterona; (B) Correlação entre AUC/120min x MFF; (C) Correlação entre AUC/120min x MFN2; (D) MFF x Concentrações séricas de progesterona; (E) MFN2 x Concentrações séricas de progesterona; (F) Correlação entre Caspase-3 clivada x Concentrações séricas de progesterona; (G) MFF x Intensidade de SOD no corpo lúteo; (H) AUC/120min x Intensidade de SOD no corpo lúteo; (I) Intensidade de SOD nas células estromais x AUC/120min; (J) MFF x Intensidade de SOD nas células estromais; (K) MFN2 x Intensidade de SOD nas células estromais.

$p<0,05$ - Teste de correlação de Pearson.

4. Discussão

Neste estudo, nós avaliamos o efeito intergeracional do diabete sobre os ovários de filhas de ratas diabéticas. Além disso, nós também avaliamos como o ambiente pós-natal (consumo de DHL) pode influenciar ou exacerbar negativamente as repercussões ovarianas. Este é o primeiro estudo a investigar como esses dois fatores (diabete e DHL) interferem na secreção hormonal, função mitocondrial, reserva ovariana e estresse oxidativo nos ovários.

O diabete e/ou dietas ricas em gorduras podem causar prejuízos na função reprodutiva. Muitos estudos experimentais vêm sendo extensamente conduzidos visando ampliar o entendimento dos mecanismos fisiopatológicos envolvidos já que as investigações clínicas relacionadas à função de órgãos reprodutivos precisam ser mais aprofundadas (Hohos *et al.*, 2018). A relação entre diabete e ovário, bem como o consumo de DHL e ovário, já está estabelecida, mas as consequências entre gerações ainda são pouco pesquisadas (Chang *et al.*, 2005; Nayki *et al.*, 2016; Tsoulis *et al*., 2016; Patel e Shah 2018; Mehrabianfar *et al*., 2020). Nós demonstramos que a hiperglicemia materna comprometeu o metabolismo glicêmico das filhas. Ao analisarmos o teste oral de tolerância à glicose (TOTG), observamos que houve maior quantidade de glicose circulante nos grupos FC/DHL, FD/DP e FD/DHL. Nossos resultados mostram que tanto o consumo de DHL quanto o diabete materno foram responsáveis por aumentar a glicemia destas filhas. As filhas de ratas diabéticas desenvolveram diabetes e, quando associado ao consumo de DHL, a proporção de ratas diabéticas foi ainda maior.

Previamente em nosso laboratório, foi verificado que filhas de ratas diabéticas, tendo ou não consumido DHL desde o desmame até a vida adulta, apresentaram diminuição da concentração de insulina sérica, além de aumento de células marcadas para insulina no pâncreas, sugerindo que a secreção de insulina e não a sua sinalização estaria comprometida nessas ratas (Paula *et al*., 2022). Então, para analisarmos a influência da associação do diabete e da dieta hiperlipídica em marcadores de resistência à insulina, a expressão de proteínas de transcrição relacionadas às vias de sinalização insulínica PI3K/Akt e MAPK (ERK1/2) foram avaliadas nos homogenatos de ovários.

O grupo controle que consumiu a dieta hiperlipídica (DHL) apresentou aumento da expressão de PI3K. Já foi demonstrado que as vias PI3K/Akt e MAPK (ERK1/2) promovem o transporte de glicose, proliferação celular e diferenciação celular, apoptose folicular, interagem com as gonadotrofinas para modular a secreção de hormônios ovarianos (Dupont & Scaramuzzi, 2016; Das & Arur, 2017; Rostamtabar *et al*., 2021), além de estarem relacionadas

à função mitocondrial no ovário (Zhang *et al*., 2019). A maior expressão de PI3K no grupo controle que consumiu DHL pode indicar aumento da ativação dessa via, enquanto que o contrário pode ser visto no grupo de filhas de mães diabéticas que consumiram DHL. A via da PI3K está relacionada com a esteroidogênese (Chen et al., 2007) e os grupos de filhas de mães diabéticas apresentaram diminuição na secreção de progesterona, além de alterações em proteínas responsáveis pela fissão e fusão mitocondrial.

A dinâmica mitocondrial tem papel central para sobrevivência dos folículos ovarianos bem como na produção de esteroides (Stocco & Clark, 1996; Morohaku *et al*.,2013) e a hiperglicemia, por si só, pode causar alterações no desenvolvimento ovariano bem como na função mitocondrial (Chang *et al*., 2005; Shao *et al.,* 2019; Yuan *et al.,* 2020). De acordo com nossos achados, os grupos de filhas de ratas diabéticas apresentaram alterações em proteínas responsáveis pela fissão e fusão mitocondrial. Há escassez de estudos sobre a relação entre as proteínas responsáveis pela função mitocondrial e ovários. Além disso, não foram encontradas investigações da expressão de MFN2 ou MFF nos ovários da prole adulta de ratas diabéticas. A literatura mostra que tanto o diabete como DHL podem prejudicar a expressão de proteínas da dinâmica motocondrial em outros tecidos. Em um modelo utilizando combinação de DHL e baixas doses de *streptozotocin* para indução de diabete em ratos machos, os animais apresentaram disfunção mitocondrial no tecido cardíaco com baixa expressões de MFN1, outra importante mitofusina que, junto com MFN2, controla a fusão mitocondrial (Shao *et al*.,2019). A diminuição da expressão de MFN2 também já foi vista em biópsia do músculo esquelético de pacientes com DM2 (Zorzano et al., 2009). Em nosso laboratório, foi verificado que filhas de ratas diabéticas aos 150 dias também apresentaram diminuição da expressão de MFN2 e MFF no músculo esquelético (Kloppel *et al.,* 2022 – *dados submetidos à publicação*).

MFN2 desempenha um papel importante na manutenção da integridade do DNA mitocondrial e sua anormalidade pode afetar a função das células da granulosa e o desenvolvimento dos oócitos (Chen *et al*., 2015). Assim, sugerimos que a hiperglicemia foi responsável pela baixa expressão de MFN2 e MFF e, consequentemente, levou a alterações nas células foliculares que comprometeu a síntese de progesterona ovariana, confirmada pela baixa concentração de progesterona sérica nas filhas de ratas diabéticas, independente do consumo de DHL. Nossas análises de correlação confirmaram que o aumento da concentração de glicose no TOTG teve impacto na diminuição da expressão de MFN2, MFF, bem como, a concentração de progesterona.

A função reprodutiva da fêmea depende do controle da esteroidogênese ovariana que é necessária para o desenvolvimento folicular, ovulação e manutenção da gestação (Drummond, 2006). A primeira etapa da esteroidogênese ocorre na mitocôndria a partir do colesterol, que é transferido da parte interna para a externa da membrana mitocondrial, com sua metabolização no citocromo P450 em pregnolona, a qual é transportada para fora das mitocôndrias e convertida em progesterona pelas células da granulosa (An *et al*.,2012; Hu et al., 2010). Além disso, estudos *in vitro* utilizando linhagem de células com bloqueio de MFN2 (Duarte *et al*., 2012) e também, *in vivo*, utilizando um modelo onde camundongos tratados com cisplatina apresentaram baixa expressão de MFN2 nos ovários (Chen *et al*., 2015; Hashem *et al*., 2019) e estavam associados à diminuição da produção de hormônios esteroides (Hashem *et al*., 2019). Estas informações reforçam ainda mais o fato de que a baixa expressão de MFN2 influenciou na menor concentração de progesterona nas filhas de diabéticas. Em nosso modelo, as correlações mostram que a diminuição de progesterona parece estar associada a disfunção mitocondrial, e isso pode refletir em prejuízo para a reprodução dessas ratas, por ser um hormônio essencial para a manutenção da prenhez (Morel *et al*.,2016). De fato, em outro estudo utilizando o mesmo modelo, foi verificado aumento de perdas de embriões antes da implantação em filhas de mães diabéticas, que consumiram ou não DHL, e diminuição do número de implantações e de fetos vivos nas filhas de mães diabéticas que consumiram DHL (Sinzato *et al*., 2022).

Funções reprodutivas anormais podem vir acompanhadas de ciclo estral anormal e são frequentemente encontradas em modelos animais de DM2 e obesidade (Ziarniak *et al*., 2018). Embora nossos resultados não mostrem diferenças nas concentrações de 17β-estradiol, houve uma maior permanência na fase estro do ciclo estral no grupo FD/DP. De acordo com outros autores, a maior permanência na fase estro frequentemente é acompanhada por aumento nas concentrações de estradiol e maior recrutamento de folículos a se desenvolverem (Connor *et al*., 2012; Zhou *et al*., 2019). Uma possível justificativa para nosso achado é o fato de que a dosagem de 17β-estradiol ocorreu com os animais na fase diestro do ciclo estral, caracterizada por diminuição das concentrações desse hormônio justamente por se tratar de uma fase progestogênica (Marcondes *et al*., 2002). Esse ponto pode ser considerado como uma limitação do nosso estudo.

A sobrevivência dos folículos ovarianos depende de vários fatores de sinalização que regulam a proliferação e a morte celular (apoptose) (Annie *et al*., 2020). A apoptose é um processo complexo que envolve vias intrínsecas e extrínsecas de ativação por proteínas pró e

anti-apoptóticas que são coordenadas pela função mitocondrial normal (Cai *et al*.,2002). Já foi estabelecida a relação entre hiperglicemia e a indução de apoptose e estudos relataram a presença de apoptose em excesso nas células ovarianas de ratas diabéticas (Asadikaram *et al*., 2013; Nayki *et al*., 2017; Fraunhoffer *et al*., 2018) e nos ovários de ratas que consumiram DHL pelo aumento da expressão de Bax e caspase-3 além de outros marcadores apoptóticos (Jungheim *et al*., 2010; Cheong *et al*.,2014; Tsoulis *et al*.,2016; Wu *et al*., 2017). No entanto, nossos resultados são controversos. Pudemos verificar que a expressão de Bcl-2 e Caspase-3 nos ovários foi menor nas filhas de ratas diabéticas e o grupo FD/DHL apresentou expressão de Bax ainda menor. O fato das filhas de ratas diabéticas exposta à DHL terem apresentado a diminuição de marcadores pró e anti-apoptóticos pode estar relacionado a um possível comprometimento da proliferação celular, visto que a expressão de PCNA, que atua na síntese, reparo e replicação de DNA (Boehm, *et al*., 2016), foi menor nos grupos de filhas de diabéticas.

Em outros modelos já foi evidenciado que a disfunção mitocondrial leva à diminuição na expressão de PCNA e prejuízos na proliferação celular (Hong *et al*., 2021). Além disso, a baixa expressão de MFF prejudica a proliferação das células pelo fato de dificultar a fissão de seus núcleos e o processo de divisão por mitose (May-Panloup *et al*., 2016). Nossas observações são inéditas na literatura e nenhum dado está disponível acerca do mecanismo de ação de PCNA na função mitocondrial nos ovários. Porém, recentemente, PCNA foi reconhecido como um importante marcador de foliculogênese, por estar presente em maior concentração nas células da granulosa de folículos secundários e antrais (Vatanparast *et al*., 2021), o que justifica a relação entre baixa expressão proteica de PCNA e falhas na proliferação das células foliculares e redução na síntese de progesterona em nosso estudo.

No ovário, o desenvolvimento folicular depende de um balanço entre apoptose e proliferação celular (Liu *et al*., 2019). Assim, níveis baixos da expressão de caspase-3 e PCNA em filhas de ratas diabéticas, independente do consumo de DHL, podem indicar que até mesmo a apoptose que deveria ocorrer de forma fisiológica parece não estar ocorrendo, o que poderia ser causa ou consequência da baixa proliferação celular nos ovários. Em um estudo utilizando um modelo de diabete grave induzido por aloxana, foi estabelecida uma relação direta entre hiperglicemia e baixa expressão de PCNA nas células do miométrio de ratas, sendo que a disfunção hormonal causada pela hiperglicemia foi a responsável pela baixa proliferação celular (Favaro *et al*., 2010). Foi encontrada correlação positiva entre a baixa expressão de caspase-3 clivada nos ovários e a baixa concentração de progesterona e, já foi demonstrado que, a produção de progesterona parece ser dependente do aumento da ativação de caspase-3 nas

células da granulosa de folículos pré-ovulatórios (An *et al*., 2012). A relação dos hormônios ovarianos com a proliferação celular já foi relatada em outros estudos e estrogênios e progesterona parecem regular a proliferação celular através da expressão de seus receptores (Zakaria *et al*., 2000; Jaffer *et al*., 2009).

Interessantemente, o mecanismo envolvido nas respostas ao consumo de DHL por ratas controle parece seguir outro padrão. No grupo FC/DHL, houve maior expressão de PCNA, indicando aumento da proliferação celular nesse grupo, que pode ser consequência do aumento da ativação da via PI3K. O aumento da proliferação celular pode resultar em maior ativação folicular, que recruta mais folículos a se desenvolverem, mas têm impacto negativo na função reprodutiva visto que mais folículos são perdidos (Jungheim *et al*., 2010; Paula *et al*., 2022). Maior proliferação e perda folicular foi verificada em ratas alimentadas com DHL, indicando diminuição da reserva ovariana (Wang *et al*., 2014; Tsoulis *et al*., 2016). Entretanto isso não foi verificado em nosso estudo. A análise da contagem de folículos ovarianos mostrou que as ratas do grupo FC/DHL não apresentaram alterações na reserva ovariana, nem na contagem de folículos em crescimento, embora fosse verificada uma diminuição na contagem de folículos antrais. A maior ativação da via PI3K, bem como aumento da proliferação celular nesse grupo, pode ter ocorrido como um mecanismo compensatório a fim de impedir a perda folicular, o que não ocorreu nos grupos diabéticos.

O desenvolvimento folicular foi prejudicado nas filhas de ratas diabéticas independentemente do consumo de DHL. A diminuição na contagem de folículos primordiais mostra que houve perda da reserva ovariana. Isso refletiu na menor contagem de folículos em crescimento e folículos antrais. Esse resultado corrobora a literatura, pois o diabete leva à diminuição da reserva ovariana tanto em modelos experimentais quanto em estudos clínicos (Artunc-Ulkumen *et al*., 2014; Erbas *et al*., 2014; Nayki *et al*., 2016; Thong *et al*., 2020) e mostra que o efeito prejudicial é transmitido entre as gerações. A baixa expressão de MFF e MFN2 pode levar à diminuição da reserva por dificultar a fissão e fusão dos núcleos celulares e prejudicar a proliferação das células da granulosa (Chen *et al*.,2003). Camundongos *knockout* para MFN2 tiveram uma diminuição drástica de folículos primordiais e em crescimento aos 12 meses de vida, levando à insuficiência ovariana (Zhang *et al*., 2019). Assim, a perda da reserva ovariana em nosso modelo pode ser consequência direta da disfunção mitocondrial, bem como do prejuízo no processo de apoptose e proliferação celular, causados pela hiperglicemia. Além disso, esse achado também se relaciona ao aumento de estresse oxidativo nas células ovarianas.

Em nosso estudo, a análise imunoistoquímica do ovário mostrou que as ratas do grupo controle que consumiram DHL (FC/DHL) e ambos os grupos de filhas de mães diabéticas (FD/DP e FD/DHL) apresentaram diminuição da intensidade de marcação da enzima antioxidante SOD nas células estromais e corpo lúteo e aumento da marcação de peroxidação lipídica por MDA nas células estromais. Tanto a exposição intrauterina ao diabete materno quanto o consumo de DHL levaram ao estresse oxidativo nas células ovarianas. No ovário, a presença de espécies reativas de oxigênio (EROS) é necessária para a ruptura dos folículos e ovulação. Entretanto é preciso que haja um balanço entre EROS e agentes antioxidantes, pois o estresse oxidativo acelera a perda de folículos (Zhao *et al*., 2019) e, consequentemente, prejudica a secreção de hormônios ovarianos (Zaid *et al.*, 2021). A hiperglicemia está associada à alta produção de EROS pelas mitocôndrias (Yu *et al*., 2006) e o estresse oxidativo é uma das características marcantes em modelos experimentais de diabete (Gallego *et al*., 2018) e/ou DHL (Olawale *et al*., 2020; Moraes-Souza *et al*., 2021).

Foi verificada uma correlação negativa entre a área sob a curva (AUC) formada pelo TOTG e a intensidade de marcação de SOD nas células ovarianas. SOD é uma enzima antioxidante que controla EROS no ovário e influencia a produção de progesterona nas células da granulosa e células lúteas, principalmente sua isoforma SOD-2, que está localizada na matriz mitocondrial (Zaid *et al.*,2021). A SOD-1, presente no citosol, é uma metaloenzima que, em altas concentrações, está relacionada à regulação do desenvolvimento folicular, ovulação e função lútea (Fujii *et al*., 2005; Combelles *et al*., 2010). A integridade do corpo lúteo é essencial para manutenção da gestação e o estresse oxidativo nas células lúteas leva ao declínio da capacidade reprodutiva. Para estudo do envelhecimento ovariano, modelos *in vitro* e *in vivo* já demonstraram a relação entre baixa concentração de SOD e aumento de EROS em células lúteas (Quian *et al*., 2016).

Nossos achados sobre a baixa intensidade de marcação de SOD-1 nas células estromais é um dado interessante pois há pouca informação do papel do estroma na foliculogênese e no ciclo ovariano. Kinnear *et al*. (2020) mostraram que as células estromais possuem subpopulações que desempenham papeis distintos para a manutenção da dinâmica ovariana, principalmente porque a partir delas que as células foliculares e lúteas são formadas. Além disso, uma subpopulação específica, chamada de células intersticiais da teca (que posteriormente migram para se tornarem as células da teca propriamente ditas) foram reconhecidas pela capacidade de síntese de hormônios, bem como, pela presença de receptores para estes (Young & McNeilly, 2010; Rotgers *et al*., 2018). No entanto, ainda é preciso

esclarecer qual o papel do estroma na produção hormonal e os mecanismos pelos quais essas células influenciam a foliculogênese (Kinnear *et al*., 2020).

Assim, a partir dos nossos resultados e de dados já descritos na literatura, podemos sugerir que a hiperglicemia materna levou à disfunção mitocondrial na prole, prejudicou a secreção hormonal, comprometeu o processo de apoptose e proliferação celular e causou estresse oxidativo nas células ovarianas. Essas alterações foram determinantes para a perda da reserva ovariana nas filhas de ratas diabéticas, o que pode indicar prejuízo no processo reprodutivo e impacto negativo na fertilidade, como verificado anteriormente por Paula *et al*. (2022).

Embora nós esperássemos que a associação da exposição ao diabete materno e o consumo de DHL fosse produzir alterações mais exacerbadas nos ovários, o diabete materno foi determinante para as alterações metabólicas. Isso pode ser explicado pela reposta adaptativa do organismo frente às condições nutricionais e energéticas intrauterinas, que confere certa vantagem sobre condições pós-natais semelhantes (Nettle *et al*., 2013). A função mitocondrial têm sido forte candidata nos estudos intergeracionais, apesar de ainda não ser claro o mecanismo envolvido na herança do comprometimento mitocondrial, principalmente nos ovários.

Apesar de nossos dados trazerem informações valiosas a respeito da associação entre diabetes e DHL sobre a dinâmica ovariana, algumas limitações podem ser apontadas. A avaliação de parâmetros biométricos pode fornecer dados importantes para a análise de um possível crescimento *catch-up* na prole de mães diabéticas, mas estes dados não foram incluídos neste estudo. A dinâmica mitocondrial envolve outras proteínas de grande importância para sua manutenção, como Drp1, MFN1 e OPA1. Entretanto, não foi possível a análise da expressão dessas proteínas neste trabalho, o que poderia fornecer mais informações sobre as vias alteradas. Além disso, uma avaliação das demais enzimas envolvidas no quadro de estresse oxidativo nos ovários poderia fornecer informações importantes para o entendimento das vias alteradas em nosso modelo. Apesar das limitações, nosso estudo é o primeiro a fornecer informações sobre a função mitocondrial em ratas filhas de mães diabéticas e nossos dados trazem uma nova perspectiva de como a hiperglicemia interfere nas futuras gerações.

Este estudo é importante pois problemas reprodutivos são uma queixa cada vez mais frequente entre as mulheres, paralelo ao crescimento do número de mulheres diabéticas e obesas. Há indicação de que medidas preventivas com o objetivo de conscientizar os efeitos do diabete e de condições nutricionais para o desenvolvimento saudável do ovário e da função

reprodutiva são necessárias e fundamentais para melhoria da qualidade de vida das mulheres em idade reprodutiva.

5. Conclusão

Em conclusão, a disfunção mitocondrial causada pela hiperglicemia foi determinante para as alterações encontradas em nosso modelo. As repercussões negativas indicam que as filhas de ratas diabéticas, independentemente do consumo de DHL, possuem disfunção mitocondrial com prejuízo na proliferação, secreção hormonal e presença de estresse oxidativo e diminuição da reserva ovariana. Assim, nossos achados indicam um possível modelo para estudo da dinâmica ovariana e fertilidade.

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CAPÍTULO III

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RESEARCH ARTICLE

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Nonpregnant and pregnant adult female rats affected by maternal diabetes environment

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ABSTRACT

Maternal diabetes-mediated fetal programming is widely discussed, however, it is important to define the extent to which intrauterine hyperglycemia interferes with the health of female pups, along with determining whether these changes can be perpetuated across generations. This study aimed to evaluate the effects of maternal diabetes on fetal programming and the repercussions on the metabolism of pregnant and nonpregnant female pups. Diabetes status was induced (diabetic group—D) using streptozotocin (a beta cell cytotoxic drug) on the fifth postnatal day of female rats, while controls received a citrate buffer (Control—C). In adulthood, the rats were mated to obtain their female pups. At 90 days of age, half of the female pups were mated (preg) and the other half continued virgin (Npreg). Furthermore, they were distributed into four groups: OC/Npreg and OC/preg—female pups from control mothers; OD/Npreg and OD/preg—female pups from diabetic mothers. At 115 days of life and/or 17 days of pregnancy, the oral glucose tolerance test (OGTT) was performed with blood collection for insulin measurement. At 120 days of life and/or 21 days of pregnancy, the rats were anesthetized and euthanized to determine their blood oxidative stress status. The OD/Npreg group showed glucose intolerance during OGTT ($p < 0.0001$), while the OD/preg group showed increased insulin secretion during OGTT ($p < 0.0001$) and insulin resistance (IR; $p = 0.0027$). An increase in homeostatic model assessment β was shown in the pregnant groups, regardless of maternal diabetes ($p < 0.0001$). The OD/preg group presented increased thiobarbituric acid reactive substances ($p < 0.0001$) and –SH levels ($p = 0.0005$) and decreased superoxide dismutase activity ($p = 0.0063$). Additionally, small fetuses for gestational age ($p < 0.0001$) were found in these rats. In conclusion, exposure to maternal hyperglycemia compromises the glycemic metabolism of female pups before and during pregnancy and causes oxidative stress, IR, and impaired fetal growth during pregnancy.

Abbreviations: OGTT: oral glucose tolerance test; TBARS: thiobarbituric acid reactive substances; SH: reduced thiol groups; DOHaD: developmental origin of health and disease; DM: diabetes mellitus; DM2: diabetes mellitus type 2; GDM: gestational diabetes mellitus; ROS: reactive oxygen species; AUC: area under the curve; IR: insulin resistance; HOMA: homeostatic model assessment; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; CAT: catalase; H2O2: hydrogen peroxide; GSH: glutathione; MDA: malondialdehyde; STZ: streptozotocin; PND: postnatal day; ELISA: enzyme-linked immunosorbent assay; PBS: phosphate-buffered saline; EDTA: ethylenediamine tetraacetic acid; Hb: hemoglobin; SD: standard deviation; ANOVA: analysis of variance

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Introduction

The pregnancy period promotes various hormonal, immunological, and metabolic changes to allow proper fetal development and to prepare the body for delivery (Kinsley and Lambert 2006; Motosko et al. 2017; Pavethynath et al. 2019). Changes begin early in the first trimester, peak in the full-term period, and return to pre-pregnancy levels a few weeks after delivery.

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Pregnancy alterations/adaptations are tolerated well by healthy women; however, the existence of a predisposition can aggravate or unmask a preexisting illness or pregnancy-related pathophysiology (Bhatia and Chhabra 2018).

The structural and functional growth of specific tissues or organs can be stimulated or inhibited by the maternal condition and, ultimately, result in disability and illness in adult life. This effect has been termed the 'fetal origins of adult disease' (Hales and Barker 1992) and later, the 'developmental origin of health and disease' (DOHaD) (Bateson et al. 2004; Calkins and Devaskar 2011; Navarro et al. 2017). Developmental plasticity occurs during sensitive periods (i.e., when a system is moldable) and acts by altering tissue morphology (e.g., anatomy and/or microanatomy) or cellular activity within the tissues from the conceptus. Therefore, developmental plasticity encompasses processes that generate alternative phenotypes from a single genotype through environmental stimuli that act during development (Gluckman and Hanson 2007). Despite DOHaD being considered a normal biological phenomenon and a part of developmental plasticity, it can be maladaptive and lead to a higher risk of disease in some circumstances.

Transgenerational effects on metabolism and incidence of metabolic diseases were addressed by cohort studies in which children born during the winter of the Dutch famine were assessed, characterized, and monitored until adulthood. These studies have clearly demonstrated a link between maternal malnutrition and the risk of metabolic disorders such as *Diabetes mellitus* (DM) in adulthood (Lumey et al. 1993; Roseboom et al. 2001). A study carried out on the Pima indigenous population showed that before the age of 20, subjects born from indigenous women with either Type 2 *Diabetes mellitus* (DM2) or gestational *Diabetes mellitus* (GDM) had a higher risk of developing DM2 than their cohorts born from prediabetic and nondiabetic mothers (Pettitt et al. 1993). Later studies and epidemiological observations showed that unhealthy nutrition, including both malnutrition and overnutrition, during intrauterine life and early postnatal life increases susceptibility to metabolic changes such as obesity, insulin resistance (IR), and DM2 (Greco et al. 2019; Tozour et al. 2020).

Prolonged exposure to hyperglycemia during pregnancy results in increased production of free radicals, particularly in reactive oxygen species (ROS) in all tissues susceptible to glucose autoxidation and protein glycosylation. Increased ROS levels, reduced glutathione (GSH) and superoxide dismutase (SOD), and decreased production and/or increased destruction of antioxidants such as catalase (CAT) (Rahman 2012; Luc et al. 2019) are related to oxidative stress status. Antioxidant enzyme levels critically influence the susceptibility of various tissues to oxidative stress and are associated with the development of complications in diabetic status (Rahman 2012), including in the intrauterine environment, which induces fetal damage and impairs growth (Damasceno et al. 2014; Netto et al. 2018). 'Intrauterine growth restriction' describes a fetal phenotype resulting from the fetus' failure to reach its growth potential, and 'small for gestational age' refers to low birth weight from a certain cutoff point for a specific gestational age (Aerts and Van Assche 2006; Briana and Malamitsi-Puchner 2009; Haugaard and Bauer 2001). Fetal damage from an inadequate intrauterine environment can also lead to long-term consequences in glucose tolerance in adult offspring, which may extend to subsequent generations (Bequer et al. 2018).

We have already demonstrated that exposure to a diabetic intrauterine milieu leads to glucose intolerance, hyperlipidemia, and pancreatic β-cell dysfunction in *female rat pups* at 120 days of life (Paula et al. 2022). However, considering that metabolic alterations during pregnancy are the starting point to induce damage in female pups, it is also necessary to investigate how the glycemic metabolism of the adult female pups will behave during pregnancy and whether these changes can impair fetal development. Our hypothesis is that female pups from hyperglycemic mothers will present exacerbated metabolic alterations during pregnancy due to acquired fetal programming. Therefore, the aim of this study was to evaluate whether the developmental plasticity induced by maternal hyperglycemia associated with the metabolic changes necessary for pregnancy maintenance can lead to worsening glycemic, insulin, and oxidative stress levels in female pups during pregnancy.

Results

Hyperglycemia during pregnancy alters the glycemic metabolism of female pups in adulthood

To determine how the glycemic metabolism in adult rat females was affected by fetal development within a hyperglycemic uterine milieu and whether there are differences during pregnancy, we compared adult female pups from diabetic and control mothers, either pregnant or nonpregnant, using the results obtained for OGTT and area under the curve (AUC).

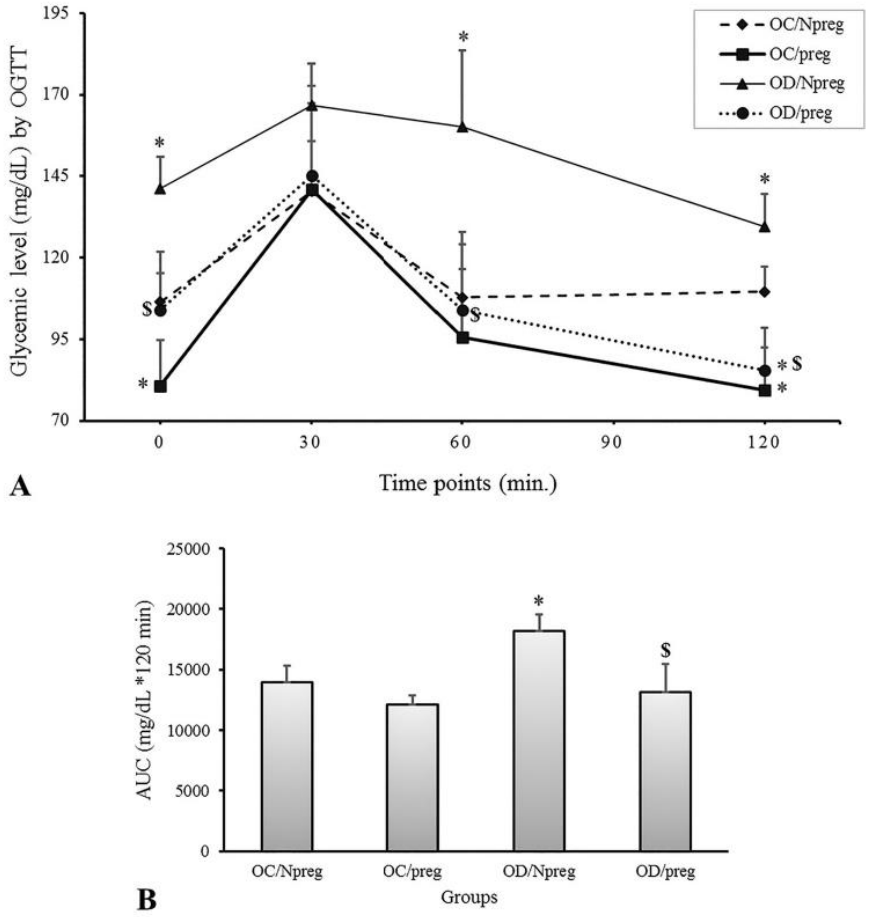


Figure 1. Oral glucose tolerance test (A) and area under the curve (B) of female rat pups born to control (OC) or diabetic mothers (OD) at 120 days of life (Npreg) or at day 17 of pregnancy (preg). Values were expressed as mean ± standard deviation (SD). $n = 6$ rats/group. $^{*}p < 0.05$—compared with the group OC/Npreg; $^{\#}p < 0.05$—compared with the group OC/preg; and $^{\$}p < 0.05$—compared with the group OD/Npreg (OGTT: two-way ANOVA and repeated measure and AUC: two-way ANOVA followed by the Tukey's multiple comparison test).

The data for the OGTT are shown in Figure 1A. Fasting glycemia was significantly higher in the OD/Npreg group and lower in the OC/preg group compared with the OC/Npreg and OD/preg groups ($p < 0.001$). Therefore, although a pregnant state results in a lower fasting glycemia for both OC (OC/preg vs. OC/Npreg, $p < 0.001$) and OD groups (OD/preg vs. OD/Npreg, $p < 0.001$), we clearly observed that when females were exposed to hyperglycemia during their development, fasting glucose values were higher, regardless of the pregnant status (OD/preg vs. OC/preg, $p < 0.001$) or not pregnant status (OD/Npreg vs. OC/Npreg, $p < 0.001$). Interestingly, at the OGTT time points of 60 and 120 min, the glycemic levels of the OD/Npreg group were still higher ($p < 0.001$) compared with the other groups. At 120 min, pregnant rats from a diabetic mother (OD/preg) or nondiabetic mother (OC/preg) exhibited reduced blood glucose levels when compared with their nonpregnant counterparts ($p < 0.001$). Based on the OGTT results, a significant increase in the AUC value was observed only in the OD/Npreg group compared with the other groups ($p < 0.001$) (Figure 1B).

IR and β-cell function changes in adult female pups from diabetic mothers

To better characterize the relationship between the changes seen in the OGTT for glucose metabolism and an impaired potential of the insulin physiology during pregnancy (i.e., pancreatic production and tissue resistance), serum insulin concentrations were also determined during all OGTT time-points (Figure 2). Pregnant pups from diabetic mothers (OD/preg) had

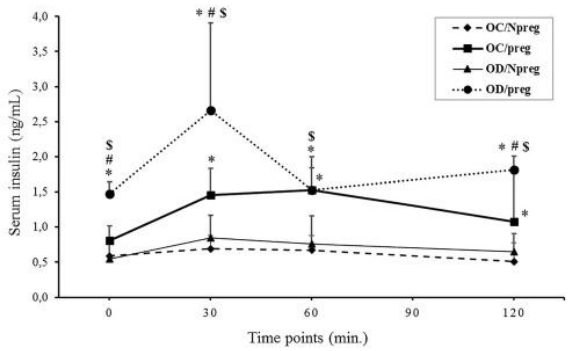


Figure 2. Serum insulin concentration during oral glucose tolerance test (OGTT) of female rat pups born to control (OC) or diabetic mothers (OD) at 120 days of life (Npreg) or at day 17 of pregnancy (preg). Values were expressed as mean ± standard deviation (SD). $n = 6$ rats/group. $^{*}p < 0.05$—compared with the group OC/Npreg; $^{\#}p < 0.05$—compared with the group OC/preg; and $^{\$}p < 0.05$—compared with the group OD/Npreg (gamma distribution test).

a significantly higher fasting insulin concentration when compared with the other groups, which were not different from each other. At the 30- and 60-min mark of the OGTT, both pregnant groups from nondiabetic (OC/preg) and diabetic (OD/preg) rats presented higher ($p < 0.0001$ in 30 min and $p = 0.003$ in 60 min) insulin concentrations compared with nonpregnant animals. Although insulin values increased during the OGTT for both pregnant groups, the raise in insulin concentration was more pronounced (see time-point 30 min in Figure 3) for the female group whose mothers were diabetic, and these values remained higher even after 120 min of the test ($p = 0.002$). On the contrary, the nonpregnant groups (OC/Npreg and OD/Npreg) showed no insulin concentration alterations during all of the OGTT time points evaluated here.

The results for blood glucose and insulin concentration during fasting were used to estimate IR and beta (β)-cell function by homeostatic model assessment (HOMA) evaluation. Females from diabetic mothers showed significantly higher values for HOMA-IR during pregnancy ($p = 0.003$) (Table 1), with approximately a twofold change compared with the other groups. In turn, the HOMA-β was similarly high for both pregnant groups, regardless of being the adult female pups of a nondiabetic (OC/preg) or diabetic mother (OD/preg) ($p < 0.001$) (Table 1). Although the HOMA-β index was lower in nonpregnant animals, the nonpregnant females from diabetic mothers (OD/Npreg) showed a reduced ($p < 0.001$) HOMA-β index compared with the OC/Npreg group, whose mother was not diabetic.

Oxidative stress is higher during the pregnancy of adult female pups from diabetic mothers

Figure 3 shows the thiobarbituric acid reactive substances (TBARS), thiol group (–SH), and peroxide hydrogen (H_2O_2) concentrations and the activity of the antioxidant enzymes SOD, glutathione peroxidase (GSH-Px), and CAT. For control females with no diabetic mothers, pregnancy was shown to reduce the –SH concentrations ($p = 0.005$), SOD ($p = 0.006$), and CAT ($p = 0.007$) activities (OC/preg vs. OC/Npreg). The OD/Npreg presented lower –SH ($p = 0.005$) and H_2O_2 ($p = 0.003$) concentrations compared with the OC/Npreg. Pregnant females from diabetic mothers showed higher TBARS ($p < 0.001$) and –SH ($p = 0.005$) concentrations, and lower SOD activity compared with other groups. There was no significant difference in CAT and GSH-Px activities or H_2O_2 concentrations in pregnant females.

Newborn weight is lower in rat pups from diabetic mothers

Table 2 presents the weight of newborns and placental weight of control and diabetic mothers. In the OD/preg group, the weights of newborns were lower compared with the OC/preg group ($p < 0.001$). There were no differences regarding placental weight but placental efficiency calculated in the OD/preg group was lower compared with the OC/preg group ($p = 0.001$).

Discussion

Maternal metabolic changes, such as diabetes, have negative consequences on offspring (Cheong et al. 2016; Hoffman et al. 2021; Lawlor et al. 2011; Perrettini et al. 2020). In this study, we not only assess the effects of diabetes-induced fetal programming on the metabolism of adult female pups, but we also show how pregnancy adaptations act on preexisting metabolic disturbances.

Adult female pups from diabetic dams presented glucose intolerance confirmed by the OGTT. The fetal exposure to an environment of glucose overload was induced by maternal diabetes, which triggered adaptive mechanisms. On one hand, these adaptations are essential to guarantee fetal development. On the other hand, however, they can lead to harmful consequences during the offspring's adult life (Reusens et al. 2007), including glucose intolerance and metabolic syndrome (Hanafi et al. 2016). We observed that maternal glycemia in female pups of nondiabetic mothers (control) decreased, which was expected since most of the

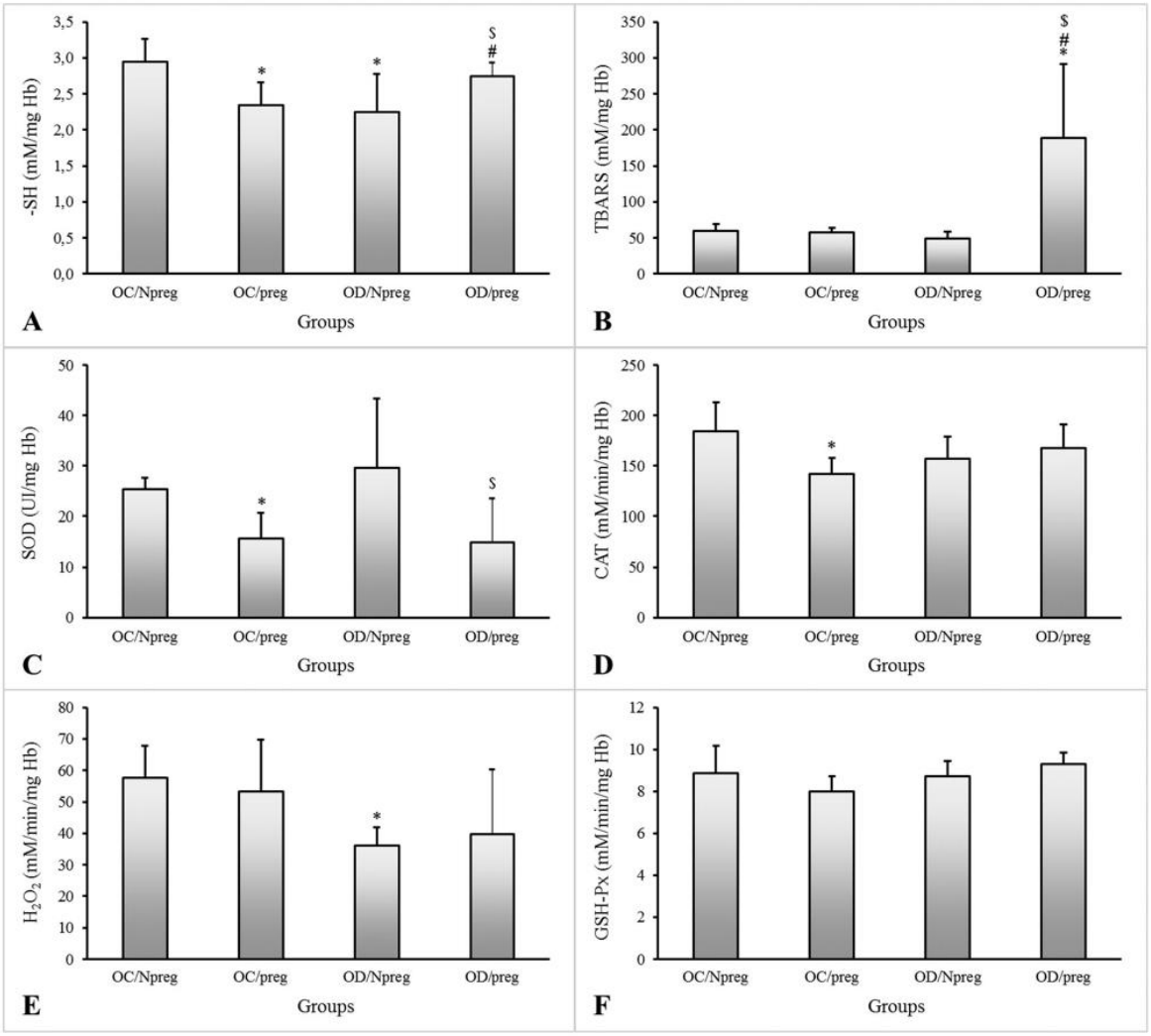


Figure 3. Concentrations of thiobarbituric acid reactive substances (TBARS—A), total reduced thiol groups (–SH—B), hydrogen peroxide (H_2O_2—C), and activities of superoxide dismutase (SOD—D), glutathione peroxidase (GSH-Px—E), and catalase (CAT—F) of female rat pups born to control (OC) or diabetic mothers (OD) at 120 days of life (Npreg) or at day 17 of pregnancy (preg). Values were expressed as mean ± standard deviation (SD). $n = 6$ rats/group. $^{*}p < 0.05$—compared with the group OC/Npreg; $^{\#}p < 0.05$—compared with the group OC/preg; and $^{\$}p < 0.05$—compared with the group OD/NPreg (two-way ANOVA test followed by the Tukey's multiple comparison test).

Table 1. HOMA-IR and HOMA-β indexes for female offspring from control (OC) or diabetic mothers (OD) at 120 days of life (Npreg) or at day 17 of pregnancy (preg).

Groups	HOMA-IR	HOMA-β
OC/Npreg	4.46 ± 1.25^{a}	$142.4\ 0 \pm 35.25^{a}$
OC/preg	4.09 ± 1.56^{a}	430.25 ± 191.31^{b}
OD/Npreg	4.77 ± 2.10^{a}	62.89 ± 22.73^{c}
OD/preg	9.43 ± 2.16^{b}	360.40 ± 122.46^{b}

Values are expressed as mean ± standard deviation. $n = 6$ rats/group.
$^{a,b,c}p < 0.05$—comparison between groups (gamma distribution test).

Table 2. Newborn weight, placental weight, and placental efficiency on the day 21 of pregnancy of female offspring from control (OC/preg) or diabetic mothers (OD/preg).

Variables	OC/preg	OD/preg
Newborn weight (g)	5.90 ± 0.29^{a}	5.36 ± 0.65^{b}
Placental weight (g)	0.57 ± 0.01^{a}	0.59 ± 0.07^{a}
Placental efficiency	10.65 ± 1.47^{a}	9.06 ± 0.97^{b}

Values are expressed as mean ± standard deviation. $n = 6$ rats/group.
$^{a,b}p < 0.05$–*t*-test.

maternal glucose is redirected to the fetus (Baeyens et al. 2016; Lain and Catalano 2007). Contrastingly, the adult female pups of diabetic mothers behaved differently. Instead of experiencing the normal drop in glycemia during pregnancy, their blood glucose levels remained high, which resulted in hyperglycemia

during pregnancy. It is important to highlight that the persistent maternal hyperglycemia observed in this study was equally harmful to the mothers as it was to their female pups.

In this study, both pregnant groups from nondiabetic and diabetic mothers responded with a higher insulin level when challenged in the OGTT. The high insulin concentrations were accompanied by increased pancreatic β-cell function in both groups. Despite the lack of differences between these rats, however, the pregnant pups from diabetic dams showed IR confirmed by an increased HOMA-IR. In response to glucose change in the fetus, the placenta secretes hormones, which increase maternal IR and hepatic glucose production, raising glucose availability in maternal circulation to compensate for fetal consumption (Nolan and Proietto 1994; Lain and Catalano 2007). To prevent excessive glucose delivery to the fetus, increased physiological resistance to maternal insulin must be balanced by an increase in the ability of maternal β-cells to respond to feeding (Baeyens et al. 2016). In rats, a higher β-cell proliferation and volume in the pancreatic islets have been observed, especially at the end of pregnancy (Chen et al. 2011; Hanafi et al. 2016). Nevertheless, due to the chronic hyperglycemic state of the rats in our investigation, this balance did not occur in the pregnant pups from diabetic mothers, and, despite the increased β-cell function to compensate for this metabolic status, the IR ceased to be physiological. Interestingly, when we previously evaluated the adult female pups from nonpregnant diabetic rats when they consumed a high-fat diet or standard diet, insulin secretion was impaired while insulin synthesis was increased (Paula et al. 2022). Pregnancy appears to exert an adaptive mechanism to increase the release of insulin for the improvement of the hyperglycemic response in the adult female pups from diabetic rat mothers.

Pregnancy per se is a state of oxidative stress due to increased metabolic activity in placental mitochondria and reduced scavenging power of antioxidants (Myatt and Cui 2004). The increased oxygen demand during gestation results in an increase of peroxide products proportionally to the mechanisms of antioxidant defense (Gitto et al. 2002; Spada et al. 2014). The ROS elimination is carried out by the action of three main enzymes: SOD, CAT, and GSH-Px, which differ in their distribution and type of reaction catalyzed (Cederberg et al. 2000). The SOD catalyzes the conversion of superoxide ions to oxygen and hydrogen peroxide (Weydert and Cullen 2010; Liu et al. 2015). On the other hand, the CAT enzyme, mainly distributed to peroxisomes, catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. The GSH-Px activity reduces hydrogen peroxide to water using GSH (Cederberg et al. 2000).

We observed a decrease in reduced thiol group levels in the pregnant rats in the control group, indicating protein oxidation and a decreased antioxidant power. The latter is in accordance with the finding that the female rats also exhibited decreased SOD and CAT activities. Despite showing reduced scavenging power, there was no increase in H_2O_2 levels and lipid peroxidation, indicating that SOD and CAT were likely consumed to prevent cellular damage due to excessive ROS.

In diabetic animals, chronic exposure to hyperglycemia results in decreased antioxidant enzymes and increased lipoperoxidation (Damasceno et al. 2011; Gallego et al. 2018; Bueno et al. 2020) and the same can be observed in children who exhibit IR and redox imbalance (Jawerbaum and White 2017; Yessoufou et al. 2006). Our study showed that in the female pups from diabetic mothers whose blood glucose is abnormally high, the physiological level of oxidative stress during pregnancy was not properly maintained. These animals presented a significant increase in TBARS, indicating high lipoperoxidation, which could lead to the disturbance of many biochemical processes and to the impairment of fetal growth. Interestingly, no decrease in –SH nor in the antioxidant enzymes was observed.

Concerningly, fetuses from diabetic dams had low birth weight and lower placental efficiency. Based on our model, we suggest that fetal hyperglycemia, a consequence of the hyperglycemic intrauterine environment, as well oxidative stress (characterized by maternal lipoperoxidation) and/or maternal IR may be related to decreased fetal weight. This is explained by maternal hyperglycemia-induced fetal hyperglycemia that overstimulates pancreatic beta cells, which deplete their insulin stores (Van Assche et al. 2001). After some time, these cells become exhausted, leading to hypoinsulinemia. When associated with a reduced number of insulin receptors, this leads to reduced glucose uptake. This causes decreased protein mass growth, which causes fetal microsomia (Canavan and Goldspink 1988; Sousa et al. 2020). In addition, lower placental efficiency also contributed to impaired oxygenation and nutrition exchange for the lower weight of the female pups (Sinzato et al. 2012). The exact nature of the relationship between maternal oxidative stress and intrauterine growth restriction is not yet clear (Rashid et al. 2018). In humans, mothers of

growth-restricted fetuses have increased malondialdehyde (MDA) levels (Kellogg et al. 2017) and reduced total antioxidant capacity in their plasma (Saker et al. 2008).

The consequences of low birth weight are of great interest and have been widely studied, as there is accumulated evidence of a higher predisposition for the development of adiposity, DM2, altered lipid metabolism, and hypertension in adulthood (Nusken et al. 2011). Our model shows that the effects of fetal programming due to diabetes, apart from being harmful to female pups, also compromise second-generation fetal growth, confirming the transgenerational effect of diabetes in our model. In our laboratory, studies are being conducted to assess the damage to the metabolism of diabetic rat granddaughters. Some limitations can be pointed out, such as the lack of biochemical and morphological determinations (lipid profile and endocrine pancreatic analysis) during pregnancy, as previously analyzed in virgin female pups at 120 days of life (Paula et al. 2022). This information might help to clarify the mechanisms involved in metabolic impairment during pregnancy of their pups.

In conclusion, our data show that the fetal programming caused by maternal diabetes was instrumental to induce glucose intolerance in the female pups in adulthood. Furthermore, maternal diabetes not only influences the metabolism of pre-pregnant pups, but also exacerbates changes that occur during pregnancy, such as oxidative stress and IR, and compromises fetal growth. This contributes to the perpetuation of the cycle of damage that is being transmitted among generations. It is important that further studies explore the mechanisms involved in the impairment of fetal growth and in the perpetuation of the effects of diabetes.

Methods

Animals

Female and male Sprague–Dawley rats weighing ~150 and 250 g, respectively, were adapted and housed at the local laboratory vivarium. In adulthood (90 days of life), these animals were mated to obtain offspring for diabetes induction or for the nondiabetic group (Control). Male and female rats (parental and generations) were maintained under standard laboratory conditions (23 ± 2 °C, humidity 50 ± 10%, 12-h light/dark cycle), fed with a standard diet (Kcal content: 28.54% protein, 62.65% carbohydrate, 8.7% fat, Purina®, Brazil). The animals had free access to food and water. A schematic representation of the experimental design is shown in Figure 4.

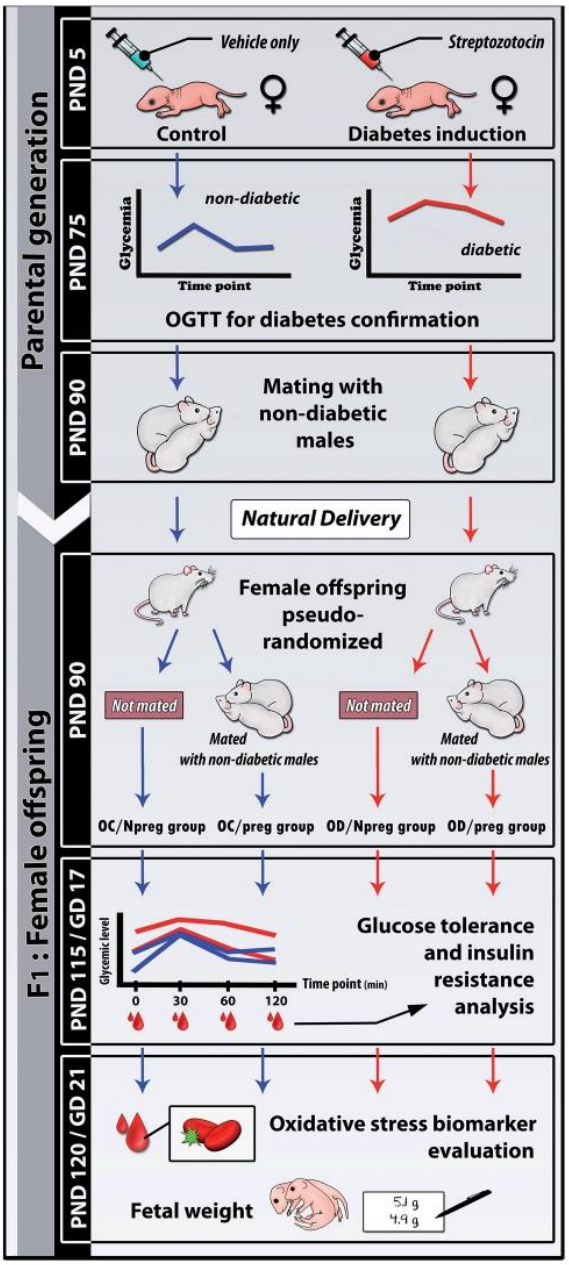


Figure 4. Experimental design to obtain female rat pups from control (OC) or diabetic mothers (OD) at 120 days of life (Npreg) or at day 17 of pregnancy (preg). PND: postnatal day; GD: gestational day; OGTT: oral glucose tolerance test.

Diabetes induction

To obtain female rats for induction of diabetes, healthy male and female rats were mated overnight

and the following morning a vaginal smear was performed to confirm pregnancy. The offspring were obtained by vaginal delivery for later procedure to study the fetal programming effect. The induction of diabetes (D) was performed using the beta-cytotoxic drug, named streptozotocin (STZ) (Sigma Aldrich®, MA, USA—70 mg/kg dose, subcutaneous route) on the fifth day of postnatal life. The nondiabetic group (Control) received only the vehicle (citrate buffer—0.01 M, pH 4.5) at the same volume, administration route, and day used for the diabetic group (Paula et al. 2022).

At 75 days of life, rats from the diabetes-induced and control groups were submitted to the oral glucose tolerance test (OGTT) for inclusion in the experimental groups. Considering the inclusion or exclusion criteria for control or diabetic mothers according to predefined glycemic values related to the values from American Diabetes Association (ADA 2021) and Sinzato et al. (2021), there was no randomization at this stage. Successful diabetes induction was confirmed based on the OGTT results and. Rats with blood glucose $\geq$200 mg/dL in at least one test point were included in the diabetic group. For the control group, only rats with blood glucose $<$140 mg/dL in at least three points of the OGTT curve were included. Females who received STZ and did not show changes in OGTT points and female rats from the nondiabetic group that showed changes in OGTT were excluded from the study. The OGTT of diabetic and control rats are shown in Supplementary Figure S1.

Mating, pregnancy, and lactation

In adulthood (90 days of life), diabetic ($n=8$) and control ($n=6$) rats were mated overnight with normoglycemic males (90 days of life). The next morning the males were removed and vaginal smear was performed with cotton swabs soaked in autoclaved distilled water. Only the region containing the cotton was introduced into the vagina for sample collection. After collection, this sample was transferred to a slide and visualized under an optical microscope to verify the presence of keratinized cells from the estrus phase and spermatozoa, which confirmed positive diagnosis of pregnancy. The pregnant rats were kept under the same conditions in individual cages and with free access to water and food throughout gestation and lactation. After birth, litters were maintained with eight pups per mother, at least four females and four males until weaning (PND 22). Litters were reduced to eight pups per lactating dam, prioring female newborns, with free access to the nipples and rat diet. The standardization of rodent litter size to eight pups was performed in an attempt to control the variability in developmental rate, reduce pup mortality and litter-size-induced variability on pup growth thereby increasing the statistical power (Agnish and Keller 1997) to detect maternal diabetes-related effects. When the litter was superior to eight pups, males and females were at random chosen. When the litter was up to eight pups, if both genders were present, we did not manipulate the litter. But, if the litter was inferior to eight pups per mother, this mother and its litter were excluded from our experiment but maintained in our vivarium for another study. After weaning, female pups were prioritized for the analysis of pregnancy related to intergenerational effects, and males were relocated to another experiment or donated to another investigator.

Experimental groups

In adulthood (postnatal day 90—PND 90), a maximum of two female pups from each diabetic mother (D) (total of 16 female pups) or control (C) (total of 12 female pups) were pseudorandomized to compose the experimental groups of female offspring from control rat (OC) and female offspring from diabetic rats (OD) to maintain the heterogeneity of the experimental groups. Pseudorandomization method was employed as convenience measure for different interventions (to obtain diabetic or not) to successive allocation based upon the date of birth (the female pups from first dam to delivery were not induced to diabetes, and the female pups from next dam received beta-cytotoxic drug for diabetes induction). This method successively occurred (Smith et al. 2015). After this, in all of the steps the randomization by drawing of numbered papers representing each animal was performed to distribute the animals for analysis. For the composition of nonpregnant groups: OC/Npreg—female pups from control mothers ($n=6$) and OD/Npreg—female pups from diabetic mothers ($n=10$). The OC/Npreg and OD/Npreg groups were euthanized by overdoses of anesthesia (sodium thiopental, 120 mg/kg) followed by laparotomy at PND 120. For the composition of the pregnant groups, the composition was as follows: OC/preg—female pups from control mothers ($n=6$) and OD/preg—female pups from diabetic mothers ($n=6$). To obtain the pregnant status, the female pups were mated in adulthood (PND 120) as described for the parental generation. After confirmation of successful mating, this

morning was considered as gestational day 0—GD0). During pregnancy, the female rat pups were kept in individual cages under standard conditions

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

The OGTT was performed at PND 115 for the nonpregnant rats and at the 17th gestational day in pregnant groups, to assess glucose intolerance in offspring from diabetic ($n = 16$) and control mothers ($n = 12$). To avoid severe metabolic complications in pregnant rats, fasting glucose was evaluated after 6 h of fasting (Corvino et al. 2017). The glycemia of each animal was determined by lancing the tail vein to obtain a drop of blood sample using conventional glucometer, without the need for anesthesia. Fasting blood glucose was considered at time 0. The rats then received an intragastric (gavage) glucose solution (0.2 g/mL) at a dose of 2.0 g/kg body weight and 30, 60, and 120 min after glucose loading, blood glucose levels were determined as described above. In this same test, ~200 µm of blood was collected from the tail of the animals at each test point for subsequent insulin measurement. The total glucose response was assessed by calculating the AUC using the trapezoidal method (Tai 1994).

Homeostatic model assessment

During the OGTT test, blood samples (controls $n = 12$; diabetics $n = 16$) were also collected at different time points to measure serum insulin using an ELISA commercial kit (Crystal Chemical® Code: 90060, USA). Data for fasting blood glucose and serum insulin concentrations were used to determine HOMA, which uses a mathematical model to evaluate the degree of IR (HOMA-IR) and pancreatic β-cell function (HOMA-β) (Aref et al. 2013; Mathews et al. 2010). For HOMA-IR, the value was calculated using the following equation: [fasting insulin (µU/mL) × fasting glucose (mmol/L)]/22.5. The HOMA-β cell function, which expresses the beta-cell function, was calculated using the following equation: [20 × fasting insulin (µU/mL)]/[fasting glucose (mmol/L) – 3.5].

Blood collection, laparotomy, and evaluation oxidative stress markers

After 6 h of fasting, the nonpregnant (at PND 120) and pregnant groups (gestational day 21) were anesthetized with sodium thiopental (120 mg/kg) followed by decapitation (Faria-Neto and Santos 2008). Blood samples (controls $n = 12$; diabetics $n = 16$) were collected and processed to obtain washed erythrocytes that were used to assess oxidative stress markers. Samples were collected in heparinized tubes and immediately centrifuged at 185 × g for 10 min at 4 °C. Plasma was discarded and the erythrocytes were then washed with PBS (pH 7.4) and centrifugation at 1575 × g for 1 min at 4 °C. The supernatant was discarded. This procedure was repeated three times, and then the washed erythrocytes were divided into two aliquots for further analysis. One aliquot was diluted (1:20) into purified water to measure TBARS (lipoperoxidation biomarker), H_2O_2 (reactive species of oxygen metabolism), and–SH (a protein oxidation marker) levels. For SOD, CAT, and GSH-Px, measurements, another aliquot was diluted into stabilizing solution of 2.7 mM EDTA and 0.7 mM 2-mercaptoethanol (1:20). Hemoglobin (Hb) concentration was determined with Drabkin's solution and according to the diluent used (purified water or stabilizing solution) (Tentor and Salvati 1981). The levels of TBARS, SH, H_2O_2, SOD, CAT, and GSH-Px were measured following procedures modified from de De Souza et al. (2010).

The uterine horns were exposed and the fetuses were removed. Following, the fetuses were weighed, sexed and their respective placentas were also weighed. The fetal weight and its respective placental weight [fetal weight (g)/placental weight (g)] were used to calculate the ratio denominated as placental efficiency (Hayward et al. 2016).

Statistical analysis

The sample size calculation was performed taking into account the mean and standard deviation values previously obtained from the SOD activities, using the SAS -STATISTICAL ANALYSIS SYSTEM Software version 9.4, 2021. Based on the SOD activity values, using 90% power and 5% type I error, the effect size was determined by a specialist in Biostatistics at our Institution, with a minimum of six rats per group. The comparative analyses for OGTT glycemic values, two-way ANOVA and the repeated measures were performed. AUC and oxidative stress were performed using the two-way ANOVA test followed by Tukey's multiple comparison test. Due to asymmetric distribution of the data, the insulin levels, HOMA-IR and HOMA-β values were compared using the gamma distribution test. The Fisher's exact test was used to proportion values. For newborn weight, placental weight and placental efficiency, Student's t-test was performed. For all statistical comparisons, a minimum

confidence limit of 95% ($p < 0.05$) was considered and results are presented as mean ± standard deviation (SD).

Ethics approval

The present study was approved by the institution's Animal Research Ethics Committee (Protocol CEUA Number: 1218/2017), and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and all efforts were made to minimize animal suffering.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contributions

Conception and design, data acquisition, analysis and interpretation, manuscript writing: VGP; Interpretation, manuscript writing: MRS; Conception and design, data acquisition, analysis and interpretation: YKS; Manuscript writing and revision: AISBV; Data analysis and interpretation: JEC; Data analysis and interpretation, manuscript writing: GTV; conception and design, data acquisition, analysis and interpretation, manuscript writing: DCD.

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CONCLUSÃO FINAL

Considerando os resultados obtidos, através do uso de animais de laboratório como modelo para melhor compreensão da associação entre diabete e dieta hiperlipídica (DHL), podemos observar que com os resultados dos 6 capítulos redigidos nessa tese, o consumo de DHL do desmame até os 120 dias de vida levou à hiperglicemia e aumentou a probabilidade de as ratas desenvolverem diabete. A exposição ao diabete intrauterino foi determinante para causar disfunção mitocondrial, prejuízo na proliferação e secreção de hormônio e impactou diretamente a reserva ovariana. Com isso, durante a prenhez das ratas filhas de mães diabéticas, essas alterações se exacerbaram e, de fato, comprometeram a reprodução. Além disso essas ratas apresentaram resistência à insulina, estresse oxidativo e crescimento anormal de seus filhotes.

Assim, nosso modelo experimental se mostra eficaz para o estudo intergeracional dos efeitos do diabete sobre a fertilidade em ratas. Isso mostra a importância de levarmos em consideração que o número de mulheres diabéticas com sobrepeso e/ou obesidade cresce em todo mundo. Além disso estudos clínicos sobre reprodução são escassos e complexos. Este estudo contribui cientificamente e socialmente, ao evidenciar as consequências que o diabete e o consumo de dietas ricas em gordura podem causar prejuízo ao processo reprodutivo e mostra a importância de se conscientizar a população, principalmente as mulheres, sobre o cuidado com a saúde reprodutiva e estilo de vida saudável.

APÊNDICE

TABELA 1. Composição bromatológica da dieta padrão e dieta hiperlipídica.

Composição	Dieta	
	Padrão	**Hiperlipídica**
Energia (cal/g)	4,022	4,925
Umidade (%)	7,45	3,65
Matéria Seca (%)	92,55	96,35
Matéria Mineral (%)	6,66	2,84
Proteína Crua (%)	25,76	26,77
Extrato Etério (%)	3,49	15,19
Fibra Crua (%)	43,63	45,42

A dieta padrão foi obtida comercialmente (Purina®). A dieta hiperlipídica foi preparada na Unidade de Pesquisa Experimental (UNIPEX) da Faculdade de Medicina de Botucatu (FMB/UNESP). Foram utilizados os seguintes ingredientes: farelo de soja, sorgo, casca de soja, amido de milho, açúcar refinado, banha de porco, minerais (ferro, biotina, vitamina B12, vitamina D, vitamina B6, vitamina E, vitamina A, selênio e potássio.), sal. Estes ingredientes foram moídos, misturados e oferecidos na forma de pellets. Após o preparo, a ração foi mantida sob refrigeração até o momento do consumo.



FIGURA 1. Fotografia ilustrativa da forma da ração hiperlipídica.

Fonte: da autora

ANEXOS

ANEXO 1. Aprovação da Comissão de Ética no Uso de Animais (CEUA)/ FMB_Unesp

Projeto de pesquisa “guarda-chuva”



UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

unesp

FMB

Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO Nº 1218/2017 -CEUA

Certificamos que a proposta intitulada: **"Avaliação de descendentes expostas ao diabetes moderado intraútero submetidas à dieta hiperlipídica no período pós-natal e tratadas com mistura de cálcio e vitamina D durante a prenhez"**, conduzida pela Pesquisadora Principal: **Profa. Dra. Débora Cristina Damasceno Meirelles dos Santos**, com a colaboração de Gustavo Tadeu Volpato, Antônio Carlos Lalla, Arlindo Sérgio Gabriel, Carlos Roberto Gonçalves Lima, Danilo Chaguri, Franciane Quintanilha Gallego Souza, Marcos Rogério Pimentel, Raianne Queiroz de Moraes Souza, Thaigra de Sousa Soares, Verônyca Gonçalves Paula e Yuri Karen Sinzato, com o nº 1218/2017, **Subprojeto I** : "**Análise do perfil lipídico e estresse oxidativo de ratas expostas á hiperglicemia intraútero, dieta hiperlipídica no período pós-natal e tratadas com cálcio e vitamina D na prenhez"**, conduzido pela Pesquisadora: **Thaigra de Sousa Soares**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n. 11.794, de 08 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi APROVADA pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião de 27 de abril de 2017.

Inclusão do Subprojeto I – Aprovado em 12 de dezembro de 2018.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	29 de abril de 2019
Espécie/LinhagemRaça	Rato Sprague Dawley
Nº de animais	412
Idade/Peso	4 a 400 gramas / Recém-nascidos e adulto
Sexo	Macho e Fêmea
Origem	Biotério Central da Unesp - Botucatu

Graziela Nogueira Bertani
Secretária da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

Prof. Ass. Guilherme Antônio Moreira de Barros
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fones: (14) 3880.1608 e 3880.1609 E-mail Secretaria: ceua@fmb.unesp.br

ANEXO 2. Aprovação CEUA / FMB_Unesp

Projeto de pesquisa da aluna Verônyca Gonçalves Paula



UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO Nº 1334/2019 – CEUA

Certificamos que o projeto intitulado: **"Avaliação da função reprodutiva ovariana de ratas expostas à hiperglicemia materna intraútero e submetidas à dieta hiperlipídica após desmame."**, conduzido pela Pesquisadora: **Verônyca Gonçalves Paula** – Orientadora: Profa. Dra. Débora Cristina Damsceno – Coorientador: Profa. Dra. Yuri Karen Sinzato e Prof. Dr. Gustavo Tadeu Volpato, registrada com o **nº 1334/2019**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei n. 11.794, de 08 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi APROVADA pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião extraordinária de 29 de janeiro de 2020.

Obs.: Informamos que o certificado anterior 1334/2019 – Com espécie Camundongo Isogênicos foi cancelado.

Finalidade	( ) Ensino (**X**) Pesquisa Científica
Vigência da autorização	01 de janeiro de 2022
Espécie/Linhagem/Raça	Sprague Dawley
Nº de animais	08 Machos e 44 Fêmeas
Idade/Peso	120 dias – 250 gramas
Origem	Biotério

Sara Rosa Stanley Sampaio
Secretária da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

Profa. Associada Bertha Furlan Polegato
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

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Publicações durante o Doutorado relacionadas ao projeto de pesquisa

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OXFORD

Research Article

Metabolic changes in female rats exposed to intrauterine hyperglycemia and postweaning consumption of high-fat diet†

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Abstract

We evaluated the influence of the hyperglycemic intrauterine environment and postweaning consumption of a high-fat diet (HFD) on the glycemia, insulin, lipid, and immunological profile of rat offspring in adulthood. Female rats received citrate buffer (Control—C) or Streptozotocin (a beta cell-cytotoxic drug to induce diabetes—D) on postnatal day 5. In adulthood, these rats were mated to obtain female offspring, who were fed a standard diet (SD) or HFD from weaning to adulthood ($n = 10$ rats/group). OC/SD and OC/HFD represent female offspring of control mothers and received SD or HFD, respectively; OD/SD and OD/HFD represent female offspring of diabetic mothers and received SD or HFD, respectively. At adulthood, the oral glucose tolerance test (OGTT) was performed and, next, the rats were anesthetized and euthanized. Pancreas was collected and analyzed, and adipose tissue was weighted. Blood samples were collected to determine biochemical and immunological profiles. The food intake was lower in HFD-fed rats and visceral fat weight was increased in the OD/HFD group. OC/HFD, OD/SD, and OD/HFD groups presented glucose intolerance and lower insulin secretion during OGTT. An impaired pancreatic beta-cell function was shown in the adult offspring of diabetic rats, regardless of diet. Interleukin (IL)-6 and IL-10 concentrations were lower in the OD/HFD group and associated to a low-grade inflammatory condition. The fetal programming was responsible for impaired beta cell function in experimental animals. The association of maternal diabetes and postweaning HFD are responsible for greater glucose intolerance, impaired insulin secretion and immunological change.

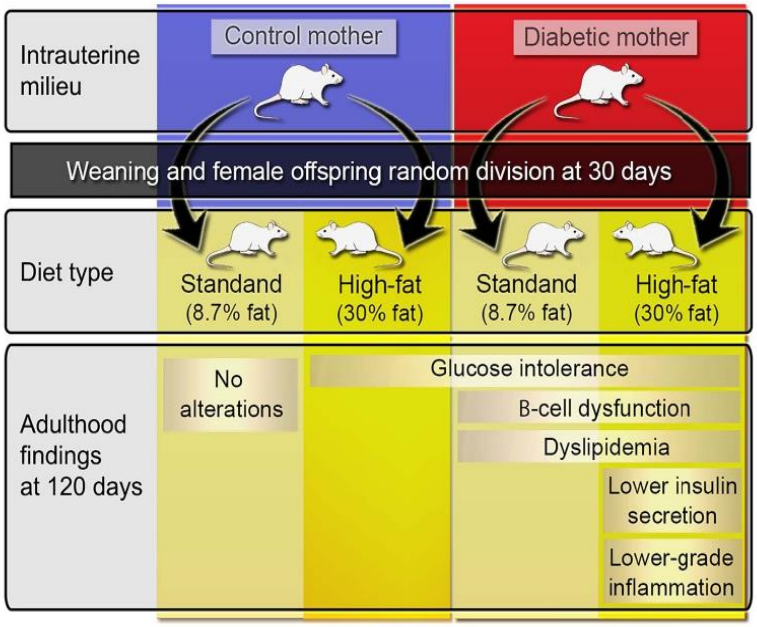
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Summary sentence

Summary Sentence The association of maternal diabetes and postweaning HFD causes glucose intolerance due to β-cell disarrangement and impaired insulin secretion, and immunological changes in adult offspring.

Graphical Abstract



Key words: fetal programming, high-fat diet, diabetes, rat.

Introduction

During pregnancy, physiological adaptations in the metabolism are necessary, and one of them is the insulin resistance that appears around week 14 of pregnancy and may reach a 2-fold increase at the end of the pregnancy [1]. However, ~5–12% of pregnant women in North America have insufficient sensitivity to insulin and/or impaired pancreatic β-cell reserve to maintain euglycemia [2]. Gestational *Diabetes mellitus* (GDM) is diagnosed in the second or third trimester of pregnancy (that was not clearly overt diabetes prior to gestation) [3, 4]. There is evidence of a high rate of pregnancy loss, maternal morbidity, macrosomia and fetal and neonatal death related to pregestational diabetes [5–7], and malformations, stillbirth, birth injury, hypoglycemia and respiratory distress syndrome related to GDM [8, 9].

In addition to the GDM, preexisting Diabetes mellitus (DM) also compromises maternal and fetal health, and it presents some classes, such as type 1 DM (DM1), which appears due to autoimmune β-cell destruction, usually leading to absolute insulin deficiency; and type 2 DM (DM2) related to a progressive loss of adequate β-cell insulin secretion frequently on the background of insulin resistance. Both DM classes are diagnosed before pregnancy. Preexisting diabetes impairs fetal development, as it alters the intrauterine environment and programs the offspring to be more susceptible to metabolic disorders [10, 11], as well as an increased risk for the DM2 development and obesity at adulthood [12].

Streptozotocin (STZ) is a chemical used in laboratory animals for diabetes induction [13]. This drug destroys pancreatic β-cells and leads to poor insulin synthesis, and consequently hyperglycemia [14, 15]. It may be used in different doses and routes of administration, lineage, and age of animals to reach different blood glucose levels, reproducing glycemia found in DM2 and GDM. Then, the diabetes induction can be performed in the neonatal period [16, 17], during pregnancy [18–21] or in both periods [22]. In these models, the rats present glucose intolerance and an oxidative stress status at adulthood [23, 24]. In addition, maternal diabetes damages fetal development and enhances chances for onset of metabolic modifications of the offspring at adulthood [25–27].

Similarly, exposure to a high-fat diet (HFD) in the postnatal period may determine that rodents are more susceptible to metabolic diseases [28–30]. Animal models fed HFD presented hyperglycemia, glucose intolerance, insulin resistance [31–35] and the presence of inflammatory processes [31, 36, 37].

Hyperglycemia promotes the release of proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) [38]. Immune cells are activated and infiltrate various tissues, contributing to a state of low-grade chronic inflammation [39], which impairs glucose and insulin metabolism, promoting a vicious cycle of hyperglycemia [40]. The diabetic rats that received HFD showed metabolic features similar to those of human diabetes [41–44]. In addition, the HFD consumption negatively affects the

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reproductive process of mothers and offspring [45–47]. However, in the database used in this study, there are no reports on the effects of intrauterine diabetes and postnatal HFD consumption on the offspring at their adult life.

Considering the high incidence of pregnant women with diabetes and their daughters exposed to a lifestyle with a high consumption of HFDs, investigations are necessary to explain how the interaction of these insults can affect the metabolism of offspring at reproductive age. Therefore, we hypothesized that female newborns from diabetic dams will present impaired metabolism due to intrauterine programming and, after a second insult (HFD from weaning until adulthood), these metabolic changes will be exacerbated. Then, the objective of this study is to evaluate whether the developmental plasticity induced by maternal hyperglycemia combined to HFD consumption after weaning lead to abnormal glycemia, impaired lipid and immune profiles, and oxidative stress in adult female offspring at reproductive age.

Methods

Animals

Sprague–Dawley rats (150–250 g) were mated with healthy males to obtain female offspring for diabetes induction. These animals were maintained at the local laboratory under controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 10%), and light/dark cycle (12 h) in polypropylene cages lined with wood shavings. Filtered water and regular chow were offered ad libitum. As a form of environmental enrichment, paper balls were used in the cages [48]. The procedures and animal handling were performed following 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 Ethics Committee for the Use of Animals of our Institution approved all the methods adopted in this study (Protocol CEUA Number: 1218/2017).

Experimental approach

The experimental approach is summarized in Figure 1.

Diabetes induction. Female rats at postnatal day (PND) 5 were injected with streptozotocin (Sigma Aldrich, MA, USA—70-mg/kg dose, subcutaneous route) for diabetes induction. Nondiabetic (Control) female rats received equivalent volume of vehicle (citrate buffer—0.01 M, pH 4.5) as previously described [49]. The blood glucose levels in oral glucose tolerance test (OGTT) were classified in accordance to American Diabetes Association [4] and Sinzato et al. [13]. Shortly, the rats were considered diabetic when presenting glycemia ≥200 mg/dL at least in one-time point during OGTT. In the control group, only rats with glycemia <140 mg/dL at least in three-time points during the OGTT were included. Female rats not accomplishing with the abovementioned characteristics were anesthetized, euthanized, and excluded from this study.

Mating, pregnancy, and lactation. At adulthood, diabetic (D) and control (C) female rats were mated overnight with nondiabetic normoglycemic male rats. The following morning when spermatozoa were found in the vaginal smear was designated as day 0 of pregnancy. Female rats that did not mate after 15 consecutive days were considered infertile and excluded from this study [50]. The offspring was obtained through vaginal delivery. After birth, male newborns were euthanized, while female newborns were kept at a maximum eight pups per litter until the weaning (PND 22). Female rats were prioritized for analysis of the transgenerational effects related to pregnancy.

Experimental groups and dietary patterns

Upon weaning, female offspring of diabetic (OD) and control (OC) mothers were pseudo-randomized by lot respecting a maximum of four females per mother (with two sisters being in the same group). Still, OD and OC groups were further split in two other groups according to the diet they were fed with, whether a standard diet (SD, Kcal content: 28.54% protein, 62.65% carbohydrate, 8.7% fat, Purina, Brazil) or HFD (Kcal content: 23.43% protein, 46.63% carbohydrate, 30% fat, using lard as fat source). Additional bromatological comparison between the diets is shown in Supplementary Table S1. Four experimental groups were distributed into: OC/SD—female offspring from control mothers and fed SD; OC/HFD—female offspring of from control mothers and fed HFD; OD/SD—Female offspring from diabetic mothers and fed SD; and OD/HFD—Female offspring from diabetic mothers and fed HFD.

Based on previous experiments conducted in our laboratory in relation to Area Under Curve (AUC) obtained from the OGTT glycemic values, using 90% power and error type I of 5%, the effect size was determined by Research Support Office (EAP) of Botucatu Medical School. Then, the sample size was determined in 10 rats per group. All the groups were followed up from weaning to PND 120. The HFD was handmade at our Institution, adequately supplemented with vitamins and minerals, and maintained under refrigeration until the time of use. Given the fact that the diets had visual characteristics that easily allow their distinction, random housing and blinding of caregivers and/or investigators were not possible. Food intake was determined weekly from PND 45 to 120. Energy intake was calculated as food mean consumption × dietary crude energy [51].

Oral glucose tolerance test

At PND 115, OGTT was performed for glucose tolerance evaluation as previously described [52, 53]. In this same test, blood samples were collected at the different times to insulin measurements using a commercial kit (Crystal Chemical Code: 90060, USA) by ELISA.

Homeostatic model assessment and TyG index

Insulin resistance [Homeostatic model assessment (HOMA)-IR] and beta-cell function (HOMA β-cell function) were calculated [54]. For HOMA-IR, the value was calculated using the following equation: [fasting insulin (μU/mL) × fasting glucose (mmol/L)]/22.5. HOMA-β cell function expresses the beta-cell function and was calculated using the following equation: [20 × fasting insulin (μU/mL)/(fasting glucose (mmol/L) − 3.5]. For TyG index, the value was calculated using the following equation: TyG = Ln [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2] [55].

Blood, pancreas, and adipose tissue collection

At PND 120, after 6 h of fasting, the rats were intraperitoneally anesthetized by sodium thiopental (Thiopentax, Cristália, Brazil—120-mg/kg dose) and decapitated. The blood samples were collected in anticoagulant-free test tubes, incubated in ice for 30 min, and then centrifuged at 1575 *x g* for 10 min at 4 °C. The serum was collected and stored at −80 °C until further analysis of the biochemical and immunological parameters. The pancreas was collected to immunohistochemical analysis (IHC). The visceral and periovarian fat pads were removed and weighed for calculation of relative weight, which was expressed in grams/100-g body weight.

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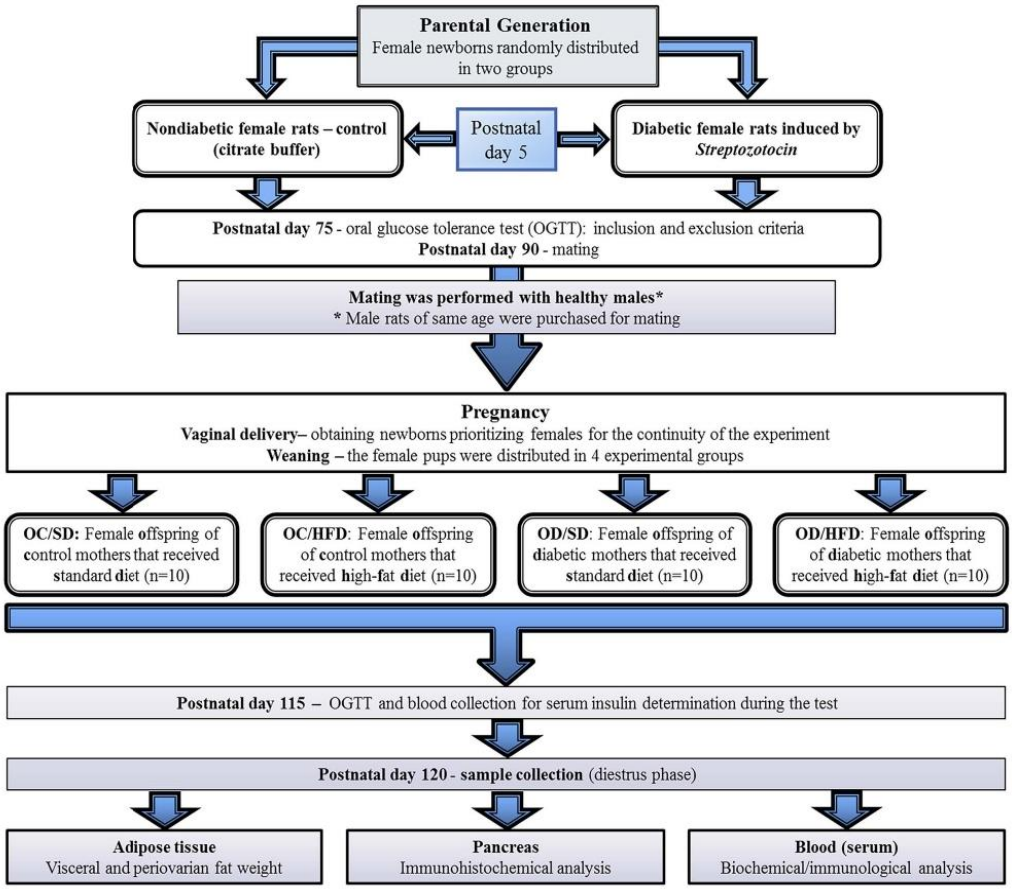


Figure 1. Experimental design.

Evaluation of biochemical profile

From here, all procedures were performed with the investigator blinded to the analyses. The serum cholesterol and triglyceride concentrations were estimated by enzymatic method using commercial kits (Winer, Argentine). Very-low-density lipoprotein (VLDL)-cholesterol values were calculated from the triglyceride concentrations [56].

Evaluation of immunological profile

The serum concentration of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10) were measured by ELISA using commercial kits (R&D Systems, MN, USA).

Immunohistochemical analysis

After its dissection, each pancreas was weighed and fixed in 10% formaldehyde for 24 h, dehydrated in progressive alcohol concentrations and embedded in paraffin. For the IHC technique, the pancreas was cut into sections of 5 μm each. Anti-insulin (Abcam-ab8304, CA, USA) was used for the primary antibody. The antigen retrieval was performed and endogenous peroxidase (peroxidase inhibitor containing hydrogen peroxide and 15-mM sodium azide—Dako, CA, USA) blockade was performed for 40 min at room temperature for all antibodies. For the blockade of nonspecific proteins, Protein Block (0.25% casein in PBS, containing carrier protein and 15-mM sodium azide—Dako, CA, USA) was used for 30 min in an oven at 27 °C. After incubation of the primary antibody, the secondary antibody (Histofine, Germany) was added for 30 min in an oven at 27 °C. For the development of peroxidase, the chromogen DAB (3,3 diaminobenzidine) was used for 3 min at room temperature. Then, the slides were counterstained in Harri's hematoxylin and mounted. The images were captured through the computerized image system (Software KS-300, version 3.0, Zeiss, Germany), which receives a digital camera image (CCD-IRIS/RGB, Sony, Japan), coupled under a microscope (DMR, Leica, Biosystems, IL, USA). The cells of the pancreatic islets were identified and classified by the presence of cytoplasmic labeling for the antibody insulin. The cell counts were performed using Image J software. The percentage of cells present in the pancreatic islet was calculated by the ratio between the number of immunolabeled cells and the number of total cells [57, 58].

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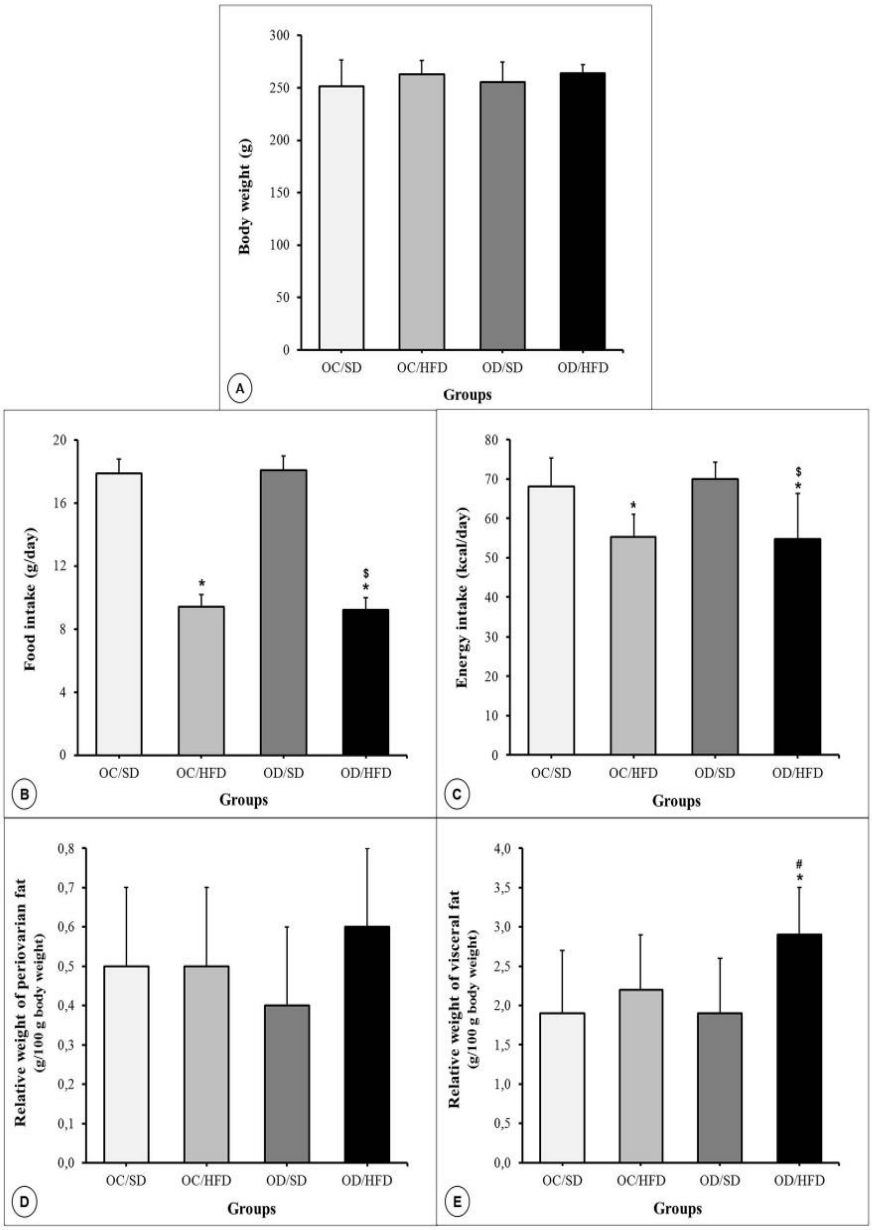


Figure 2. Body weight (A), food (B) and energy intake (C), and relative weight of periovarian (D) and visceral (E) fat of female offspring of control mothers (OC) or diabetic mothers (OD) given an SD or HFD after weaning up to day 120 of postnatal life. *Values are expressed as mean ± SD, n = 10 rats/group. *P < 0.05—compared with the group OC/SD; #P < 0.05—compared with the group OC/HFD (One-way ANOVA test followed by Tukey's Multiple Comparison Test).*

Statistical analysis

Data were presented as mean ± *SD*. For IHA, the calculation was estimated in five animals/group, with a minimum of 10 islets/animal/pancreas. For comparative analysis of glycemia of OGTT, AUC, and HOMA, the Gamma Distribution Test was used because the distribution of the data was not symmetric. A factorial scheme 2 × 2, two factors for the group (OC or OD) and two factors for diet (SD or HFD) with group × diet interaction, was used for body weight, food intake, and fat weight. For serum insulin and cytokine concentrations, and the ratio between imunostained cells per total number of cells, the One-way ANOVA test followed by Tukey's Multiple Comparison Test was used. For all statistical

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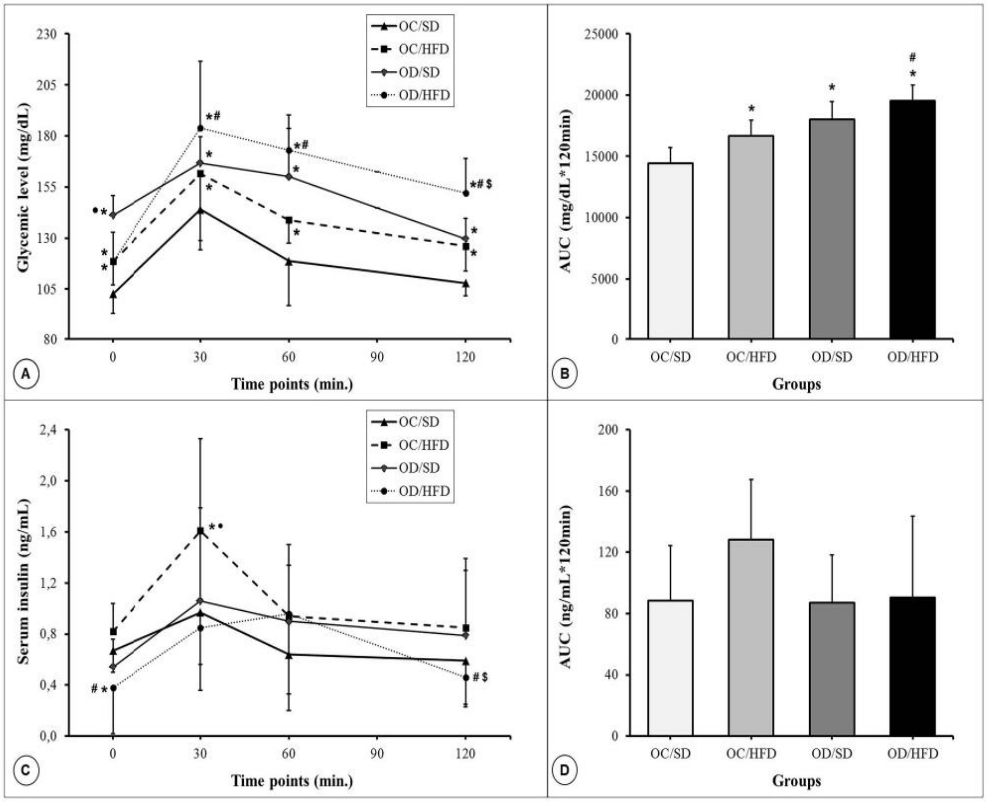


Figure 3. Blood glucose (A—OGTT; B—Area under the curve) and insulin levels (C—Serum insulin concentration during OGTT; D—Area under the curve) at day 115 of life of female OC mothers or OD mothers and received an SD or HFD after weaning up to day 120 of postnatal life. *Values were expressed as mean ± SD. n = 5 rats/group. *P < 0.05—compared with the group OC/SD; #P < 0.05—compared with the group OC/HFD; $P < 0.05—compared with the group OD/SD; • P < 0.05—compared with the group OD/HFD (Gamma Distribution Test).*

comparisons, a minimum confidence limit of 95% ($P < 0.05$) was considered.

Results

HFD consumption increases adiposity without increasing body weight

At PND 120, there were no changes in body weight, fat weight, and food consumption in OD/SD group in relation to OC/SD rats. However, the OD/HFD group showed decreased food consumption compared with the OC/SD group, increased visceral fat weight compared with the OC/SD, and OD/SD group and relative visceral fat weight compared with the OC/SD group (Figure 2).

Exposure to intrauterine diabetes causes glucose intolerance and hyperlipidemia, and the HFD consumption exacerbates glucose intolerance and impairs insulin secretion

At PND 115, OGTT showed that OC/HFD, OD/SD, and OD/HFD groups had higher fasting blood glucose compared with the OC/SD group, and the fasting glycemia of OD/SD was higher than OD/HFD. During the test, at 30, 60 and 120 min, glycemic levels remained higher in both groups of diabetic offspring compared with the OC/SD group. At the end of the test (120 min), the diabetic rats had higher blood glucose levels compared with the OC/SD and OC/HFD, but the glycemic increase was more evident in OD/HFD, with no glycemic return to fasting values (Figure 3A). There was an increased AUC in OD/SD and OD/HFD groups compared with the OC/SD group, confirming glucose intolerance (Figure 3B).

There were no changes in serum insulin concentrations in OD/SD; however, the OD/HFD group had decreased fasting insulin secretion compared with the OC/SD group, and at 120 min of the test compared with the other groups (Figure 3C). Regarding the lipid profile (Figure 4), the OD/SD and OD/HFD groups showed higher serum levels of total cholesterol, VLDL-cholesterol, and triglycerides compared with the OC/SD group, showing a hyperlipidemia in these animals.

Exposure to intrauterine diabetes causes insulin resistance and impairs the function of β-pancreatic cells

Despite not showing changes in the HOMA-IR index, the OD/SD and OD/HFD groups showed insulin resistance confirmed by the TYG index, which was higher compared with the OC/SD group. The β-cell function showed an impairment in both groups of diabetic offspring, confirmed by decreased HOMA-β index compared with

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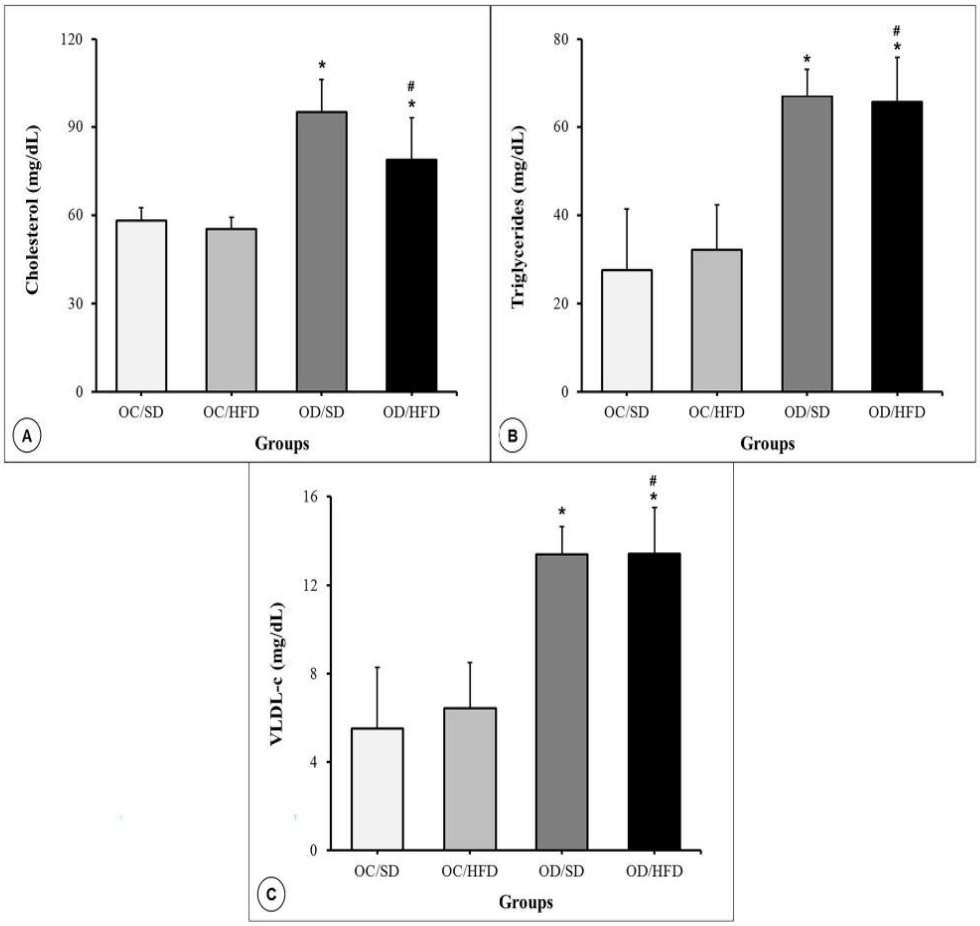


Figure 4. Lipid profile (A—Cholesterol, B—Triglyceride, and C—VLDL-cholesterol) at day 120 of life of female OC mothers or OD mothers and received an SD or HFD after weaning up to day 120 of postnatal life. *Values were expressed as mean± SD. n= 10 rats/group.* $^{*}P < 0.05$*—compared with the group OC/SD;* $^{\#}P < 0.05$*—compared with the group OC/HFD (One-way ANOVA test followed by the Tukey's Multiple Comparison Test).*

the OC/SD group (Figure 5). There was an increase in the ratio between the number of immunostained cells for insulin and the total number of cells in the OD/SD and OD/HFD groups compared with the OC/SD group (Figure 6).

Association between intrauterine diabetes and postweaning HFD consumption impairs the immunological profile

There were no changes in interleukin concentrations in OD/SD group. However, the OD/HFD showed a lower IL-6 concentration compared with the OC/SD and OC/HFD rats and decreased IL-10 level as compared with the OD/SD rats. There was no difference in TNF-α concentration between the groups (Figure 7).

Discussion

The influence of an impaired intrauterine environment caused by diabetes (OD/SD) associated to postnatal nutritional insult represented by postweaning consumption of HFD (OD/HFD) was showed in this study. This study is unique because it is one of the first to provide information on the relationship between fetal programming and postnatal environment in the metabolism of female offspring in reproductive age. Intrauterine exposure to pre-existing diabetes was metabolically able to program the offspring to respond to the second insult (HFD), being more impactful for metabolic changes, even though HFD has exacerbated the impairment of insulin secretion. These alterations aggravated the already established metabolic disorder.

There was no change in body weight between the groups. The HFD fed animals showed higher visceral adipose tissue accumulation and greater adipocytes area (unpublished data), in spite of decreased food intake. The increased relative weight of visceral fat in OD/HFD can be explained due to the fact that HFD is responsible for greater fat deposition [59, 60]. Although results on the amount of HFD ingested by animals are conflicting, our findings corroborate studies in which food intake was lower in the high-fat group [61–64]. The literature is not clear in explaining how HFD can interfere with the energy homeostasis process [61–64]; however, some hypotheses might be suggested, especially regarding the HFD palatability, which

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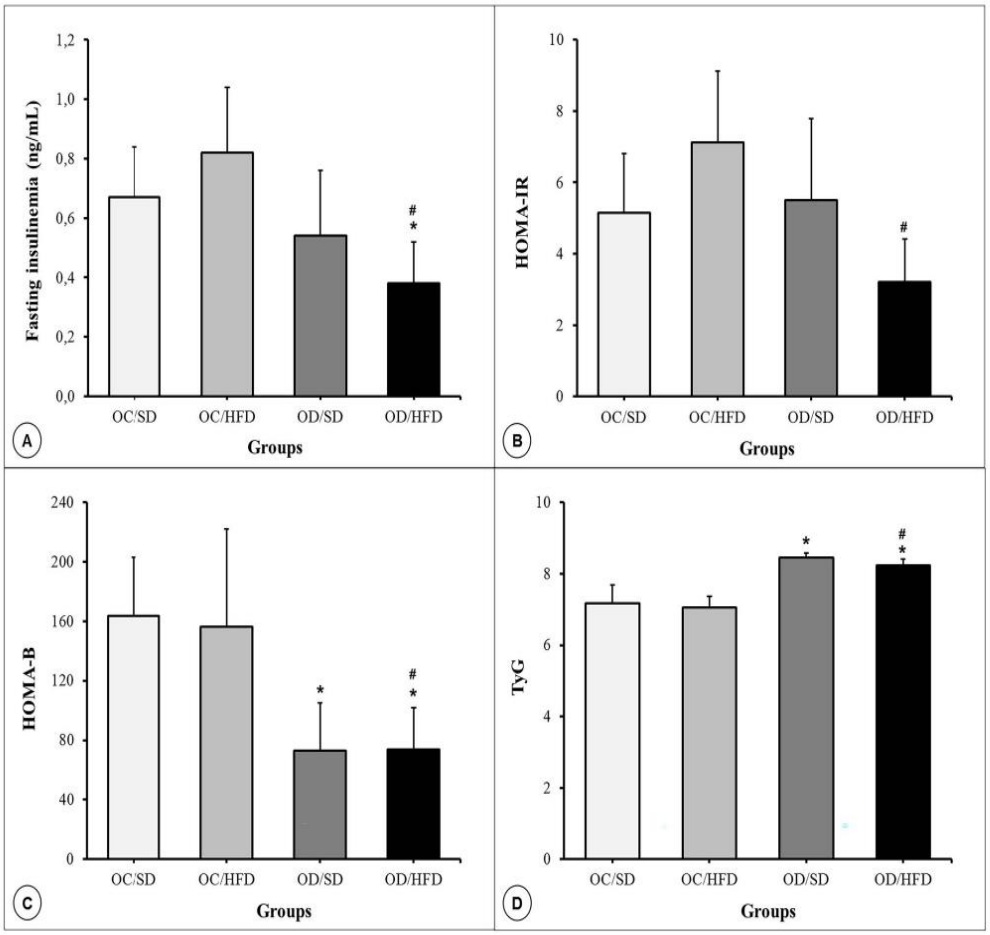


Figure 5. Fasting insulinemia (A) HOMA-IR (B), HOMA-β (C), and TyG index (D) of female OC mothers or OD mothers given an SD or HFD after weaning up to day 120 of postnatal life. *Values are expressed as mean ± SD. n = 10 rats/group. * $P < 0.05$—compared with the group OC/SD; # $P < 0.05$—compared with the group OC/HFD. (Gamma Distribution Test for HOMA-IR and One-way ANOVA test followed by the Tukey's Multiple Comparison Test for fasting insulinemia, HOMA-β, and TyG).*

may be responsible for the lower energy consumption [65]. The source of fat and the amounts of salt and sugar must be considered [66, 67]. Oliva et al. [65] verified a decreased HFD consumption due to its palatability compared with the cafeteria diet. Despite the lower energy consumption in our study, we cannot affirm whether rats consumed an adequate amount of calories and whether there was a lesser energy expenditure.

The greater fat deposition caused by HFD might contribute for worsening glucose metabolism, since visceral fat is an important risk factor for metabolic syndrome and insulin resistance [68], indicating a relationship between adiposity and glucose intolerance since excessive blood glucose levels can impair in lipolytic and lipogenic pathways, particularly in white adipose tissues [69]. An HFD-induced obesity is associated with high levels of lipids (fats) in the blood, including cholesterol and triglycerides, which is also called "hyperlipidemia" [70]. The offspring from diabetic mothers, regardless of the type of diet, presented hyperlipidemia as confirmed by the greater triglyceride, total cholesterol, VLDL-cholesterol concentrations. This result indicates that the maternal diabetes-induced fetal programming, which was a main responsible for abnormal lipid metabolism.

Our results show that the diabetic intrauterine environment and postnatal HFD consumption were responsible for glucose intolerance in the OC/HFD, OD/SD, and OD/HFD groups, being more exacerbated in the OD/HFD group. Even with the attempt of a compensatory mechanism to increase insulin synthesis, evidenced by the increase in insulin-immunolabeled cells, to reduce blood glucose levels, there was no change in the condition of glucose intolerance in the offspring, suggesting that rats of diabetic mothers, fed or not with HFD, may be presenting damage to the insulin secretion process. This can be confirmed by the higher levels of triglycerides and cholesterol, leading to a lipotoxicity, lower serum concentration of insulin and HOMA-β, showing dysfunction of beta-pancreatic cells even with insulin synthesis is increased. This fact may be related to the increased free fatty acid (FFAs) levels, which is related to an impaired insulin secretion [71].

Elevated levels of plasma FFAs are observed in obese and/or diabetic type 2 patients [72] and also when there is an increase in

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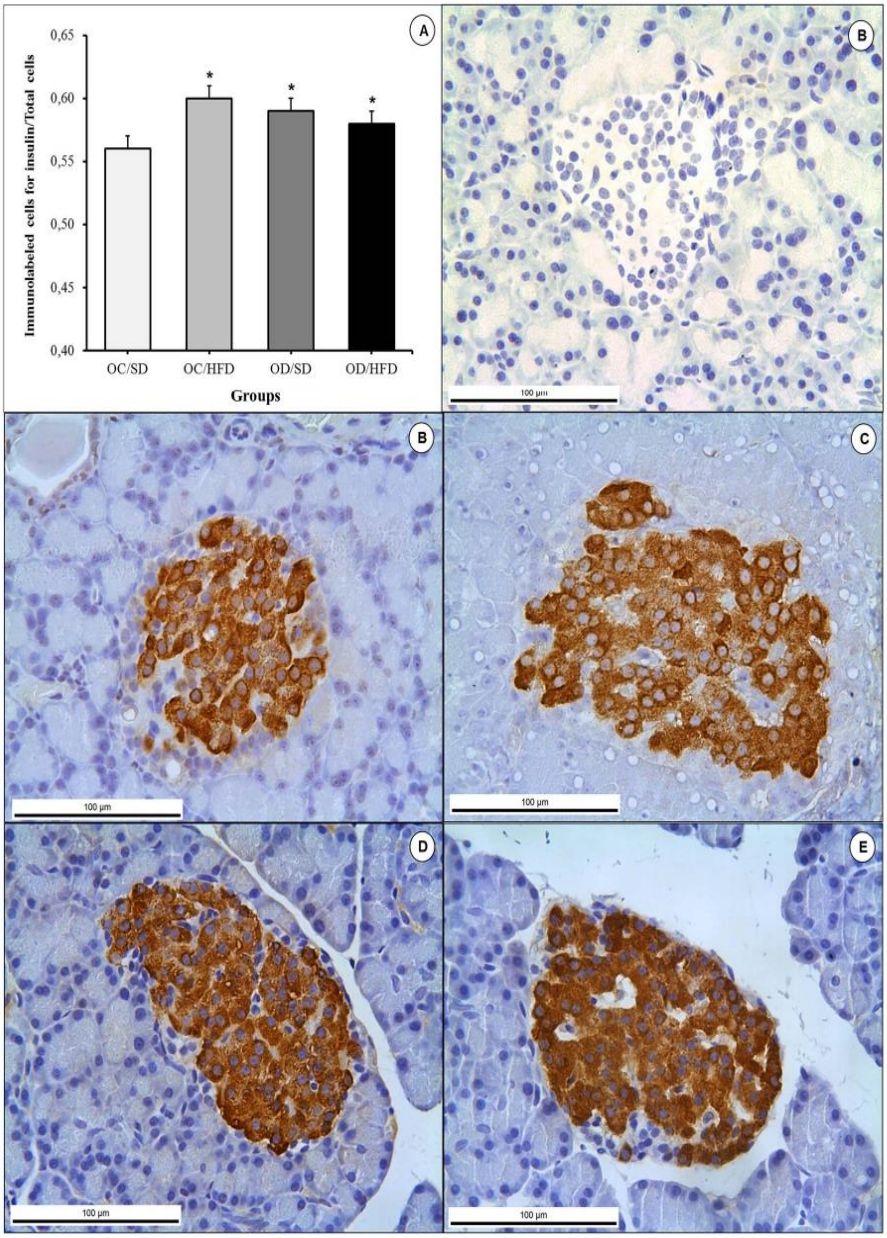


Figure 6. Insulin-immunolabeled pancreatic islet (A) of female offspring from OC or OD mothers given an SD or HFD after weaning up to day 120 of postnatal life. B—Negative control; C—OC/SD group; D—OC/HFD group; E—OD/SD group; F—OD/HFD group. Magnification: 40×. *Values are expressed as mean± SD. n= 100 islets/group.* $^{*}P < 0.05$*—compared with the group OC/SD (One-way ANOVA test followed by Tukey's Multiple Comparison Test).*

dietary lipid intake [73]. Fat is normally stored in adipocytes in the form of triglycerides, but when plasma levels of FFAs are too high, adipocytes are overloaded and fat accumulates in the cells of other organs such as pancreas [74, 75]. Although we have not evaluated the FFAs, the literature shows that diabetic animals and animals that consumed HFD have increased FFA and can compromise insulin secretion, thus ultimately leading to the destruction of β-cells [71, 76, 77].

It is suggested that HFD is not a determining factor for this β-cell dysfunction in the offspring of diabetic rats. Therefore, the main interference was fetal programming induced by maternal intrauterine hyperglycemia. Although the HOMA-IR index did not change,

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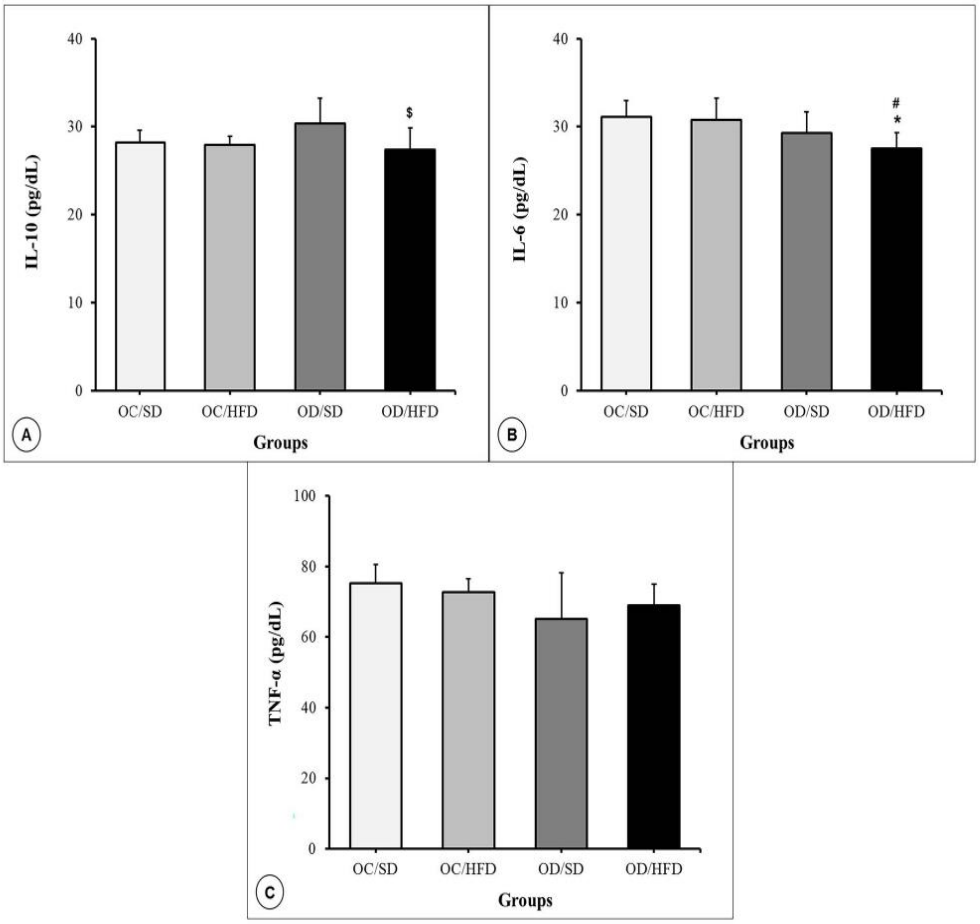


Figure 7. Cytokine concentrations [A—Interleukin-10 (IL-10), B—Interleukin-6 (IL-6), and C—Serum Tumor Necrosis Factor-alpha (TNF-α)] at day 120 of life of female OC mothers or OD mothers and received an SD or HFD after weaning up to day 120 of postnatal life. *Values were expressed as mean ± SD. n = 10 rats/group. * $P < 0.05$—compared with the group OC/SD; # $P < 0.05$—compared with the group OC/HFD; • $P < 0.05$—compared with the group OD/SD (One-way ANOVA test followed by the Tukey's Multiple Comparison Test).*

insulin resistance was confirmed by the TyG index. It is important to emphasize that the TyG index takes into account the relationship between an increase in triglyceride levels and an increase in glucose levels, and studies have already demonstrated its efficacy as more tangible marker for metabolic syndrome and insulin resistance being better than markers already widely used, such as HOMA-IR [78], which can be a better indicator of insulin resistance in the liver [79, 80]. These results are important because although the statement of insulin resistance seems contradictory, since there is a serum decrease in insulin in the OD/HFD group, the fact that there is β-cell dysfunction shows that in our experimental model, OD/SD and OD/HFD animals have passed the resistance phase and are already in pancreatic failure.

The β-cell dysfunction may also be related to diabetic status regardless of insulin resistance [81]. In addition, it was suggested that increased glucose levels could activate the hexosamine pathway, resulting in excess generation of reactive oxygen species and the inhibition of insulin gene transcription and insulin secretion [82, 83]. This information corroborates our results since adult animals from diabetic rats presented oxidative stress (unpublished data).

Metabolic disorders are known to induce low-grade inflammation during the development [84, 85] and this condition causes a change in the production of inflammatory cytokines related to obesity, insulin resistance, and diabetes [86]. In addition, inflammation is one of the consequence of lipotoxicity [75]. In our study, there were no changes in TNF-α concentrations, but the OD/HFD group showed a decreased in IL-10 and IL-6 concentration. There is evidence showing that IL-6 presents an ambivalent role in the development of metabolic disorders [87]. Studies show mice with deleted IL-6 develop inflammation [88–90]. HFD-fed IL-6 knockout rats presented a worse insulin resistance and cardiac lipotoxicity [88], glucose intolerance, insulin resistance, and increased liver inflammation [91], suggesting that IL-6 can mitigate metabolic disorders and organ damage. However, the functions of IL-6 in HFD exposure are not yet fully understood.

Although a more evident response was expected with the association of hyperglycemic intrauterine life and consumption of an

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HFD, which did not happen, this can be explained by the predictive adaptive response [92, 93]. It is a form of phenotypic plasticity against nutritional and energetic intrauterine conditions that confer a short-term survival advantage when environmental conditions remain similar to that of the previous ones. However, with increasing longevity, many diseases can arise from this adaptive response [92]. Since these changes were found in female rats of reproductive age, there may be future impairment in the reproductive process of these animals.

Thus, our findings show that models of laboratory animals submitted to hyperglycemia present metabolic changes that are also observed in the future generation, regardless of the presence of an HFD. In conclusion, fetal programming caused by maternal hyperglycemia is a determinant for causes glucose intolerance and beta-pancreatic cell dysfunction in offspring at adulthood, regardless of the type of feeding submitted from weaning to adulthood. The exposure to an HFD is more related to increased adiposity, abnormal insulin secretion, and immune responses in adult offspring. In addition, both interferences (hyperglycemic intrauterine environment and inappropriate postnatal diet) negatively exacerbate the glucose tolerance, insulin secretion, and immunological profile. More studies might be conducted to explore other consequences in different organs and tissues related to these insults as our research group is leading to better understand the pathophysiological mechanisms involved in this cascade of transgenerational effects.

Supplementary material

Supplementary material is available at *BIOLRE* online

Data availability

All data are incorporated into the article and its online supplementary material.

Authors' contributions

V.G.P.—Conception and design, data acquisition, data analysis and interpretation, manuscript writing; Y.K.S.—Conception and design, data acquisition, data analysis and interpretation, manuscript writing; R.Q.M.S.—Data acquisition and interpretation; T.S.S.—Data acquisition and interpretation; F.Q.G.S.—Data acquisition and interpretation; B.K.—Manuscript writing and English review; A.M.A.P.—Data analysis and interpretation, manuscript writing; J.E.C.—Data and statistical analysis; D.C.D.—Data analysis and interpretation, manuscript writing; G.T.V.—Conception and design, data acquisition, data analysis and interpretation, manuscript writing.

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Conflict of interest

No competing interests declared.

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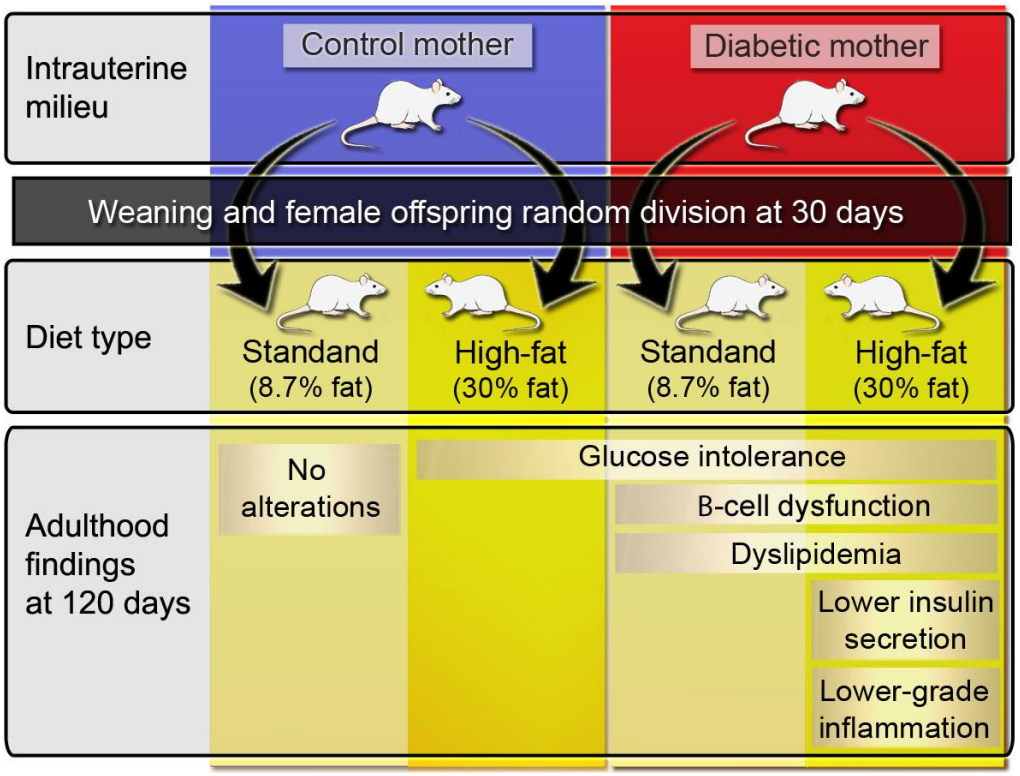
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On behalf of the SSR Publications Committee, I am pleased to inform you that your graphical abstract for your paper "Metabolic Changes in Female Rats Exposed to Intrauterine Hyperglycemia and Post-weaning Consumption of High-Fat Diet" was selected through our online contest as the **"People's Choice" winner** in Biology of Reproduction's 2022 Graphical Abstract contest.

Your abstract was rated highly for its effectiveness in conveying a meaningful scientific message, and visual appeal. It is an example of excellence for graphical abstracts in our journal.



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Research Article

Oxidative Stress Profile of Mothers and Their Offspring after Maternal Consumption of High-Fat Diet in Rodents: A Systematic Review and Meta-Analysis

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Maternal exposure to the high-fat diet (HFD) during gestation or lactation can be harmful to both a mother and offspring. The aim of this systematic review was to identify and evaluate the studies with animal models (rodents) that were exposed to the high-fat diet during pregnancy and/or lactation period to investigate oxidative stress and lipid and liver enzyme profile of mothers and their offspring. The electronic search was performed in the PUBMED (Public/Publisher MEDLINE), EMBASE (Ovid), and Web of Science databases. Data from 77 studies were included for qualitative analysis, and of these, 13 studies were included for meta-analysis by using a random effects model. The pooled analysis revealed higher malondialdehyde levels in offspring of high-fat diet groups. Furthermore, the pooled analysis showed increased reactive oxygen species and lower superoxide dismutase and catalase in offspring of mothers exposed to high-fat diet during pregnancy and/or lactation. Despite significant heterogeneity, the systematic review shows oxidative stress in offspring induced by maternal HFD.

1. Introduction

During gestation, the developing fetus is totally dependent on the maternal environment for nutrition [1]. The intrauterine environment is a crucial determinant in the fetal programming of chronic diseases in adulthood. This concept is called Fetal Origin of Adult Diseases (FOAD) [2]. However, after several studies, this term has been extended to DOHaD (Developmental Origins of Health and Disease) [3] and encompasses from the pregestational state (oocytes), gestation, and postnatal periods involving the entire period of postnatal development and maturation from childhood to adolescence [4] although it is controversial. There is also other evidence about these periods which the DOHaD includes that spread worldwide through the "First 1000 Days" campaign, which supports the importance of the nutritional status of infants and nursing mothers during the fetal and neonatal periods until two years after birth comprising between 280 days before birth and approximately 730 infantile days after birth [5]. Although there is no single consensus, research-involving DOHaD thematic purposes to raise awareness about nutrition and health have been investigated [4].

According to the World Health Organization (WHO), malnutrition refers to deficiencies, excesses, or imbalances in a person's intake of energy and/or nutrients [6], leading to undernutrition or overnutrition [7]. The population is leaving traditional diets that are rich in fibers and grain for diets that include increased levels of sugars, oil, and animal fats [8]. There are five times more obese than malnourished adult people worldwide [6].

The excess of high-fat diet (HFD) consumption is associated with the establishment of permanent state of inflammation [9] and an increased availability of some nutrients, such as free fatty acids, and the glucose overloads the whole cascade of the electron transport chain and consequently increases the production of reactive oxygen species [9, 10]. The increased oxidative environment can be a vicious cycle between inflammatory processes [11]. These disorders in the organism can contribute to the establishment of metabolic diseases [9, 11]. Furthermore, the excessive ROS causes cumulative oxidative damage to macromolecules, including DNA, proteins, and membrane lipid [12].

Maternal consumption of HFD is an important factor that causes harm to both mothers and their offspring [13, 14]. In the last decades, epidemiological evidence has shown that intrauterine life conditions influence growth, body composition, and the risk of developing chronic diseases [15]. Animal studies also indicate that overnutrition during pregnancy induces phenotypic changes that can enhance susceptibility to diseases in adult offspring [16, 17], such as hyperglycemia [18], obesity [19–21], and metabolic syndrome [22]. The maternal HFD consumption also causes oxidative stress on offspring [23, 24]. However, the mechanisms are largely unknown. Lin et al. [25] suggested that the maternal redox state affects the placenta and consequently the fetal development changing transcription factors and abnormal gene expression of antioxidant defenses of the fetuses. In addition, the adverse effects of oxidized molecules, such as lipids and proteins, at critical windows of the fetal development (prenatal or postnatal) "program" the susceptibility to the metabolic syndrome [23].

Epidemiological studies in humans are limited in their ability to assess the influence of diet during pregnancy to offspring phenotype because it is difficult to distinct the effects of intrauterine and post-natal maternal exposure and genetic factors [24]. Therefore, research involving adequate experimental models is relevant, not only for ethical reasons but also due to uncontrollable variables, such as lifestyle, socioeconomic, nutritional, and genetic factors. Hence, the objective of this systematic review was to identify and evaluate the studies with animal models (rodents) that were exposed to the HFD during pregnancy and/or lactation period to investigate oxidative stress of mothers and their offspring.

2. Methods

2.1. Literature Search. This systematic review was undertaken in accordance with the PRISMA [26] and registered on PROSPERO-International Prospective Register of Systematic Reviews (Protocol number CRD42019120418). The literature search was performed on April 30, 2020, on titles, abstracts, and keywords in PUBMED (Public/Publisher MEDLINE), EMBASE (Ovid), and Web of Science databases. The following Medical Subject Headings (MeSH) and their synonyms were used in different combinations and variations with the Boolean operators "OR" and "AND" to yield a sensitive and comprehensive, yet relevant collection of possible articles "high-fat diet," "oxidative stress," "triglyceride," "cholesterol," "low-density lipoprotein," "high density lipoprotein," "Alanine transaminase," "alanine aminotransferase," and "rodent" (see Supplementary Table S1 for complete search strategy). Our primary outcome was to evaluate oxidative stress levels of mothers and their offspring. The secondary outcomes were to investigate the lipid and liver enzyme profile of mothers and their offspring. Besides the electronic search, other sources were used, such as hand searching and screening of reference lists.

Additional records were included from review articles and author-based searches. The searches were restricted to original studies that were published in the English language in scientific journals submitted to the peer-review process without year restriction. Two reviewers (RQMS and VPG) independently screened the titles, abstracts, and full-text manuscripts. Disagreements were resolved in consensus discussions with a third reviewer (DCD).

2.2. Eligibility Criteria. Original animal studies were included in the data set only if they fulfilled the following criteria:

(1) Types of participants: these are rats and mice of any age; nonrodents, spontaneously obese; and genetically modified animals; ex vivo and *in vitro* studies involving human subjects were excluded.

(2) Types of intervention: studies on dams are subjected to an HFD around gestation (before and/or during the whole or any part of pregnancy) or lactation. HFD was considered chow-based HFD from any fat type (e.g., lard and vegetable oils). The % of fat and time of diet exposure were not limited. Custom-made diet (i.e., cafeteria), high-fiber diet, high-calorie diet, high-glucose diet, low-fat diet in short, and any other diets than non-high-fat diet were excluded.

(3) Comparisons: animals that were fed a standard diet were included. The evaluation of articles presenting other forms of manipulation (i.e., surgery, drugs, stress, and exercise) was not considered.

(4) Types of outcome measures: the included primary outcomes were oxidative stress of the dams and their offspring.

(i) Oxidative stress status: malondialdehyde/thiobarbituric acid reactive substances (MDA/TBARS) (lipid oxidation), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, 8-hydroxy-2′-deoxyguanosine (8-OHdG-DNA oxidation), quantification, and scavenging reactive oxygen species (ROS)

Secondary outcomes included the following:

(i) Lipid profile: triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) concentrations

(ii) Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities

2.3. Data Extraction. Pairs of reviewers (RQMS and GV) independently extracted data into an excel spreadsheet. The following information was extracted from studies presenting eligibility criteria: publication characteristics (first author, title, publication year, and journal), animal strain, intervention, and control diets (nutrient content, period and time of administration, proportion of Kcal, and age of the start of intervention), specific methods used for assessment of oxidative stress, lipid and liver enzyme profile, and maternal and offspring outcomes [sample size (n), mean, standard deviation (SD), and standard error (SE)]. When data were provided in graphical images, we extracted data using WebPlotDigitizer 4.2 (https://apps.automeris.io/wpd/). If relevant data were unclear, we contact authors to provide further information.

2.4. Risk of Bias Assessment. Risk of bias for animal studies was assessed using the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE's tool), which was evaluated in ten steps: three of selection (random group allocation, group similar at baseline, and blinded group allocation); two of them on performance (random housing and blinded intervention); two of detection (random and blinded outcome assessment); one of attrition bias (reporting of drop-outs); one reporting (selective outcomes); and one to other potential bias [27]. Included studies were assessed independently by two reviewers (RQMS and GV), and any discrepancies were solved by discussion. The items were classified as low, unclear, or high risk of bias (see Supplementary Figure S2). The score of all the articles was defined as the percentage of 0 to 100% and each category [27]. We assessed the risk of bias of studies included in meta-analysis and did not exclude studies based on high risk of bias.

2.5. Statistical Analysis. Statistical analysis and forest plots were conducted using Review Manager [28]. Studies were considered for meta-analysis if interventions were considered to be similar in terms of period and length of exposure, more than two studies were available, all outcome data could be obtained, and assessment of outcomes were considered comparable. We presented separate pooled effects for dams and their offspring. If a study using the same methods for intervention and control groups reported outcome data separately for sex, the respective groups were pooled using the recommendations in the Cochrane Handbook [29]. When the outcome was measured in different age cohorts, we then considered more than one outcome from the same study. In case of when the outcome was assessed in multiple tissues in the same animal (e.g., blood, liver, and mesentery), only one assessment was included in the meta-analysis to avoid double counting the sample size. We conducted meta-analysis on the levels of oxidative and antioxidative stress markers for continuous variables, and the effect sizes were pooled and presented as standardized mean difference (SMD) since the outcomes were measured in different units across the included studies. Forest plot was generated by the software to illustrate the individual and pooled effect sizes along with 95% confidence interval (CI) using random effects models due to anticipated heterogeneity. The association of percentage of fat and death age and primary outcomes was assessed a using random effects metaregression model. All metaregression results were generated using R version 1.3.1093 (The R Foundation, Vienna, Austria). Between-study heterogeneity was calculated using I^2 statistics, and we considered any degree of heterogeneity. We defined according to I^2 cut-offs of low for <40%, moderate for 30-60-%, substantial for 50-90%, and considerable for >75% [24]. p value less than 0.05 was considered to be statistically significant. Publication bias was not accessed in the included studies because there were an insufficient number of studies for this assessment (i.e., less than 10 studies included in the meta-analysis) [29].

3. Results

3.1. Search Results. Initial electronic searching using three databases yielded a number of 2372 of citations. In addition, 33 articles were added from other sources. The removal of 662 duplicates resulted in 1710 individual articles to be subjected to inclusion and exclusion criteria. Firstly, the inclusion and exclusion criteria were imposed on title and abstract (removal of 1515) and secondly on study design and methods (removal of 119). Finally, 77 citations were selected for review and are shown in Figure 1 [13, 14, 20, 21, 23, 25, 30–100]. Of these studies, 68 evaluated lipid and hepatic enzyme profile and 21 evaluated oxidative stress profile with 12 overlaps (i.e., studies that presented both outcomes).

3.2. Characteristics of Studies That Evaluated Stress Oxidative Profile. The first reports assessing the effects of maternal HFD on oxidative stress of dams and/or offspring were published in 2009 [40]. All studies were published in the last ten years. The characteristics of the selected maternal results are shown in Table 1. Data could be retrieved from 4 studies with six comparisons that provided sufficient data for meta-analysis [14, 25, 44, 47]. Only two rodent species have been used in the included studies: mice (C57Bl/6) [14 48] and rats (Sprague–Dawley and Wistar) [25, 44]. Fat content in maternal HFD was 40% [30], 45% [14], and 49% [44] calories from fat (control group 10 and 11%), and the main source was the animal-derived fats (lard). The duration of the intervention was 19 (pregnancy only) [25], 42 (pregnancy and lactation) [44], 63 (premating period, pregnancy, and lactation) [14], and 113 (premating period, pregnancy, and lactation) [47] days. Feeding was reported as *ad libitum* in the included studies. All studies reported the MDA levels as outcome, one investigated the maternal scavenging capacity on reactive oxygen species [25], and other two studies [44, 47] showed antioxidant enzymes as outcome. Different

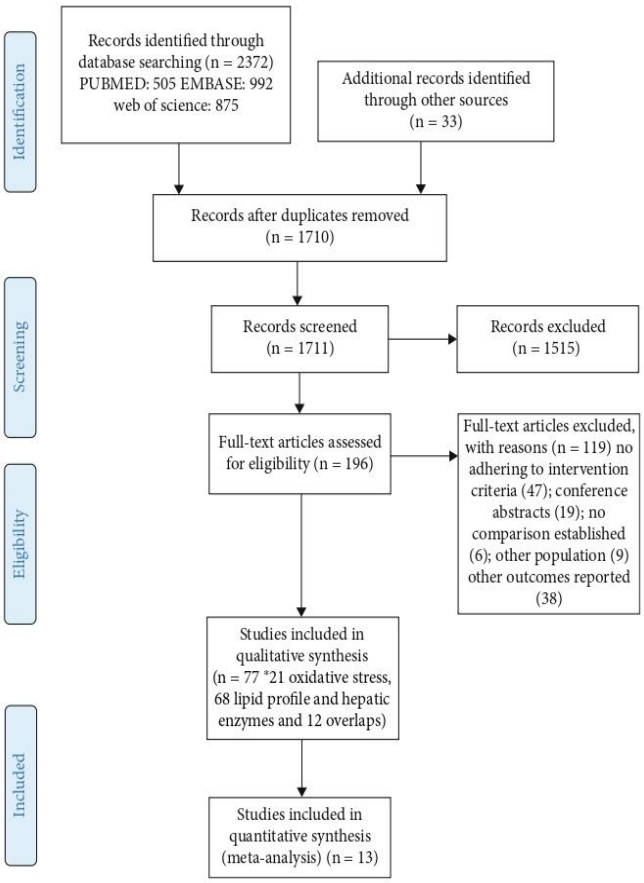


Figure 1: Flow diagram of selection of articles based on PRISM guidelines (http://www.prisma-statement.org).

samples were tested in the included studies, all studies used blood samples [14, 25, 47], two used the liver [14, 25], one used the mammary tissue [47], and one used placenta [25].

Table 2 provides an overview of the study characteristics and outcome measures of the offspring effects. We extracted data from 18 studies that described 49 independent comparisons; however, not all necessary data for meta-analysis could be extracted from papers [23, 25, 30–42, 45, 46]. Out of 18 selected studies, ten [23, 25, 33–38, 45, 46] used rats (four Sprague–Dawley and six Wistar) and eight studies [30–32, 34, 39–41, 43] used mice (C57BL/6). Data from eleven studies were obtained from male offspring [30–33, 37–40, 42, 43, 46], and six studies used groups of mixed sex [25, 34–36, 41, 45], whereas only one study represented data obtained from females [23]. The death age of the offspring was between one day after birth [25] and 650 days old [38, 40]. Among the included studies, there were no consistent patterns with respect to characteristics of HFD. Fat content in maternal HFD ranged from 29% [36] to 62% [41] calories from fat and the control group from 10 to 20%. The lard was the main fat component used by animal-derived fats in the twelve studies [23, 25, 30, 33, 36–39, 41, 43, 45, 46]; other two studies [34, 35] used vegetal oils; and four studies did not report the fat component used [31, 32, 40, 43]. The duration of maternal HFD exposure ranges from 19 [25] to 141 days [38, 45], while the offspring HFD exposure ranges from 1 [25, 35, 40] to 650 postnatal day [38, 45].

The largest number of comparisons was reported on offspring's levels of MDA (36/49), SOD activity (25/49), and GPx (24/49) while a limited number of studies reported comparisons of ROS (14/49), CAT activity (12/49), 8-OHdG (4/49), and Thiols groups (2/49). The oxidative stress levels were evaluated in sixteen (33%) liver samples; blood samples were assessed in fifteen assays (31%). Other samples were also used, three (6%) used sperm, three (6%) used tests, four (8%) used mesentery, two (4%) tested islet, two (4%) used femoral artery, two (4%) tested kidney, one used mesentery vessels (2%), and another one (2%) used cardiomyocytes (Table 2).

3.3. Characteristics of Studies That Evaluated Lipid and Hepatic Enzyme Profile. Supplementary Table S2 shows the period of maternal exposure to diet and the assessments of

Table 1: Maternal oxidative stress repercussions.

References	Animal	Kcal of fat/ main fat source	Maternal HFD consumption (days)	Outcomes of dams						Sample evaluated
				MDA	ROS	SOD	CAT	GPX	Scavenging capacity of reactive oxygen species	
Lin et al., [25][a]	Rats (Sprague–Dawley)	40%/lard	19	↑	NM	NM	NM	NM	↓	Blood
Lin et al., [25][b]	Rats (Sprague–Dawley)	40%/lard	19	↑	NM	NM	NM	NM	↓	Placenta
Gonçalves et al., [44][a]	Rats Wistar	71%/lard	42	↔	NM	↔	↔	NM	NM	Liver
Gonçalves et al., [44][b]	Rats Wistar	71%/lard	42	↔	NM	NM	NM	NM	NM	Blood
Kim et al., [14][a]	Mice (C57BL/6)	45%/lard	63	↑	NM	NM	NM	NM	NM	Blood
Kim et al., [14][b]	Mice (C57BL/6)	45%/lard	63	↑	NM	NM	NM	NM	NM	Liver
Harphoush et al., [47][a]	Mice (C57BL/6)		113	↑	NM	↓	↓	↓	NM	Blood
Harphoush et al., [47][b]	Mice (C57BL/6)		113	↑	NM	↓	↓	↓	NM	Mammary tissue

MDA: malondialdehyde; ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; ROS: scavenging capacity of reactive oxygen species; NM: not measured.

maternal lipid and hepatic biomarkers. The HFD exposure ranges from 19 to 141 days [25, 61]. The biochemical parameters analyzed were TG, TC, HDL, LDL, and ALT. Of the 21 assessments on maternal TG level, 16 (76%) presented increased levels [14, 20, 25, 35, 43, 48, 51, 52–60], four (19%) showed no change [21, 49, 53, 55], and one (5%) showed a decreased level [50]. The maternal TC level was presented in ten evaluations, eight (80%) were increased [14, 20, 21, 40, 56, 60, 61], one (10%) showed no change [55], and another (10%) was decreased [20]. Of the four analyses of maternal HDL, two (50%) presented an increase [56], and the other two (50%) presented no change [14, 25]. Two studies (100%) evaluated maternal LDL assessments and showed higher levels of this biomarker [56]. The only paper with maternal analysis of ALT showed no change [14].

The period of maternal diet exposure, characteristics of offspring (sexes and death age), and biochemical measurements of the offspring are presented in Supplementary Table S3. The HFD exposure ranges from 21 to 154 days [23, 37, 61–64, 74, 88, 89]. In relation to gender, 31 articles verified both genders [21, 35, 36, 41, 50, 54, 57, 58, 60–62, 64–66, 68, 71, 72, 80–82, 84–92, 94, 96, 99]; 23 studies analyzed males [14, 18, 30–33, 37, 40, 42, 48, 51–53, 55, 56, 63, 74, 75, 77, 78, 83, 91, 93, 100], 11 evaluated females [23, 59, 67, 70, 73, 76, 79, 87, 88, 95, 97], and only one did no report on the offspring sex [69]. The ranged age for the offspring was between one day after birth [21, 30, 35, 40, 50, 52, 53, 55, 62, 89] and 360 days old [71]. The observed biochemical parameters were TG, TC, HDL, LDL, ALT, and AST. Of the 141 assessments about TG, 65 (46%) verified higher levels, 71 (50%) showed no change, and five (4%) presented lower levels. Of all 86 evaluations about TC, 21 (24%) showed increased levels, 58 (68%) verified no change, and seven (8%) observed lower concentrations. There were 33 HDL assessments in the offspring. Of these, three (9%) were increased, 26 (79%) presented no abnormal HDL levels, and four (12%) had decreased concentrations. Furthermore, in 20 analyses of LDL of the offspring, seven (35%) presented higher levels, 12 (60%) of them showed no change, and one (5%) observed lower level. The AST enzymatic activity of the liver of the offspring was increased in one article (12.5%), and in seven (87.5%), no change was observed. Of the 11 studies about ALT measurements, four (36%) presented higher activity, and seven of them (64%) had no change. Given the substantial level of heterogeneity in the studies that assess lipid and hepatic enzyme profile, we did not present a quantitative analysis for this outcome.

3.4. Effects of HFD on Stress Oxidative Status in Dams and Offspring. Four studies were included in the meta-analysis on MDA levels in dams that received HFD compared with controls [14, 25, 44, 47]. The MDA levels of included studies were measured from day 19.5 of pregnancy to the end of lactation. The effect size of MDA was not different in mothers exposed to HFD compared to control, SMD 2.15 (95% CI: -0.21 to 4.52, $p < 0.07$; $I^2 = 89\%$) (Figure 2). Two studies were included in the meta-analysis on SOD (SMD: -2.62; 95% CI: -9.15 to 3.90, $p = 0.43$; $I^2 = 94\%$) and CAT (SMD: -0.73; 95% CI: -1.56 to 0.09, $p = 0.08$; $I^2 = 92\%$) were not different in mothers exposed to HFD compared to control (Supplementary Figure S1).

Data on MDA levels of the offspring were available from five studies [23, 37, 42, 43, 45] between 21 and 650 days of life. Two studies were included two times in meta-analysis as the MDA levels were analyzed in two separate age cohorts (90 and 180 days) [23, 37]. Another study was included

Table 2: Oxidative stress repercussions from offspring.

References	Animal	Kcal of fat/main fat source	Maternal HFD consumption (days)	Sex offspring	Death age (days)	Outcomes of offspring							Sample evaluated
						MDA	8-OHdG	ROS	SOD	CAT	GPX	Thiols	
Lin et al., [25]	Rats (Sprague–Dawley)	40%/lard	19	M/F	1	NM	NM	NM	↓	NM	NM	NM	Liver
Emiliano et al., [23][a]	Rats (Wistar)	47%/lard	21	F	90	↔	NM	NM	↓	↓	↔	NM	Blood
Emiliano et al., [23][b]	Rats (Wistar)	47%/lard	21	F	180	↑	NM	NM	↓	↓	↓	NM	Blood
Emiliano et al., [23][c]	Rats (Wistar)	47%/lard	21	F	90	↑	NM	NM	↓	↓	↓	NM	Mesentery
Emiliano et al., [23][d]	Rats (Wistar)	47%/lard	21	F	180	↑	NM	NM	↓	↓	↓	NM	Mesentery
Resende et al., [37][a]	Rats (Wistar)	47.40%/lard	21	M	90	↑	NM	NM	↓	↓	↓	NM	Blood
Resende et al., [37][b]	Rats (Wistar)	47.40%/lard	21	M	180	↑	NM	NM	↓	↓	↓	NM	Mesentery
Resende et al., [37][c]	Rats (Wistar)	47.40%/lard	21	M	90	↑	NM	NM	↓	↓	↓	NM	Blood
Resende et al., [37][d]	Rats (Wistar)	47.40%/lard	21	M	180	↑	NM	NM	↓	↓	↓	NM	Mesentery
Zhang et al., [42][a]	Rats (Sprague–Dawley)	45%/lard	42	M	84	↔	NM	NM	NM	NM	NM	NM	Blood
Zhang et al., [42][b]	Rats (Sprague–Dawley)	45%/lard	42	M	84	↑	NM	NM	NM	NM	NM	NM	Liver
Mdaki et al., [35]	Rats (Sprague–Dawley)	40%/oil vegetable +animal fat	49	M/F	1	↑	NM	NM	NM	NM	NM	NM	Cardiomyocyte
Gray et al., [33]	Rats (Sprague–Dawley)	45%/lard	52	M	140	NM	NM	↔	NM	NM	NM	NM	Mesenteric vessels
Miranda et al., [36][a]	Rats (Wistar)	29%/lard	98	M	180	NM	NM	NM	↓	↓	↓	↓	Liver
Miranda et al., [36][b]	Rats (Wistar)	29%/lard	98	F	180	NM	NM	NM	↓	↓	↔	↔	Liver
Oliveira et al., [46]	Rats (Wistar)	28.6%/lard	98	M	46	NM	NM	NM	↔	↔	↔	NM	Liver
Rodriguez-Gonzalez et al., [38][a]	Rats (Wistar)	46%/lard	141	M	110	↑	NM	↑	↑	NM	↑	NM	Tests
Rodriguez-Gonzalez et al., [38][b]	Rats (Wistar)	46%/lard	141	M	110	↔	NM	NM	↔	NM	↔	NM	Sperm
Rodriguez-Gonzalez et al., [38][c]	Rats (Wistar)	46%/lard	141	M	450	↑	NM	↑	↑	NM	↑	NM	Tests
Rodriguez-Gonzalez et al., [38][d]	Rats (Wistar)	46%/lard	141	M	450	↑	NM	NM	↓	NM	↓	NM	Sperm
Rodriguez-Gonzalez et al., [38][e]	Rats (Wistar)	46%/lard	141	M	650	↑	NM	↑	↑	NM	↑	NM	Tests
Rodriguez-Gonzalez et al., [38][f]	Rats (Wistar)	46%/lard	141	M	650	↑	NM	NM	↓	NM	↓	NM	Sperm
Rodriguez-Gonzalez et al., [38][a]	Rats (Wistar)	46%/lard	141	M	110	↑	NM	NM	NM	NM	NM	NM	Blood

Table 2: Continued.

References	Animal	Kcal of fat/main fat source	Maternal HFD consumption (days)	Sex offspring	Death age (days)	Outcomes of offspring							Sample evaluated
						MDA	8-OHdG	ROS	SOD	CAT	GPX	Thiols	
Rodriguez-Gonzalez et al., [38][b]	Rats (Wistar)	46%/lard	141	M	450	↑	NM	NM	NM	NM	NM	NM	Blood
Rodriguez-Gonzalez et al., [38][c]	Rats (Wistar)	46%/lard	141	M	650	↔	NM	NM	NM	NM	NM	NM	Blood
Rodriguez-Gonzalez et al., [38][d]	Rats (Wistar)	46%/lard	141	F	110	↔	NM	NM	NM	NM	NM	NM	Blood
Rodriguez-Gonzalez et al., [38][e]	Rats (Wistar)	46%/lard	141	F	450	↑	NM	NM	NM	NM	NM	NM	Blood
Rodriguez-Gonzalez et al., [38][f]	Rats (Wistar)	46%/lard	141	F	650	↔	NM	NM	NM	NM	NM	NM	Blood
Rodriguez-Gonzalez et al., [38][g]	Rats (Wistar)	46%/lard	141	M	110	↑	NM	↔	↔	NM	↑	NM	Liver
Rodriguez-Gonzalez et al., [38][h]	Rats (Wistar)	46%/lard	141	M	450	↑	NM	↑	↔	NM	↓	NM	Liver
Rodriguez-Gonzalez et al., [38][i]	Rats (Wistar)	46%/lard	141	M	650	↑	NM	↑	↔	NM	↓	NM	Liver
Rodriguez-Gonzalez et al., [38][j]	Rats (Wistar)	46%/lard	141	F	110	↑	NM	↔	↑	NM	↔	NM	Liver
Rodriguez-Gonzalez et al., [38][k]	Rats (Wistar)	46%/lard	141	F	450	↑	NM	↑	↔	NM	↔	NM	Liver
Rodriguez-Gonzalez et al., [38][l]	Rats (Wistar)	46%/lard	141	F	650	↑	NM	↑	↓	NM	↓	NM	Liver
c[a]	Mice (C57BL/6)	45%/unidentified	42	M	21	↑	NM	↑	NM	NM	NM	NM	Liver
Cao et al., [43][b]	Mice (C57BL/6)	45%/unidentified	42	M	84	NM	NM	↔	NM	NM	NM	NM	Liver
Ito et al., [34][a]	Mice (C57BL/6)	31%/oil vegetable +animal fat	42	M/F	21	↔	NM	NM	NM	NM	NM	NM	Liver
Ito et al., [34][b]	Mice (C57BL/6)	31%/oil vegetable +animal fat	42	M/F	77	↔	NM	NM	NM	NM	NM	NM	Liver
Yokomizo et al., [41][a]	Mice (C57BL/6)	62.20%/lard	42	M	140	NM	↑	NM	NM	NM	NM	NM	Islet
Yokomizo et al., [41][b]	Mice (C57BL/6)	62.20%/lard	42	F	140	NM	↔	NM	NM	NM	NM	NM	Islet
Torrens et al., [39][a]	Mice (C57BL/6)	45%/lard	70	M	105	NM	NM	↑	NM	NM	NM	NM	Femoral artery
Torrens et al., [39][b]	Mice (C57BL/6)	45%/lard	70	M	210	NM	NM	↑	NM	NM	NM	NM	Femoral artery
Tozuka et al., [40][a]	Mice (C57BL/6)	57.50%/unidentified	79	M	1	↔	NM	NM	NM	NM	NM	NM	Blood
Tozuka et al., [40][b]	Mice (C57BL/6)	57.50%/unidentified	79	M	10	↑	NM	NM	NM	NM	NM	NM	Blood
Tozuka et al., [40][c]	Mice (C57BL/6)	57.50%/unidentified	79	M	21	↑	NM	NM	NM	NM	NM	NM	Blood
Tozuka et al., [40][d]	Mice (C57BL/6)	57.50%/unidentified	79	M	70	↔	NM	NM	NM	NM	NM	NM	Blood

Table 2: Continued.

References	Animal	Kcal of fat/main fat source	Maternal HFD consumption (days)	Sex offspring	Death age (days)	Outcomes of offspring							Sample evaluated
						MDA	8-OHdG	ROS	SOD	CAT	GPX	Thiols	
Glastras et al., [32]	Mice (C57BL/6)	43%/unidentified	84	M	224	NM	↑	NM	NM	NM	NM	NM	Kidney
Glastras et al., [31]	Mice (C57BL/6)	43%/unidentified	84	M	224	NM	↑	NM	NM	NM	NM	NM	Kidney
Bringhenti et al., [30]	Mice (C57BL/6)	49%/lard	98	M	10	NM	NM	NM	↓	↔	↔	NM	Liver

MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; NM: not measured.

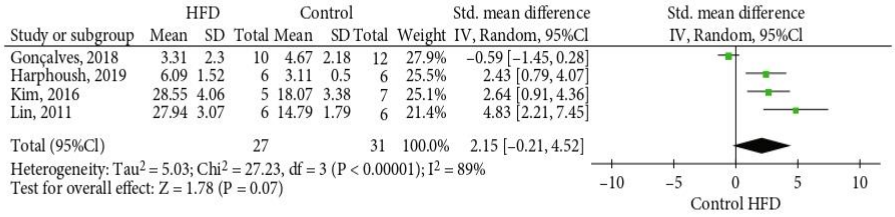
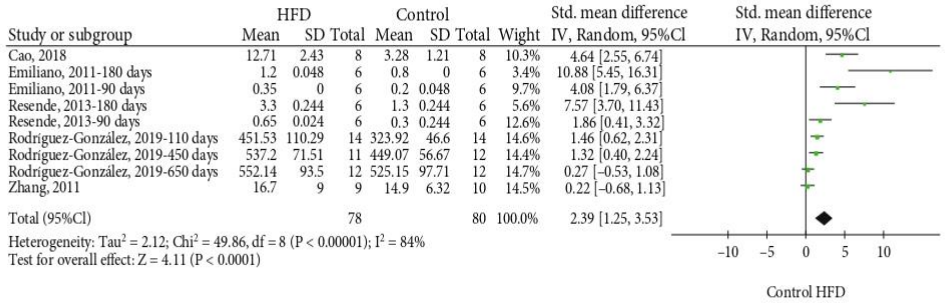
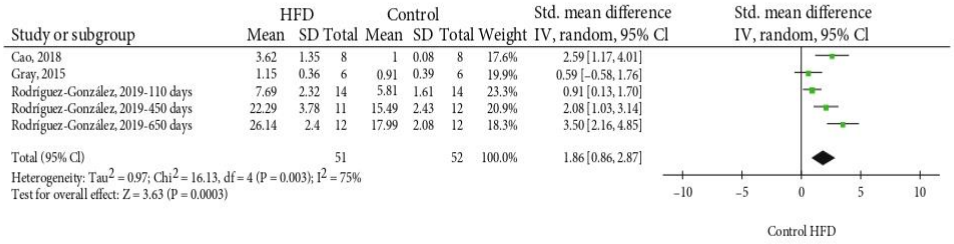


FIGURE 2: Meta-analysis of HFD maternal consumption on MDA levels compared with controls. HFD: high-fat diet; 95% CI: 95% confidence interval; IV: inverse variance.



(a)



(b)

FIGURE 3: Meta-analysis of MDA levels (a), ROS levels (b) of the offspring from mothers exposed to HFD. HFD: high-fat diet; 95% CI: 95% confidence interval; IV: inverse variance.

according to the separate age cohorts (110, 450, and 650) [40]. The MDA levels of the offspring were higher in dams exposed to HFD compared to a standard diet (SMD 2.39) (95% CI: 1.25 to 3.53, $p < 0.0001$; $I^2 = 84\%$) (Figure 3(a)).

The evaluation of ROS occurred in three studies [33, 43, 45] between 21 and 650 days of life [33, 43, 45]. One study was included three times due to assessment in different age cohorts (110, 450, and 650 days) [40]. The ROS assessment of the offspring was higher in dams exposed to HFD compared to a standard diet (SMD 1.86) (95% CI: 0.86 to 2.87, $p = 0.0003$; $I^2 = 75\%$) (Figure 3(b)).

SOD activity was obtained from seven studies between 1 and 650 days of life [25, 30, 46]. Two studies were included two times in meta-analysis as the MDA levels were analyzed in two separate age cohorts (90 and 180 days) [23, 37]. Furthermore, another study was included according to the separate age cohorts (110, 450, and 650) [45]. SOD activities were decreased in the offspring of dams exposed to HFD compared to standard diet. The effect size was -2.11 (95% CI: -3.23 to -0.99, $p < 0.0002$; $I^2 = 87\%$) (Figure 4(a)).

10 comparisons were included the GPX activity analysis from six studies [23, 30, 36, 37, 45, 46] between 10 and 650 days of life. [30, 36, 46]. Two studies were included two times in meta-analysis because GPX activity was detected in different age cohorts (90 and 180 days) [23, 38]. Another study was stratified according to separate age cohorts (110, 450, and 650 days) [41]. GPX activity was not different in the offspring of dams exposed to HFD compared to those of dams given a standard diet. The effect size was -0.69 (95% CI: -1.56 to 0.18, $p = 0.12$; $I^2 = 84\%$) (Figure 4(b)).

CAT activity was obtained from five studies between 10 and 180 days of life [23, 30, 36, 37, 46]. Two studies were included two times in meta-analysis since they detected the CAT activity in different age cohorts (90 and 180 days) [23, 37]. CAT activity was lower in the offspring of dams exposed to HFD compared to those of mothers given a standard diet. The effect size was -1.17 (95% CI: -2.32 to -0.02, $p = 0.05$; $I^2 = 82\%$) (Figure 4(c)).

Eight studies could not be included in the pooled analysis because of the lack of information of sample size [30–34,

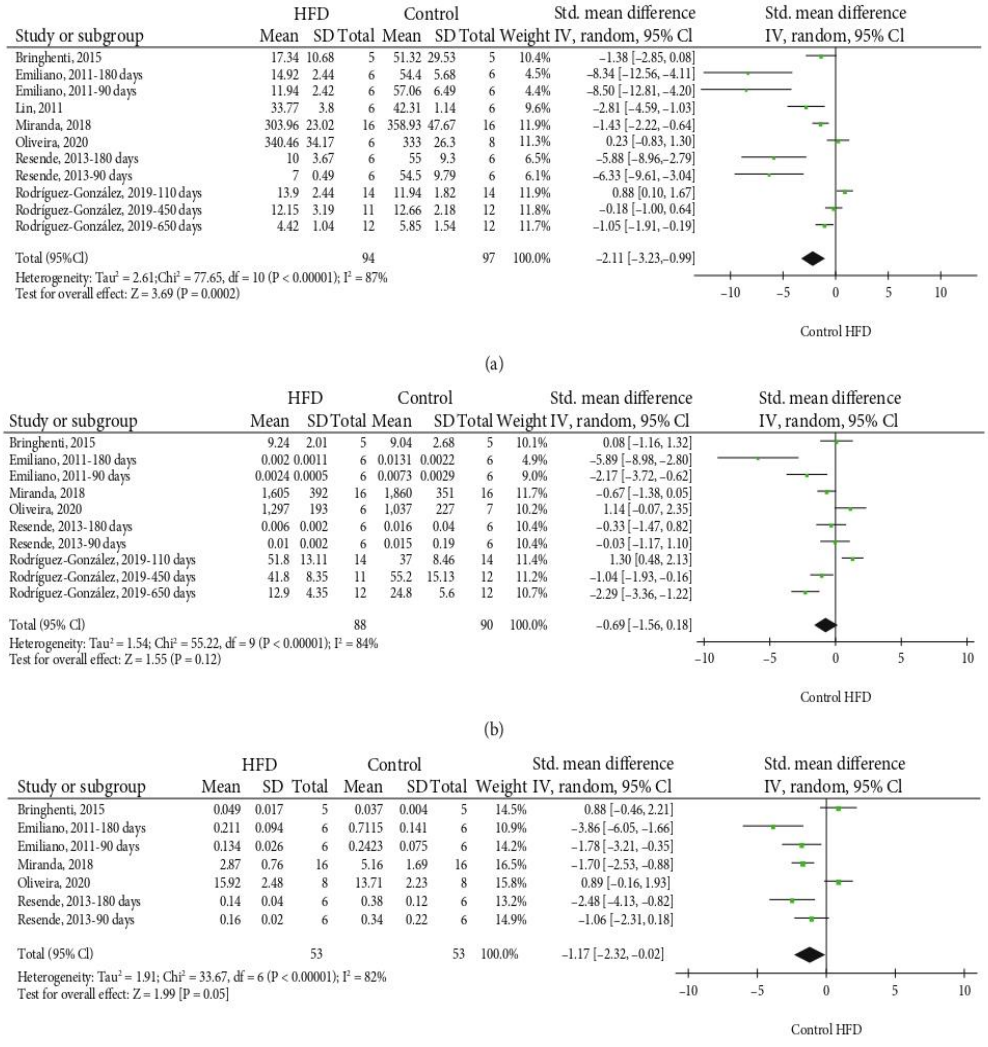


FIGURE 4: Meta-analysis of SOD activity (a), GPx activity (b), and CAT activity (c) of the offspring from mothers exposed to HFD. HFD: high-fat diet; 95% CI: 95% confidence interval; IV: inverse variance.

35, 38–41]. One study could not be pooled as it was the single study that evaluate the 8OHdG levels showed higher levels in male offspring of mothers exposed to HFD during pregnancy and lactation (SMD: 2.83; 95% CI: 1.04 to 4.62, $p = 0.002$); however, the female offspring comparison showed no difference between group (SMD: 0.51; 95% CI -0.65 to 1.66, $p = 0.39$) [41].

3.5. Metaregression. The results of metaregression show that with increasing of age, the GPx ($p = 0.0032$) and CAT ($p < 0.0001$) levels significantly decrease and the ROS levels significantly increase ($p = 0.047$) (Table 3).

3.6. Risk of Bias in Studies. All included studies were assessed on risk of bias. The results can be found in Figure 5 and in Supplementary Figure S2 in the supplemental material in more detail. Following the results of the SYRCLE Risk of Bias tool, most of the included papers had an overall unclear risk of bias because of poor or even absence of reporting of essential information. Information about random sequence generation was absent in four studies (19% high risk, 81% unclear risk). Although 14 of the included studies reported the baseline characteristics (14 studies—67% were low risk), and seven studies omitted this information (33% were unclear risk). There is no description about concealment of the allocation sequence in the included studies (21 studies—100% were unclear risk). Information about performance bias, such as animals randomly housed, were unclear in 21 studies (21 studies—100% were unclear risk); care and blinded investigation of intervention/exposure of each animal was deficient (21 studies—100% of them were unclear risk). Furthermore, detection bias was considered

Table 3: Summary of findings of metaregression analysis across all offspring outcomes.

Outcome	Covariate	Number of comparison	Coefficient	95% CI		SE	z	p value	R^2	I^2
MDA	% fat	12	0.572	-0.0898	1.2337	0.3376	1.6941	0.0902	0	77.20%
	Death age	12	-0.0037	-0.008	0.0006	0.0022	-1.6906	0.0909	0	78.88%
SOD	% fat	15	-0.0637	-0.1945	0.0671	0.0667	-0.9542	0.34	0	84%
	Death age	15	0.002	-0.0024	0.0064	0.0023	0.8852	0.376	0	84.00%
GPx	% fat	14	-0.0392	-0.1502	0.0718	0.0567	-0.6922	0.4888	0	81.32%
	Death age	14	-0.0052	-0.0087	-0.0018	0.0018	-2.9465	0.0032	37.38%	71.40%
CAT	% fat	8	-0.0226	-0.1511	0.106	0.0656	-0.3442	0.7307	0	82.86%
	Death age	8	-0.0207	-0.0288	-0.0126	0.0041	-5.0183	<0.0001	96.93%	10.79%
ROS	% fat	8	0.543	-1.6153	2.7013	1.1012	0.4931	0.622	0	69.65%
	Death age	8	0.0035	0	0.0069	0.0017	1.9866	0.047	32.96%	55.47%

CI: confidence interval; SE: standard error; HFD: high-fat diet.

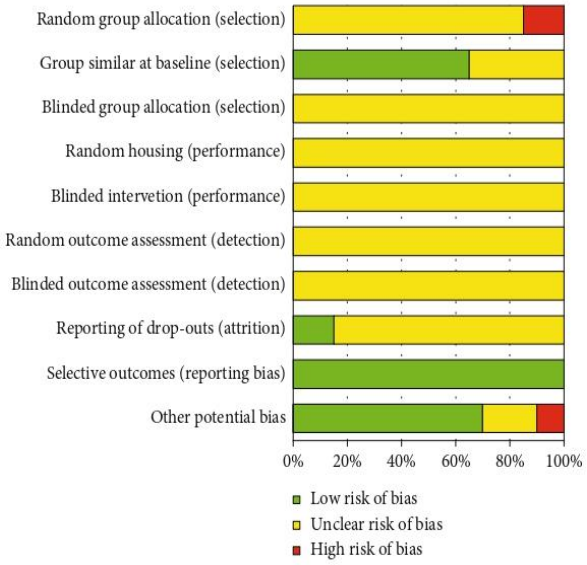


Figure 5: Risk of bias score for each risk item in animal studies, as assessed using the SYRCLE tools.

unclear risk due to no information of random selection for outcome assessment and blinding outcome assessor (21 studies—100%). While more than 86% (18 studies were unclear risk) of the not included studies reported unclear attrition bias, only 14% correctly described incomplete outcome data (3 studies were low risk). The reporting bias was classified as low risk (21 studies—100%). A low risk of other bias was scored for fifteen studies (71%), the other four studies were unclear (19%), and two studies showed conflict of interest (10% were high risk).

4. Discussion

4.1. Overview of Findings. This review is aimed at studying and summarizing the literature regarding oxidative stress and lipid and hepatic profile induced by maternal high-fat diet (HFD) consumption on the dams and their offspring. We found 68 studies that evaluated lipid and hepatic enzymatic profile and 21 studies about the effects of HFD on oxidative stress markers in rats and mice. Our pooled analysis of oxidative stress levels suggests that HFD consumption during pregnancy and/or lactation significantly increases MDA levels in dams and their offspring. Furthermore, there were increased ROS and decrease of SOD, GPx, and CAT activities in the offspring. Another factor that can influence the results is age of descedants; older animals have decreased in antioxidant enzymes activities and increased ROS. These studies included pooled analysis; all offspring were fed a standard diet after weaning, so the changes observed in the levels of stress oxidative markers were independent on offspring's diet. Systematic reviews are commonly used for human studies [101, 102]. However, in reviews using animal

models, predominantly, rodents have been highlighted [103–109]. Rodents (mice and rats) are ideal models to induce metabolic alterations [107] and suitable for exploring the mechanisms related to DOHaD [108]. Considering these and other advantages, rodents were employed in this review.

The HFD exposure is associated with dyslipidemia, which involves higher triglycerides, total cholesterol, and LDL concentrations, as well as a reduction in HDL-cholesterol levels [109, 110]. Furthermore, the excessive energy and hypertriglyceridemia can cause the increased hepatic content of triglycerides in the liver [36, 113]. Then, an overload in the liver results in increased ALT and AST activities [112, 113], which are enzymatic biomarkers of the hepatic damages [36]. After the consumption of HFD, the mothers showed abnormal lipid profile, and most studies reported increased TG and TC concentrations. However, the results in offspring are divergent in relation to these biomarkers. The heterogeneity of the experimental design (such as time of maternal exposure to HFD, amount of fat in the feed, and age of offspring death) was a determining factor for the divergence of the outcomes found in these studies. Therefore, it was not possible for the meta-analysis to be performed. We found that LDL, HDL, ALT, and AST levels were lower in the studies using HFD. Although the consumption of HFD can lead to dyslipidemia and liver damage, it was not evident in most studies. However, it is possible that the association of these biomarkers and the model used are not sufficient to translate the damage caused by maternal consumption of HFD.

To establish a broad search, there was no year limitation of the included studies. In this context, the articles were related to the last ten years and this might be explained because the investigations on developmental plasticity and fetal programming have been started in the last years [3, 114].

4.2. Variability of Diets Used in the Researches. In this review, only articles that used diets with a higher fat content than the control group were evaluated. The major source was lard, which mainly consists of nonessential fatty acids [115]. Some studies have used plant-originated fat, which contains essential fatty acids (polyunsaturated) [116, 117]. According to Tellechea et al. [115], maternal exposure to the diet rich in lard is directly related to metabolic syndrome-related phenotypes in offspring rats. Besides that, essential fatty acids contain fundamental nutrients to fetal and postnatal development and normal cell function [118]. However, an excess may injure and have adverse consequences to offspring [118]. The different sources, concentrations, and periods of exposure to HFD might be responsible for the heterogeneity of results on reproductive and biochemical parameters [107]. Several authors present the energy from fat (% Kcal) and others in centesimal composition. For this review, to standard these comparisons, the fat values were presented in % Kcal. Considering that carbohydrate provides four calories/gram, protein provides four calories/gram and fat provides nine calories/gram; these values were considered in our review [119].

4.3. Oxidative Stress. The redox status, nutritional and environmental factors play an important role in the susceptibility to oxidative stress and other metabolic alterations [120]. Oxidative stress occurs due to increased production of reactive oxygen species (ROS) and/or failure of the antioxidant system [30]. In our meta-analyses, this imbalance was observed in offspring, in which malondialdehyde (MDA) and ROS were shown to be elevated and the antioxidant enzymes decreased. In the qualitative analysis, MDA, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and ROS were analyzed as the prooxidants or lipid peroxidation products included in this review. MDA is the final product of lipid peroxidation measured by the quantification of thiobarbituric acid reactive substances (TBARS) [121]. 8-OHdG is one of the major products of DNA oxidation [122]. These biomarkers represent a detrimental environment for both mothers and their offspring [123–125]. The association between HFD and higher prooxidant levels can be explained by endothelial dysfunction [35] and increased inflammatory process [31, 42].

The enzymatic antioxidant system composed of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), the three main endogenous antioxidants, is triggered according to the organism requirement to protect itself against the oxidative insult caused by maternal HFD exposure [23]. The lower antioxidant profile observed in this meta-analysis might be due to the enzymatic rapid consumption and depletion [126]. The reduction of antioxidants represents an attempt to stabilize ROS [127].

The balance between prooxidants and antioxidants is the key of organism homeostasis, but there are several factors to be considered with aging and senescence that stress is directly associated with phenomenon of oxidative stress [128]. The exact mechanism of oxidative stress-induced aging is still not clear, but probably increased ROS levels lead to cellular senescence, a physiological mechanism that stops cellular proliferation in response to damages that occur during replication [129]. Furthermore, according to the results of this review, we believe that intrauterine insults may highlight oxidative stress in aging.

It is important to note that several outcomes are evaluated in blood samples for biochemical analysis (plasma or serum) [14, 23, 25, 40, 42]. Blood is an effective material for the evaluation of biochemical profile because it informs the health state at the collection time [130]. The second type of sample most used is the liver [14, 25, 30, 34, 36, 42]. The hepatic tissue undergoes maturation stages during late gestation and early postnatal life. Hence, the liver is highly susceptible to a maternal inadequate nutrition [131]. There were also few determinations in other samples, such as the mesentery [23, 33, 37], kidney [31, 32], placenta [25], islet [41], sperm and testis [38], cardiomyocytes [35], and femoral artery [39]. The nonuniformity of the samples is related to the objectives of each research.

An inadequate feeding during the prenatal period likely increases the risk to chronic diseases, such as diabetes and metabolic changes, during adult offspring life [132, 133]. The overnutrition during pregnancy is a risk factor for the mother and their offspring because insults may generate later life physiological and metabolic changes in the offspring [134], corroborating the DOHaD theory [3].

4.4. Risk of Bias and Gaps in the Literature. The selected articles were evaluated with an appropriate assessment

instrument for bias risk, which was applied to experimental models [27]. A design with low-risk of bias describes the process of randomization, such as bias origin and their influence in the results [135]. It was verified most articles only cited randomization of animals; however, they correctly described no process. The blindness of researchers and data analysis were also an argument for bias, which was neglected in the studies. This fact probably occurs because of the difficulty for blinding during management with animals and diet. Then, the implementation of more appropriate methodologies could reduce the bias, contributing to improving the reliability and interpretation of results [106].

The contribution of our systematic review was the identification of gap in the existing review about maternal HFD consumption and oxidative stress. Other reviews only show how maternal HFD consumption has an effect on blood glucose [104, 106], body weight [47], metabolic syndrome [116], cardiometabolic parameters [136], and growth [137] of the offspring.

4.5. Limitations and Strengths. In this review, there are methodological limitations of the included studies. Firstly, the complete description of diet composition, in both the control and HFD groups, because they are at times ignored by the authors or not clearly reported. However, by neglecting this information, the investigators hinder the interpretations and make the impractical reproducibility of these studies [138]. Secondly, the selected articles present a variability of the standard diet (control group) characteristics, which causes difficulty for comparison among the experimental groups and control groups from different studies, showing that there is no consensus in the researches involving HFD. The American Institute of Nutrition (AIN) published the use of formula to standard chow for experimental rodents, AIN-93G, which shows all the necessary nutrients to be used during the early growth phase and during reproduction [139]. Despite these limitations, this review presents with strengths, such as an extensive view of the literature. We used different databases with a large number of terms and keywords to increase the number of searches. In addition, we also showed the consequences for both mothers and their offspring with exclusion of confounding postnatal diet effects.

5. Conclusion

The current systematic review suggests that maternal HFD causes oxidative stress in offspring influencing the prooxidants and antioxidants in a mother and offspring. Although the writing of a definitive conclusion is difficult given the substantial heterogeneity found in the included studies, we found that maternal exposure to HFD with 40% fat for 19 days during the pregnancy period can negatively impact the oxidative stress levels in maternal organism, which "programs" the offspring and leads to the inadequate repercussions. When the an evaluation is performed in older offspring (around 90 days old), the time of maternal HFD consumption would need to be a little longer and the amount of fat in the diet would have to approximately be 47% to show effects on oxidative stress levels in offspring and to obtain results with translational importance. Therefore, if the interest is to evaluate the maternal outcomes using HFD on their offspring, it is necessary to introduce this diet with a higher exposure time along with a higher proportion of fat. HFD impairs not only the mother but also the offspring postnatal life during their adulthood. Studies that highlight these findings are important for the development of intervention measures for the treatment/prevention of this condition.

Data Availability

All data is available in Supplementary Materials.

Conflicts of Interest

The authors have declared that no competing interests exist.

Authors' Contributions

RQMS is responsible for the conceptualization, data curation, formal analysis, investigation, methodology, validation, writing—original draft preparation, and writing—review and editing. GV is involved in the conceptualization, data curation, formal analysis, investigation, methodology, and writing—review and editing. VGP and TSS participated in data curation, investigation, and writing—review and editing. YKS contributed in the data curation, investigation, validation, and writing—review and editing. RBG also contributed in validation and writing—review and editing. GTV and DCD are also responsible for the conceptualization, data curation, formal analysis, investigation, methodology, validation, supervision, writing—original draft preparation, and writing—review and editing. All authors read and approved the final manuscript.

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Supplementary Materials

Supplementary 1. Table S1: file search strategy.

Supplementary 2. Table S2: maternal biochemical repercussions.

Supplementary 3. Table S3: biochemical repercussions of offspring.

Supplementary 4. Figure S1 File: meta-analysis of HFD maternal consumption on SOD and CAT activities compared with controls. HFD: high-fat diet.

Supplementary 5. Figure S2 File: risk of bias summary.

Supplementary 6. Figure S3 File: PRISMA 2009 checklist.

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Exposure to maternal hyperglycemia and high-fat diet consumption after weaning in rats: repercussions on periovarian adipose tissue

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Abstract

Clinical and epidemiological studies show that maternal hyperglycemia can change the programming of offspring leading to transgenerational effects. These changes may be related to environmental factors, such as high-fat diet (HFD) consumption, and contribute to the comorbidity onset at the adulthood of the offspring. The objective of this study was to evaluate the hyperglycemic intrauterine environment, associated or not with an HFD administered from weaning to adult life on the periovarian adipose tissue of rat offspring Maternal diabetes was chemically induced by Streptozotocin. Female offsprings were randomly distributed into four experimental groups (n = 5 animals/group): Female offspring from control or diabetic mothers and fed an HFD or standard diet. HFD was prepared with lard enrichment and given from weaning to adulthood. On day 120 of life, the rats were anesthetized and sacrificed to obtain adipose tissue samples. Then, the hyperglycemic intrauterine environment and HFD fed after weaning caused a higher body weight, total fat, and periovarian fat in adult offspring, which could compromise the future reproductive function of these females. These rats showed higher adiposity index and adipocyte area, contributing to hypertrophied adipose tissue. Therefore, maternal diabetes itself causes intergenerational changes and, in association with the HFD consumption after weaning, exacerbated the changes in the adipose tissue of adult female offspring.

Introduction

The prevalence of diabetes in pregnancy has increased worldwide.[1] In 2019, data from the International Diabetes Federation (IDF) showed that one in six pregnancies was affected by diabetes.[2] In addition to the increased prevalence of prepregnancy *Diabetes mellitus* (DM) (type 1 and type 2 DM) in women of reproductive age, there is a dramatic increase in the rates of gestational *Diabetes mellitus* (GDM),[3] with 13.6% of pregnancies affected by pregestational diabetes and 86.4% affected by GDM.[2] Clinical and epidemiological studies show that maternal hyperglycemia causes developmental programming leading to adverse long-term effects through the generations,[2] such as increased adiposity in offspring, regardless of birth weight,[4–6] increased susceptibility to obesity and adverse metabolic risks that can persist for as long as childhood to adulthood of offspring.[7,8] These changes, added to environmental factors such as consumption of a high-fat Western diet and low physical activity, significantly contribute to the increased prevalence of overweight, obesity, type 2 DM, GDM[9–11] and reproductive alterations[12] at the adulthood of offspring, regardless of the type of maternal diabetes (pregestational diabetes or GDM).[13]

Due to several uncontrollable variables and ethical aspects involved in studies in humans, it is necessary to use laboratory animals in an extremely ethical way to mimic the conditions of pregestational diabetes and obesity models in rats. The offspring coming from hyperglycemic intrauterine environment present alterations in the metabolic profile concerning glucose tolerance.[14] The presence of maternal hyperglycemia creates an unfavorable intrauterine environment and interferes with developmental programming. Fat deposits in the periovarian region are widely observed in rodents, but this is not observed in humans, and this fat is highly analogous to the functions of visceral omental fat.[15,16] The fat accumulation or expansion occurs through the process of hyperplasia (increased number of cells) or hypertrophy (increased area/volume of cells) of adipocytes.[17] This accumulation is found in the visceral or subcutaneous regions[18]

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and has cell specificity in each region, being more prone to hyperplasia in the subcutaneous region and hypertrophy in the visceral region.[19]

Hypercaloric or hyperlipidic diet (HFD)-induced obesity in animal models has already proven to be effective in adipose tissue changes.[9,20–22] Mothers fed chronic HFD influences the morphology of visceral white adipose tissue of their offspring (area, diameter, and the number of adipocytes), especially in the subcutaneous region of females, compared to those that maintain the consumption of the diet after birth.[23–25]

Because of the current global scenario in which maternal diabetes can interfere with fetal development plasticity and the consumption of HFD or high-calorie diet has been exponentially increasing, the study of the association of maternal diabetes and HFD consumption is relevant. We hypothesized that the offspring of diabetic mothers and those exposed to an HFD after weaning have changes in the periovarian adipose tissue. Thus, the objective of this study was to evaluate the influence of an HFD after weaning, associated or not with the hyperglycemic intrauterine environment, on the periovarian adipose tissue of adult female rat offspring.

Method

Animals

A total of 52 rats (8 males and 44 females) of the Sprague-Dawley lineage were used, acquired from the Animal Facility of the State University of Campinas (CEMIB/UNICAMP), and kept in the Animal Facility of the Laboratory of Experimental Research in Gynecology and Obstetrics, Botucatu Medical School, Unesp, under controlled conditions of temperature (22 ± 2°C), humidity (60 ± 10%) and light/dark cycle (12 h). Filtered water and food were offered *ad libitum* to the animals, which were kept in a polypropylene cage (three animals/cage) on wood shavings with environment enrichment (paper balls).[26]

Experimental sequence

An experimental sequence of parental generation (mother rats)

The experimental sequence of animals from the first generation aimed at inducing the diabetes status, which was made in females on day 5 of postnatal life (n = 10) using the beta-cytotoxic drug Streptozotocin (STZ, Sigma Aldrich®), at a dose of 70 mg/kg subcutaneously.[27] Females of the nondiabetic group (control, n = 6) received a volume of vehicle (citrate buffer) corresponding to the group that received the diabetogenic drug. On day 75 of life, the Oral Glucose Tolerance Test (OGTT) was performed to assess the glycemic values, which characterized the inclusion criteria of the rats.[28] For this, after six hours of fasting, a drop of blood was obtained from the distal end of the animal's tail, which was introduced into a conventional glucometer for glycemic determination (time 0). Following, the rats received glucose solution (0.2 g/mL) via intragastric (*gavage*) at a dose of 2.0 g/kg of body weight. After 30, 60, and 120 min of the administration of the glucose solution, the blood glucose levels were also determined. The glucose level determination with threshold values between 140–200 mg/dL for glucose intolerance status at least two-time points during OGTT, and ≥200 mg/dL at any point of the test for confirmation of diabetes. In the control group, only rats with glycemia <140 mg/dL at least three time points during the OGTT were included.[27,29] The animals that received the STZ and presented an unchanged OGTT, as well as the nondiabetic rats that presented an abnormal OGTT were excluded from the respective groups, anesthetized with sodium thiopental (120 mg/kg), and killed.

Mating period, pregnancy, and breastfeeding

In adulthood, from day 90 of life onwards, the Diabetic (D, n = 8) and Control (Con = 6) rats were mated during a maximum duration of 15 days, corresponding to at least three estrous cycles, until reaching the trial number. In each propylene cage with three female rats and one male rat was used for mating, and these cages were lined with wood shavings. At the end of the afternoon, each normoglycemic male rat was placed in each cage with three females in the vivarium. The following morning, males were removed and vaginal smears were collected. After analyzing the material deposited on the slides under a biological microscope, pregnancy confirmation was performed by the presence of sperm, which was considered day zero of pregnancy (GD0). According to previous results of our studies, about 20% of diabetic rats are infertile and, of those with a positive vaginal smear, around 10% of rats did not reach pregnancy at term due to complications caused by hyperglycemia, so we started with a larger number of rats in this group. During pregnancy, the rats were kept in individual cages and the offspring were obtained through vaginal delivery to evaluate the diabetogenic environment on developmental plasticity in the female offspring. After birth, female newborns were kept with their mothers from lactation until the weaning period (22 days of life). After weaning, these animals were distributed into the corresponding experimental groups.

Experimental sequence of offspring of diabetic or control mothers (Fig. 1)

Calculation of sample size

To calculate the sample size, the mean values and standard deviations of blood glucose levels obtained in the area under the curve (AUC) from previous experiments were considered. In addition, reliability of 95%, power of 90%, and margin of error of 5% were also considered, and the calculated sample size was 5 animals per group.

Experimental groups

After weaning, the rats were randomly distributed in a pseudo-randomized manner (respecting a maximum of two rats from each litter per experimental group) into four experimental groups (n = 5 animals/group): 1. Female offspring from control mothers and who received a standard diet (FOConSD); 2. Female offspring from control mothers and who received a high-fat diet (FOConHFD); 3. Female offspring from diabetic mothers who received a standard diet (FODSD) and 4. Female offspring from diabetic mothers and who received a high-fat diet (FODHFD).

Standard or high-fat diet

Female offspring from control and diabetic mothers received standard commercial diet Purina chow, Purina®, Brazil (28.54% Kcal of protein, 62.65% Kcal of carbohydrate, 8.7% Kcal of fat – commercial rat food, Purina®, Brazil) or high-fat diet (HFD: 23.43% Kcal from protein, 46.63% Kcal from carbohydrates, 30% Kcal from fat)[30] according to the experimental group from weaning to adulthood (120 days of life). HFD was made at our institution and lard was the source of fat used. After preparation, the food was kept under refrigeration until consumption.

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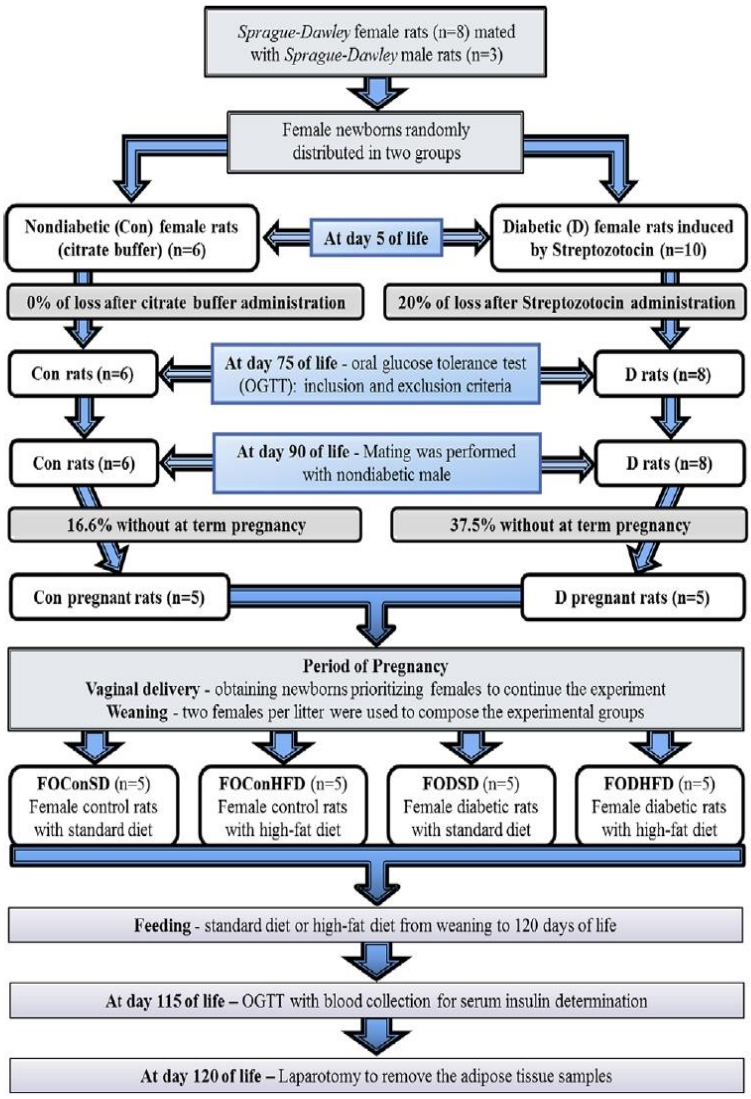


Fig. 1. Experimental design.

Oral glucose tolerance test

On day 115 of life, the OGTT was performed to assess glucose tolerance as described above. The total glucose response was assessed by estimating the AUC using the trapezoidal method.[31,32]

Animal death and morphological analysis of adipocytes

On day 120 of life, the rats were anesthetized with sodium thiopental (Thiopentax® – 120 mg/kg, intraperitoneal route) and submitted to laparotomy to remove the adipose tissue samples. The total body fat was calculated by the sum of the periovarian fat + retroperitoneal fat + visceral fat. For adiposity index was used the ratio (total body fat/final body weight) × 100.[33] The samples of periovarian adipose tissue were weighed, placed in a fixative solution (10% formaldehyde) for 24 hours, and then placed in 70% ethanol. The fragments were processed and embedded in paraffin for further cutting in a rotating microtome. The adipose tissue samples were stained with hematoxylin–eosin (H&E). After this process, the images were analyzed under a microscope at 20× magnification to allow for counting the number of adipocytes and calculating their areas using the ImageJ software. Large adipocytes were considered as those whose area was greater than or equal to 4.58 μm^2, medium those whose area was between 1.03 and 4.57 μm^2, and those whose area was lesser than or equal to 1.02 μm^2 were considered as small.[34]

Statistical analysis

For comparison of the means and standard deviation between the four experimental groups with data presenting normal distribution, Tukey´s Multiple Comparison Test was used. If the data presented abnormal distribution, Negative Binomial and Poisson Distribution with Wald's Multiple Comparison Test was carried out. Pearson´s correlation test was used for correlation analysis. $P < 0.05$ was considered as the limit of statistical significance.

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Table 1. Body weight, fat and glycemic parameters at day 120 of life of female offspring (FO) from control (Con) or diabetic (D) mothers who consumed a standard diet (SD) or high-fat diet (HFD) after weaning

	Groups			
	FOConSD	FOConHFD	FODSD	FODHFD
Body weight (g)[a]	247.20 ± 5.26	264.00 ± 13.25	262.20 ± 24.23	264.12 ± 8.34*
Total fat weight (g)[a]	4.75 ± 1.45	6.89 ± 1.94	6.02 ± 1.95	8.83 ± 3.32*
Periovarian adipose tissue weight (g)[a]	1.08 ± 0.45	1.26 ± 0.36	1.10 ± 0.58	1.65 ± 1.09*
Adiposity index[a]	1.92 ± 0.26	2.62 ± 0.29	2.27 ± 0.24	3.29 ± 0.47*
Relative weight of periovarian adipose tissue[b]	0.44 ± 0.19	0.48 ± 0.16	0.42 ± 0.18	0.62 ± 0.20
Adipocyte number/body weight (g)[b]	0.21 ± 0.01	0.25 ± 0.01	0.24 ± 0.01	0.22 ± 0.01
Adipocyte area/body weight (g)[c]	11,041.00 ± 1191.13	9,335.64 ± 851.32	10,356.00 ± 1117.23	15,372.00 ± 1513.52*
Adipocyte number/Periovarian adipose tissue weight[c]	55.50 ± 3.33	56.21 ± 2.83	66.84 ± 3.66	54.03 ± 3.00
Adipocyte area/Periovarian adipose tissue weight[a]	2,886,491.00 ± 482,760.00	2,041,313.00 ± 288,400.00	2,698,047.00 ± 451,009.00	3,336,160.00 ± 510,157.00*
AUC (glycemic level – 120 minutes)[a]	14,529.54 ± 1865.80	16,174.50 ± 2,487.10*	18,174.54 ± 1,286.00*	19,365.00 ± 1,279.50*,#,$
AUC (circulating glucose per minute)[a]	121.07 ± 15.54	134.78 ± 20.72*	151.45 ± 10.71*	161.37 ± 10.66*,#,$

AUC (area under the curve), g (gram).
Data presented as mean ± standard deviation.
*$p < 0.05$ – compared to the FOConSD group.
#$p < 0.05$ – compared to the FOConHFD group.
$$p < 0.05$ – compared to the FODSD group.
[a]Tukey´s multiple comparison test.
[b]Poisson distribution with Wald's multiple comparison test.
[c]Negative binomial distribution with Wald's multiple comparison test.

Table 2. Correlation between body weight and fat parameters at day 120 0f life of the female offspring (FO) from control (Con) or diabetic (D) mothers who consumed a standard diet (SD) or high-fat diet (HFD) after weaning

	Adipocyte number	Adipocyte area	Body weight (g)	Periovarian adipose tissue weight (g)	Total fat weight (g)	Adiposity index
Adipocyte number	r = 1	r = –0.05634	r = 1784	r = –0.11648	r = 0.06075	r = 0.05222
		p = 0.7985	p = 0.4154	p = 0.5966	p = 0.783	p = 0.8129
Adipocyte area	–	r = 1	r = 0.4766*	r = 0.65835*	r = 0.66658*	r = 0.6236*
			p = 0.0215	p = 0.0006	p = 0.0005	p = 0.0015
Body weight (g)	–	–	r = 1	r = 0.4331*	r = 0.4800*	r = 0.3640
				p = 0.0390	p = 0.0240	p = 0.0877
Periovarian adipose tissue weight (g)	–	–	–	r = 1	r = 0.80646*	r = 0.78018*
					p < 0.0001	p < 0.0001
Total fat weight (g)	–	–	–	–	r = 1	r = 0.99082*
						p < 0.0001
Adiposity index	–	–	–	–	–	r = 1

*$p < 0.05$ – Pearson´s Correlation test

Results

As shown in Table 1, significant increases in body weight, total fat, periovarian adipose tissue weight, and adiposity index were found in offspring from mothers with hyperglycemia and who were postnatally exposed to a high-fat diet (FODHFD) compared to FOConSD rats. The ratio between the periovarian adipose tissue weight and the body weight showed no significant difference between the groups, as well as the ratio between the number of adipocytes and the body weight, and the ratio between the number of adipocytes and the periovarian adipose tissue weight. There was a significant increase in the ratio between adipocyte area and body weight, and in the ratio between adipocyte area and periovarian

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adipose tissue weight in the FODHFD group compared to the FOConSD group.

The calculation of the AUC obtained during OGTT showed a significant increase in the FOConHFD, FODSD, and FODHFD groups than to the FOConSD rats, as the sum as the level of circulating glucose per minute, and the FODHFD group showed an even greater increase in these parameters compared to the other groups (Table 1).

When the correlation between the adipocyte number, adipocyte area, body weight, periovarian adipose tissue weight, total fat weight and adiposity index was evaluated, positive correlations between body weight and area of adipocytes ($r = 47\%$, $p = 0.02$), as well as between the weight of the periovarian adipose tissue and the adipocyte area ($r = 65\%$, $p = 0.0006$), the total fat weight and the adipocyte area ($r = 66\%$, $p = 0.0005$), and the adiposity index and adipocyte area ($r = 62\%$, $p = 0.0015$) were found. There was also a positive correlation between body weight and periovarian adipose tissue weight ($r = 43\%$, $p = 0.03$), and between body weight and total fat weight ($r = 48\%$, $p = 0.02$). The periovarian adipose tissue weight and total fat weight ($r = 80\%$, $p < 0.0001$), as well as the periovarian adipose tissue weight and adiposity index ($r = 78\%$, $p < 0.0001$), total fat weight and adiposity index ($r = 99\%$, $p < 0.0001$) showed a positive correlation (Table 2).

Regarding the classification of the size of adipocyte areas, there was a higher frequency of large adipocytes in the FOConHFD group compared to the FOConSD group and a higher frequency in the FODHFD group compared to the other three groups (FOConSD, FOConHFD, and FODSD). For medium-sized adipocytes, the FOConHFD rats had a higher frequency compared to the FOConSD group, but the FODHFD rats had a reduced proportion in medium-sized adipocytes compared to the other groups. As for small adipocytes, the FOConHFD group showed a higher frequency than the FOConSD group (Fig 2).

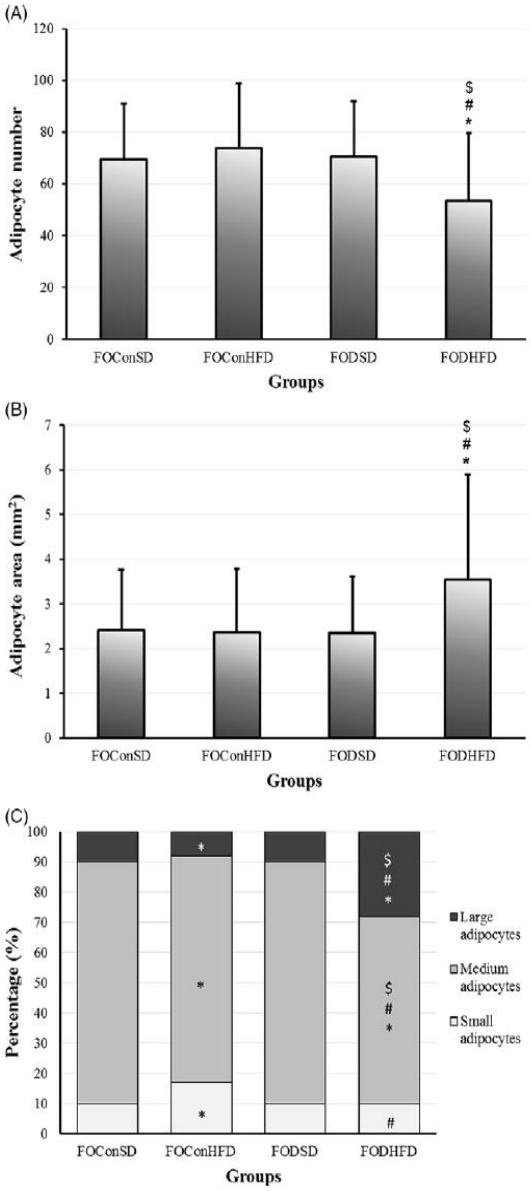


Fig. 2. Number (A) and area (B) of adipocytes, and frequency of large, medium, and small adipocytes (C) at day 120 of life of the female offspring (FO) from control (Con) or diabetic (D) mothers who consumed a standard diet (SD) or high-fat diet (HFD) after weaning. Data presented as mean ± standard deviation (A and B) and percentage (C). *$p < 0.05$ – compared to the FOConSD group, #$p < 0.05$ – compared to the FOConHFD group, $$p < 0.05$ – compared to the FODSD group (Poisson Distribution with Wald's Multiple Comparison Test).

Discussion

Animal studies show that the metabolic imprint caused by the obese or diabetic intrauterine environment can be transmitted through the generations.[35] Study carried out in Pima Indians also corroborates these findings, in which maternal diabetes increased the risk for the onset of Type 2 DM in young Pima Indians, confirming the vicious cycle postulated by which "diabetes breeds diabetes".[36] Similar data were reported among young adults in Denmark exposed to Type 1 DM in the intrauterine period, suggesting that the effects of a hyperglycemic environment at long-term are similar regardless of the type of diabetes.[37] The mechanisms responsible for these effects are not entirely clear, but much research has been carried out involving animal models to better understand the epidemiology and mechanistic effects. However, the association of a hyperglycemic intrauterine environment and post-weaning exposure to a HFD has not been explored. Thus, there was an interest in studying the fusion of these variables to comprehend the repercussions in the adult life of offspring from diabetic mothers who started to consume an HFD after weaning due to the exponential increase in women with prepregnancy or gestational diabetes and due to the excessive consumption of foods rich in saturated fat during childhood, youth, and adulthood.

The present study showed that offspring who were exposed to an unfavorable intrauterine environment (maternal hyperglycemia) and who consumed an HFD after weaning had higher total fat weight, periovarian fat weight, and adiposity index, contributing to the greater body weight. These offspring (FODHFD) also had adipocytes presenting larger areas, concerning either the body weight or periovarian fat weight. The adipose tissue alterations might be related to the increase in glycemic concentrations measured during the OGTT, which confirmed a state of glucose intolerance in these animals due to the greater values of the area under the curve. Adipocytes are recognized for adipokine (cytokine) synthesis and secretion, which act on many physiological systems including appetite, energy expenditure, insulin sensitivity, and

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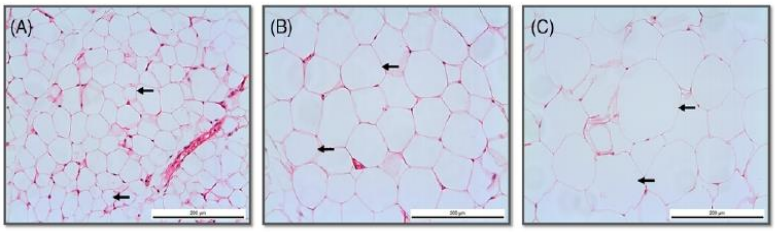


Fig. 3. Representative photomicrograph of the adipocytes. (A) Small adipocytes (pointed by arrows). (B) Medium Adipocytes (pointed by arrows). (C) Large adipocytes (pointed by arrows). Hematoxylin & Eosin staining (H&E). Magnification: 20×.

inflammation. However, an ineffective adipose expansion (or excessive visceral adipose tissue) leads to local inflammation, and insulin-resistant phenotype,[38] dyslipidemia, dysglycemia, and a predisposition to diabetes status,[39] according to our results.

There was also a greater relationship among total fat weight, the periovarian adipose tissue weight and the adiposity index, and the total body weight in the FODHFD adult offspring. This relationship was also established between the increase in adipocyte area (as evidenced by the higher percentage of medium and large-sized adipocytes) with the different types of weight (body, total fat, and periovarian adipose tissue). Furthermore, there was a significant positive correlation between adiposity index and body weight in adult FODHFD females. However, none of these parameters correlated with the number of adipocytes, showing a greater effect of adipocyte hypertrophy than hyperplasia. The increase in the area and the decrease in the number of adipocytes in the adult offspring of the FODHFD group demonstrates that as larger the area of these cells as the smaller amount of cells in this tissue. Hypertrophic obesity is characterized by having adipose tissue with macrophage infiltration, which impairs the adipogenesis process (adipocyte differentiation process). This indicates that the hypertrophied adipose tissue inhibits the signaling cascade responsible for the increase in the number of cells in this tissue.[40] In this study, we found that HFD changed the size of adipocytes, but differently depending on the intrauterine environment to which the offspring has been exposed. There was a higher percentage of medium-sized adipocytes in offspring from nondiabetic mothers and an increase in the percentage of large adipocytes from those from diabetic mothers. In general, the HFD consumption after weaning promoted an increase in the cell size of the adipose tissue of the offspring, showing tissular hypertrophy, leading to greater fat weight. Adipose tissue with hyperplasia synthesizes and secretes pro-inflammatory adipokines in physiological amounts. In addition, it synthesizes and secretes anti-inflammatory adipokines and has protective metabolic effects.[41,42] In contrast, hypertrophied adipose tissue synthesizes and secretes greater amounts of pro-inflammatory adipokines,[43] which negatively contributes to the appearance of other comorbidities in the adult female offspring.

Our study showed that this association of maternal diabetes and HFD consumption after weaning exacerbated the size of adipocytes, obtained from the periovarian adipose tissue, leading to adipocyte hypertrophy. This finding might be explained by the fact that the HFD caused an imbalance between consumption and energy expenditure,[44,45] driving more to the consumption, which resulted in the storage of energy in adipocytes in the form of triglycerides through lipogenesis.[46]

The evaluation of the molecular effects involved in the regulation of lipogenesis, especially regarding the PPAR (Peroxisome proliferator-activated receptor) pathway has been widely investigated. However, there is a need to further investigate the cascade of biochemical changes involved in lipogenesis considering the transgenerational repercussions. So, this encourages further studies to be carried out to unite the molecular and biochemical information involved in this process.

The increased size of adipocytes in females of the FODHFD group verified in our findings is related to a greater total fat weight, periovarian adipose tissue weight, adiposity index, and, consequently, an increase in body weight. Another explanation would be that, while maternal glucose is transferred to the fetus via the placenta, maternal insulin does not cross the placental membrane due to its molecular weight. Faced with maternal glycemic stimulation, the fetal pancreas responds to this glucose overload and synthesizes additional insulin. Hyperinsulinemia, in turn, acts as a fetal growth hormone leading to fetal growth and adiposity.[47,48] The relationship between maternal blood glucose and neonatal adiposity is very important for understanding and possible influence on the development of future obesity.

Excess adipose tissue in the visceral compartment leads these nonesterified free fatty acids (NEFA) to spread to non-adipose tissues such as the liver, skeletal muscle, and pancreas. In the liver and skeletal musculature, the NEFA deposit causes a reduced insulin sensitivity in these tissues. As a consequence, there is increased hepatic glucose synthesis and lower glucose uptake by skeletal muscle. NEFA can also act on the pancreas and destroy pancreatic beta cells, decreasing insulin secretion. Taken together, the cellular defects lead to insulin resistance throughout the body, progressing to hyperglycemia and later to diabetes itself. Visceral adipose tissue is also secreting lower levels of insulin-sensitizing hormones, such as adiponectin, which further contribute to peripheral insulin resistance.[49]

Children exposed to diabetes and/or maternal obesity during pregnancy are at greater risk of becoming obese and developing Type 2 DM at a young age. Many of the offspring (female) become obese and have diabetes or abnormal glucose tolerance by the time they reach the fertile stage, thus perpetuating the cycle. Over generations, this cycle likely increases the risk and/or anticipates the onset of obesity and Type 2 DM.[47] Despite growing evidence that exposure to intrauterine diabetes is associated with diabetes on the offspring and may be responsible for the early onset of a disease of adulthood, it is not known whether this exposure associated with the consumption of a HFD exacerbates the metabolic abnormalities that are precursors of DM2 and/or obesity in adulthood. In our findings, we found that female rats who consumed HFD, or who were exposed to maternal diabetes or who were subjected to the association of these two conditions had hyperglycemic peaks, with a diagnosis of glucose intolerance, confirmed by the glycemic values obtained in the calculations of the area under the curve. Impaired insulin secretion has also been proposed as a possible mechanism, but whether this is a transient phenomenon or whether it leads to impaired glucose tolerance later in life is still under discussion.

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Considering all of the information above described and the developmental critical window, especially the period of formation and development of white adipose tissue in the fetus, a maternal insult may determine vulnerability to possible environmental aggressions, such as the HFD consumption.[50]

In conclusion, the hyperglycemic intrauterine environment, associated with the HFD consumption by the offspring, increases the size of adipocytes, leading to hypertrophy, which contributes to the greater weight of periovarian fat, total fat, and, consequently, in greater body weight and its adiposity index. Furthermore, the association between maternal diabetes and postweaning HFD consumption resulted in glucose intolerance in offspring, confirming the intergenerational effect of diabetes and exacerbation after another environmental insult. Thus, it is necessary to understand the repercussions of intrauterine and postnatal insults, so that it is possible to reduce the prevalence of obesity and type 2 diabetes in the next generation and, consequently, benefits for future generations.

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Conflicts of interest. No conflicts of interest to this article have been reported.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Guide for the Care and Use of Laboratory Animals) and has been approved by the institutional committee (Protocol number CEUA 1335/2019).

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Maternal Diabetes and Postnatal High-Fat Diet on Pregnant Offspring

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Maternal diabetes-induced fetal programming predisposes offspring to type 2 diabetes, cardiovascular disease, and obesity in adulthood. However, lifelong health and disease trajectories depend on several factors and nutrition is one of the main ones. We intend to understand the role of maternal diabetes-induced fetal programming and its association with a high-fat diet during lifelong in the female F1 generation focusing on reproductive outcomes and the possible changes in physiological systems during pregnancy as well as the repercussions on the F2 generation at birth. For this, we composed four groups: F1 female pups from control (OC) or from diabetic dams (OD) and fed with standard (SD) or high-fat diet from weaning to full-term pregnancy. During pregnancy, glucose intolerance and insulin sensitivity were evaluated. In a full-term pregnancy, the maternal blood and liver were collected to evaluate redox status markers. The maternal blood, placental tissue, and fetal blood (pool) were collected to evaluate adiponectin and leptin levels. Maternal reproductive parameters were evaluated as well. Maternal diabetes and high-fat diet consumption, in isolation, were both responsible for increased infertility rates and fasting glucose levels in the F1 generation and fetal growth restriction in the F2 generation. The association of both conditions showed, in addition to those, increased lipoperoxidation in maternal erythrocytes, regardless of the increased endogenous antioxidant enzyme activities, glucose intolerance, decreased number of implantation sites and live fetuses, decreased litter, fetal and placental weight, increased preimplantation losses, and increased fetal leptin serum levels. Thus, our findings show that fetal programming caused by maternal diabetes or lifelong high-fat diet consumption leads to similar repercussions in pregnant rats. In addition, the association of both conditions was responsible for glucose intolerance and oxidative stress in the first generation and increased fetal leptin levels in the second generation. Thus, our findings show both the F1 and F2 generations harmed health after maternal hyperglycemic intrauterine environment and exposure to a high-fat diet from weaning until the end of pregnancy.

Keywords: Female reproduction, diabetes, malnutrition, fetal programming, rodent

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1 INTRODUCTION

Diabetes is a complex and chronic disease that requires continuous medical care, with strategies of multifactorial risk reduction and glycemic control (ADA, 2021a). According to the American Diabetes Association (ADA), there are three main classes of *Diabetes mellitus* (DM): type 1 DM (DM1), which is characterized by the autoimmune destruction of pancreatic beta (β)-cells, usually causing insulin deficiency; type 2 DM (DM2) that is characterized by the progressive loss of insulin secretion by β-cells, leading to insulin resistance as a background; and gestational *diabetes mellitus* (GDM) that is diagnosed in the second or third trimester of pregnancy, in which the diabetes is not manifested before pregnancy (ADA, 2021b). The prevalence of diabetes in pregnancy has increased worldwide. In 2019, the International Diabetes Federation (IDF) showed that one in six pregnancies was being affected by hyperglycemia, with 13.6% of pregnancies affected by pre-gestational diabetes and 86.4% affected by GDM. The occurrence of GDM has been described by the International Diabetes Federation as a severe and neglected threat to maternal and child health (IDF, 2019). The hyperglycemic intrauterine environment can interact with the genome of offspring, inducing long-term altered patterns of gene expression and can be transmitted transgenerationally (Gauguier et al., 1990; Boloker et al., 2002). For this reason, the IDF recommends that pregnant women with diabetes or at high risk of developing GDM should monitor their blood glucose to avoid long-term consequences for themselves and transgenerational effects for their children (IDF, 2019).

In addition to maternal glycemic control during pregnancy, adequate nutrition and specific nutrients are important in all periods of life, but they are essential during specific times, such as in intrauterine life and early postnatal life (Greco et al., 2019). Studies have shown that the first 1,000 days after conception (from intrauterine life/pregnancy to the first 2 years of the life of a child) are windows of particular sensibility to environmental factors influencing lifelong trajectories through health and disease (Barker, 2012; Simeoni et al., 2018). In this context, several epidemiologic and experimental studies show that maternal hyperglycemia and an unbalanced diet can induce health consequences several decades after exposure, leading to a higher prevalence of overweight, obesity, DM2, GDM, and reproductive disorders in the adult lives of male and female descendants (Boullu-Ciocca et al., 2008; Glavas et al., 2010; Bouchard et al., 2012; Kayser et al., 2015; Sánches-Guarrido et al., 2015; World Health Organization, 2019). However, the exposure to adverse conditions during intrauterine development is particularly important to female offspring because it can cause physiological changes that have the potential to alter both the reproductive capacity of the first generation and the health of the second generation through changes in the oocyte (Yao et al., 2021). Moreover, the sex of the embryo also plays an important role in determining how an insult might become part of the epigenome and be transmitted to future generations. In the case of female rats, as the entire repertoire of primordial follicles forms during intrauterine and early neonatal phases, the consequences may be more evident in reproductive aspects than in males (Yao et al., 2021).

Environmental factors and lifestyle have a direct influence on developmental programming and may even reverse those (Gluckman et al., 2008). Leptin and adiponectin are hormones that regulate energy balance and insulin sensitivity, playing a critical role in the establishment of this program (Parent et al., 2014; Choi, 2018). Both hormones have a key role in metabolism, maternal–fetal interaction, and metabolic abnormalities which can lead to pregnancy complications and fetal growth changes (Santos et al., 2015). Intrauterine exposure to hyperglycemia leads to the development of both leptin and insulin resistance in the placenta (Greco et al., 2019), and these alterations are related to maternal glycemic levels (Bouchard et al., 2012; Houde et al., 2013). Moreover, animal and human studies show that leptin and insulin resistance act on hypothalamic receptors and appetite circuits, leading to postnatal hyperphagia, decreased satiety, and subsequent development of metabolic syndrome (Block and El-Osta, 2017). Plasma leptin concentrations, in both dams and their offspring, may play a role in linking nutrition and development (Stocker et al., 2007). Stocker et al. (2007) verified that the impaired glucose tolerance in rats from mothers fed a high-fat diet can be prevented by the administration of leptin to their mothers, indicating the maternal leptin levels during pregnancy and lactation can affect the development of energy balance regulatory systems in their offspring.

In an attempt to assess the repercussions of fetal programming and malnutrition and to eliminate the possible confounding factors between these two simultaneous interventions during pregnancy, we first used a pre-gestational diabetes model (F0 generation), and then a high-fat diet (HFD) was offered only to the female offspring (F1 generation) from weaning until full-term pregnancy. Thus, we intend to understand the role of maternal diabetes-induced fetal programming and its association with a high-fat diet during lifelong in the female F1 generation focusing on reproductive outcomes and the possible changes in physiological systems represented by glycemic metabolism, redox status, concentrations of leptin and adiponectin, and insulin sensitivity as well as the repercussions on the F2 generation at birth.

2 METHODS

2.1 Ethics

The Ethics Committee for the Use of Animals of Botucatu Medical School approved all the methods adopted in this study (Protocol CEUA Number: 1218/2017). Animal handling was performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals promulgated by the Society for the Study of Reproduction and with the guidelines provided by the Brazilian College of Animal Experimentation.

2.2 Calculation of Sample Size

Based on previous experiments conducted in our laboratory, each rat from a different litter was used for circulating glycemic calculation obtained by area under the curve (AUC) during

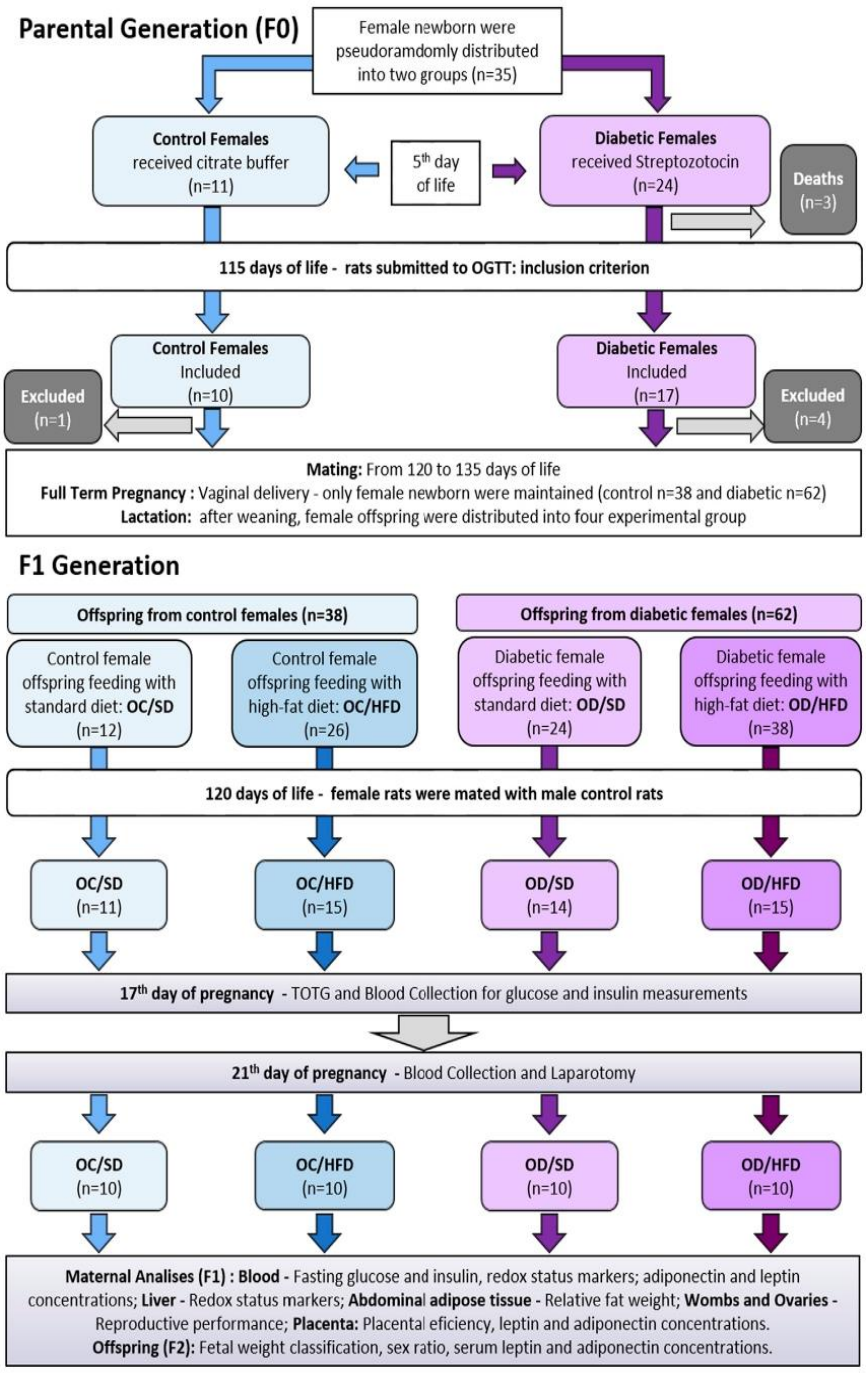


FIGURE 1 | Experimental approach for parental and offspring generation.

the oral glucose tolerance test (OGTT), and using 90% power and an error type I of 5%, the effect size was determined as 10 rats per group.

2.3 Animals

The animals were maintained at the local laboratory of Botucatu Medical School (Unesp) under controlled conditions of

temperature (22 ± 2°C), humidity (50 ± 10%), and light/dark cycle (12 h) in polypropylene cages lined with wood shavings. Filtered water and feed were offered *ad libitum*. As a form of environmental enrichment, paper balls were used in the cages (Simpson and Kelly, 2011).

2.4 Experimental Approach

2.4.1 Experimental Approach for Parental Generation (F0)

The parental generation (F0) aimed to create an inadequate intrauterine environment leading to fetal programming based on the induction of experimental diabetes. The experimental sequence was described in **Figure 1**.

Male and female Sprague-Dawley rats (200–250 g) were mated overnight to obtain female pups to induce or not induce diabetes during the perinatal period of these offspring. The following morning, when spermatozoa were found in the vaginal smear, was designated as day 0 of pregnancy. The female rats that were not mated after 15 consecutive days were considered infertile and excluded from this study (Moraes-Souza et al., 2017). The pregnant rats were randomly assigned to diabetic (D) and nondiabetic groups by draw, and the female rats were kept in individual polypropylene cages during pregnancy. On day 21 of pregnancy, the rats had a vaginal delivery. Eight pups per dam were kept with their dams until postnatal day (PND) 22, considering the highest number of females wherever possible. We prioritize female rats because our research group studies the transgenerational effects related to pregnancy. The excess pups were euthanized by decapitation (Sinzato et al., 2021).

2.4.2 Diabetes Induction

On postnatal day 5 (PND), the female pups (First Generation—F1) were injected with streptozotocin (Sigma Aldrich®, United States, a dose of 70 mg/kg, intraperitoneal route) for diabetes induction, while control female rats received an equivalent volume of vehicle (citrate buffer - 0.01 M pH 4.5), as previously described (Damasceno et al., 2011). Male offspring were maintained with their mothers until weaning and then were euthanized by decapitation or used by other investigators. The blood glucose levels were determined in female adult rats on a PND 75, as established by Gallego et al. (2018—with modifications) and classified by the American Diabetes Association (ADA, 2019). Following, female rats were considered diabetic when presenting glycemia ≥200 mg/dl (11.11 mmol/L) at least at a one-time point during the oral glucose tolerance test (OGTT). In the control group, only rats with glycemia <140 mg/dl (7.77 mmol/L) at least at a three-time point during the OGTT were included. The female rats that were not accomplishing the aforementioned characteristics were anesthetized by using sodium thiopental (Thiopentax®, Cristália, Brazil—120 mg/kg dose), euthanized, and excluded from this study.

2.4.3 Mating, Pregnancy, and Lactation

After inclusion criteria on PND 90, the diabetic (D) and nondiabetic (control—C) female rats were mated as previously described. The offspring were obtained through vaginal delivery. To avoid differences in maternal care between male and female pups (Beery and Francis, 2011) which could result in epigenetic consequences (Champagne et al., 2003), after birth, all-male newborns were euthanized by decapitation, while female newborns were kept in a range from six to eight pups per litter until weaning day 22, to maintain the milk consumption balance. The excess number of female pups was also euthanized by decapitation. When the litter had less than six female pups, it was excluded from the study and redirect to another experiment in our group. After weaning, female pups from nondiabetic (control) and diabetic dams were randomly assigned to compose the experimental groups.

Experimental approach for female pups from the maternal diabetic environment (F1 generation).

2.4.4 Experimental Groups and Dietary Patterns

Upon weaning, the female pups from diabetic (OD) and control (OC) mothers were pseudo-randomized by lot respecting a maximum of four female pups per mother (with two sisters to each group: C or D). The other females from the same litter were used in another subproject with non-pregnant rats (Protocol CEUA Number: 1218/2017). The OD and OC groups were further distributed into two other groups according to their diets: standard diet (SD - Kcal content: 28.54% protein, 62.65% carbohydrate, 8.7% fat, Purina®, Brazil) or high-fat diet (HFD - Kcal content: 23.43% protein, 46.63% carbohydrate, 30% fat, using lard as the main fat source). Additional bromatological comparison between the diets is shown in **Supplementary Table S1**. Thus, four experimental groups were established: OC/SD: female pups from control mothers and fed standard diet; OC/HFD: female pups from control mothers and fed high-fat diet; OD/SD: female pups from diabetic mothers and fed standard diet; and OD/HFD: female pups from diabetic mothers and fed high-fat diet. All the groups were followed from weaning to PND 120. The HFD was handmade at our institution, adequately supplemented with vitamins and minerals, and maintained under refrigeration until the time of use (Paula et al., 2021). Given the fact that the diets had visual characteristics that easily allow their distinction, random housing, and blinding of caregivers and/or investigators were not possible.

2.4.5 Mating

From PND 120, diabetic (D) and control (C) female rats were mated as previously described.

2.4.6 Pregnancy

2.4.6.1 Oral Glucose Tolerance Test (OGTT) and QUICK Index.

Maternal weight, water consumption, and food intake (evaluated as gram intake and energy intake) were monitored during the entire pregnancy. Energy intake was determined to observe if the moderate differences showed in body weight evolution would be related to differences in food intake (Pérez-Matute et al., 2007). On pregnancy day (PD) 17, OGTT was performed for glucose tolerance evaluation as previously described (Tai, 1994; Gallego et al., 2019). In this same test, blood samples were collected at fasting for insulin measurements using an ELISA commercial kit

(Crystal Chemical® Code: 90060, United States). The quantitative insulin sensitivity check index (QUICK index) was calculated as defined by Katz et al. (2000) as QUICKI = 1/[(log (I_0) + log (G_0)], where I_0 is the fasting plasma insulin level (microunits/mL), and G_0 is the fasting blood glucose level (milligrams per dL).

2.4.6.2 Blood Collection and Laparotomy

On PD 21, the rats were intraperitoneally anesthetized by using sodium thiopental (Thiopentax®, Cristália, Brazil—120 mg/kg dose) and decapitated. The collection of maternal blood was performed to obtain serum and washed erythrocyte samples according to the ELISA kit's instructions for leptin and adiponectin (#80570; # E-EL-R0582) assays and according to Souza et al. (2010) for redox status analyses, respectively. All samples were stored at −80°C until further analysis. Uterine horns, ovaries, newborns, and placentas were removed and weighed for evaluation of maternal reproductive performance, birth weight classification, and placental efficiency (Sinzato et al., 2021). Visceral fat pads from the abdominal cavity (periovarian, periuterine, perirenal, and retroperitoneal) were pooled and weighed to calculate the relative fat weight (grams of total visceral fat/100 g of body weight). Maternal liver and two placentas from each litter (one from female newborns and the other from one of the male newborns were randomly assigned) were removed and quickly frozen in liquid nitrogen and stored in a freezer at -80°C until homogenates were performed. The liver homogenate (modified from Spada et al., 2014) was used for redox status evaluation, and the placental homogenate (modified from Portela et al., 2021) was used for leptin and adiponectin determinations. Newborns were sexed and all male and female newborns from each litter were euthanized and killed by decapitation for blood collection (pool). Serum samples from each pool were obtained as described for maternal blood and used for leptin and adiponectin determinations.

2.4.6.3 Reproductive Outcomes and Birth Weight Classification

The mothers who presented no live newborns at the end of pregnancy were not included in the reproductive analyses. The ovaries were used to count the number of corpora lutea as an indirect parameter to assess the number of oocytes. The uterus of pregnant rats was dissected to count the number of living and dead fetuses, resorptions (embryonic deaths), and implantation sites (Sinzato et al., 2021). When the implantation sites were visually undetectable, the Salewski (1964) reagent was used as a dye. The percentage of embryonic loss before implantation (preimplantation loss rate), the percentage of embryonic loss after implantation (postimplantation loss rate), and the classification of the birth weight into small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA) were made according to Sinzato et al. (2021).

2.4.6.4 Liver Homogenates for Redox Status Analyses

Liver samples from a maternal organism (F1 generation) frozen samples were homogenized using the bullet blender system, adding zirconium beads, phosphate-buffered saline (PBS—0.1 M Na2HPO4, 0.1 M KH2PO4, 0.1 M EDTA, pH 7.8), and protease inhibitor cocktail (#P8340) for glutathione peroxidase (GSH-Px), catalase (CAT), reduced thiol groups (-SH), and hydrogen peroxide (H_2O_2—reactive oxygen species) assays. For thiobarbituric acid reactive substances (TBARS–lipoperoxidation biomarker) assay, RIPA lysis buffer (1X #92590) was used. For superoxide dismutase (SOD), a solution with 20 mM HEPES, 1 mM EGTA, 210 mM mannitol, and 70 mM sacarose (pH 7.2) was used. Then, all samples were centrifuged at 1,600 *x g* for 10 min at 4°C for GSH-Px, CAT, -SH, and H_2O_2; 240 *x g* for 10 min at 4°C for TBARS; and 1.500 *x g* for 5 min at 4°C for SOD. After centrifugation, the supernatant was stored in a freezer at -80°C until assays. Protein concentrations were analyzed by the Bradford method (Bradford, 1976).

2.4.6.5 Placental Homogenate for Leptin and Adiponectin Analyses

From here, all procedures were performed with the investigator blinded to the analyses. For the placental homogenate, out of ten samples collected from male newborns, five were randomly assigned to analyses. The same procedure has been made for the placentas from female newborns. Placental frozen samples were homogenized using the bullet blender homogenizer® (Next Advanced, NY, United States) adding zirconium oxide beads (1 mm–Code ZrOB10, and 2 mm–ZrOB20, Next Advanced, NY, United States) and lysis buffer (RIPA 1X #9806), protease/phosphatase inhibitor cocktail (1X #5872), phenylmethane sulfonyl fluoride (PMSF 1 mM #8553), and the remaining volume completed with water purified by the ultra purifying master system® (GEHAKA, São Paulo, Brazil). After homogenization, the samples were kept and incubated on ice and homogenized by vortex every 15 min for 2 hours, then centrifuged at 7,000 × g for 15 min at 4°C, and the supernatant was stored at -20°C. Protein concentration was analyzed by the Bradford method using the BSA curve as a standard. The final protein concentration in μg/μL was determined based on the BSA standard curve (Bradford, 1976) performed at each dose. Samples were normalized by the lowest protein concentration obtained after measurement.

2.4.6.6 Evaluation of redox status and hormone assays

Samples of washed red blood cells and liver homogenates were used to evaluate the redox status of a maternal organism following the protocols from Yagi (1976) and Buege and Aust (1978) for TBARS; Boyne and Ellman (1972) modified by Hu (1994) for -SH; Noble and Gibson (1970) for H_2O_2; Marklund and Marklund (1974) for SOD; Lawrence and Burke (1976) for GSH-Px; and Aebi (1984) for CAT.

Fasting serum insulin was determined using an ultra-sensitivity ELISA kit from Crystal Chemicals→, United States (Code: 90060). For maternal, placental, and fetal adiponectin and leptin determinations were used, respectively, an ELISA kit from Crystal Chemicals®, United States (Code: 80570) and an ELISA kit from Elabscience®, United States (Code: E-EL-R0582).

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TABLE 1 | Maternal reproductive performance, relative fat weight, placental and birth weight, and placental efficiency on the 21st day of pregnancy from F1 control pregnant offspring (OC) and diabetic pregnant offspring (OD) that received standard diet (SD) or high-fat diet (HFD) from weaning.

Variable	Group			
	OC/SD	OC/HFD	OD/SD	OD/HFD
Fertility rate (%)	91.7	58.3	57.7	39.5*
Non-pregnant at full-term (%)	9.1	33.3	26.7	33.3
Number of corpora lutea	13.9 ± 1.7	14.0 ± 1.6	13.8 ± 0.9	13.2 ± 1.7
Number of implantation	13.7 ± 1.8	12.9 ± 1.5	11.1 ± 4.5	7.1 ± 5.3*#
Number of embryonic deaths	1.0 ± 1.2	1.9 ± 2.0	1.4 ± 1.9	0.8 ± 1.3
Number of live fetuses	12.7 ± 1.6	10.9 ± 2.4	9.7 ± 4.9	6.6 ± 5.2*#
Preimplantation loss (%)	1.1	7.5	12.5	28.7*
Postimplantation loss (%)	7.0	15.3	18.2	12.1
Maternal weight gain (g)	126.3 ± 31.3	82.1 ± 24.5*	109.3 ± 17.7	88.0 ± 16.5*$
Litter weight (g)	99.6 ± 12.8	79.0 ± 16.3	79.5 ± 25.9	58.9 ± 29.8*
Relative fat weight (g/100 g of body weight)	2.49 ± 0.58	4.14 ± 1.02*	3.68 ± 1.43	3.18 ± 0.23
Birth weight (g)	5.84 ± 0.33	5.14 ± 0.66*	5.29 ± 0.68*	4.81 ± 0.87*#$
Placental weight (g)	0.56 ± 0.10	0.53 ± 0.08	0.60 ± 0.08*	0.51 ± 0.12$
Placental efficiency	10.74 ± 1.43	9.89 ± 1.57*	8.86 ± 1.13*	9.68 ± 2.10*$

Values expressed as mean ± standard deviation (SD). n = *10 rats/group.* *$p < 0.05$—*compared to the OC/SD group;* #$p < 0.05$—*compared to the OC/HFD group;* $$p < 0.05$—*compared to the OD/SD group (Tukey Multiple Comparison Test, and Poisson distribution test was used for corpora lutea, implantation and alive fetus numbers, and placental efficiency and chi-square test for proportions).*

2.5 Statistical Analysis

To calculate the sample size, 10 mothers of the parental generation were used, each one from a different litter, and a completely randomized design was made by the Research Support Office of Botucatu Medical School, Unesp. Approximately 10 animals/groups have been established for each group. For the analysis of the F1 generation, we used a maximum of two female rats from the same litter for the experiments. All newborns from each litter were used in the F2 generation analyses. There were no used repeated measures for any parameter. For asymmetric distribution of the data (fetal and placental weights, glycemia of OGTT and AUC, serum insulin concentrations during OGTT, and TBARS levels), the gamma distribution test followed by the Wald multiple comparison test was used. For the QUICK Index, adiponectin and leptin concentrations, other redox status markers, maternal weight gain, maternal body and litter weight, water intake, food consumption, and energy intake, one-way ANOVA followed by the Tukey multiple comparison test was used. The Poisson distribution test was used for corpora lutea, implantation and alive fetus numbers, and placental efficiency since these parameters presented the asymmetric distribution of the data. For proportion analysis (sex ratio, fetal weight classification, and pre and postimplantation loss percentages), the chi-square test was used. A minimum confidence limit of 95% ($p < 0.05$) was considered statistically significant for all statistical comparisons. All statistical analyses were performed using SAS software for Windows, v.9.4.

3 RESULTS

3.1 Fertility Rate, Maternal Weight and Water, and Food Consumption

The fertility rates were 91.7%, 57.7%, 58.3%, and 39.5% in OC/SD, OC/HFD, OD/SD, and OD/HFD, respectively. The OC/HFD and OD/SD groups presented no differences compared to the control group (OC/SD) ($p = 0.060$ and $p = 0.059$, respectively). The OD/HFD group presented a lower fertility rate compared to the OC/SD ($p < 0.05$) (**Table 1**). The percentage of female rats with positive vaginal smear did not reach full-term pregnancy is presented in **Table 1** ($p > 0.05$). These animals were not included in the statistical analysis of the preimplantation losses.

The maternal weight presented no differences between OC/HFD and OC/SD at PD0, PD_7, PD_{14}, and PD_{21} compared to other groups. The OD/HFD rats showed an increase in relative fat weight at full-term pregnancy (**Table 1**) and maternal weight PD_7 of pregnancy compared to the OC/SD, OC/HFD, and OD/HFD dams (**Supplementary Figure S2A**). There were no differences in water consumption among groups (**Supplementary Figure S2D**). The food consumption was lower in OC/HFD rats at PD_{14} and PD_{21} of pregnancy compared to the OC/SD. The OD/HFD group presented lower food intake on day 14 of pregnancy than the OC/SD rats and compared to the OD/SD group at day 21 of pregnancy (**Supplementary Figure S2B**). However, when we evaluated the energy intake, the total calories ingested (Kcal/day) in the OC/HFD and OD/HFD groups (fed with a high-fat diet) at PD0 and PD_7 had an increase compared to those in the OC/SD and OD/SD groups. At PD_{14}, only the OD/HFD rats presented a higher energy intake on the same day of pregnancy in relation to the OD/SD (**Supplementary Figure S2C**).

3.2 TOTG, AUC, Fasting Insulin, and QUICK Index

Figure 2 shows the comparison of the glycemic levels by OGTT (**Figure 2A**) and AUC (**Figure 2B**), QUICK Index (**Figure 2C**), and fasting insulin (**Figure 2D**) among groups. The OD/SD and OD/HFD groups showed increased fasting glucose levels compared to the OC/SD group. At time point 30 of TOTG, all groups had an increase in the blood glucose levels compared to time point 0, but only the OD/SD and OD/HFD groups presented

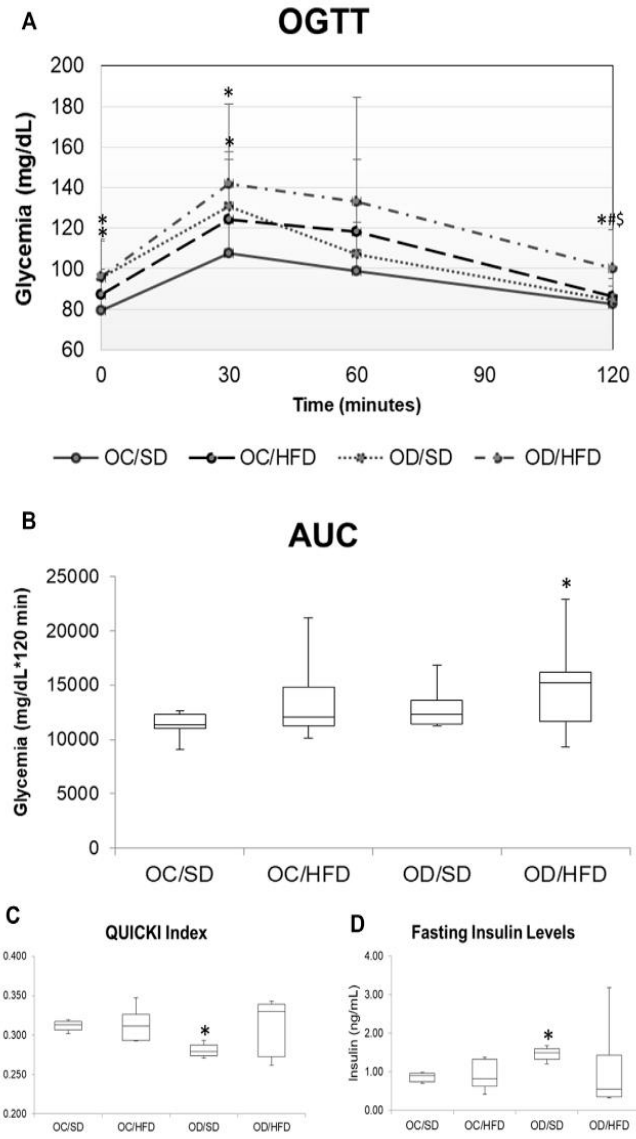


FIGURE 2 | (A) OGTT, oral glucose tolerance test; **(B)** AUC, area under the curve; **(C)** QUICK Index; and **(D)** fasting maternal insulin levels on PD17 from OC, control offspring; and OD, diabetic offspring that received a SD, standard diet; or HFD, high-fat diet from weaning. Values are expressed as mean ± SD, standard deviation. $n = 10$ rats/group. *$p < 0.05$—compared to the OC/SD group; #$p < 0.05$—compared to the OC/HFD group; $$p < 0.05$—compared to the OD/SD group. For OGTT and AUC, the gamma distribution test was used and for QUICK Index, Tukey Multiple Comparison Test was used.

glycemic levels superior to 140 mg/dl. The OC/HFD and OD/HFD groups had increased glycemia compared to the OC/SD and OD/SD rats at 60 min of TOTG. At 120 min of TOTG, the OD/HFD group showed an increase in blood glucose levels compared to the other groups (OC/SD, OC/HFD, and OD/SD). AUC showed increased total glucose during TOTG of OC/HFD, OD/SD, and OD/HFD compared to the OC/SD group. The fasting maternal insulin concentration was increased in the OD/SD and OD/HFD rats compared to the OC/SD group, and the QUICK Index (insulin sensitivity index) was decreased in the OD/SD group compared to the other groups.

3.3 Analysis of Redox Status Markers in Maternal Blood and Liver Samples

Figures 3, 4 show, respectively, redox status markers sampled from maternal washed erythrocytes and liver. There was no difference in SOD activity, H_2O_2, and -SH concentrations among groups in

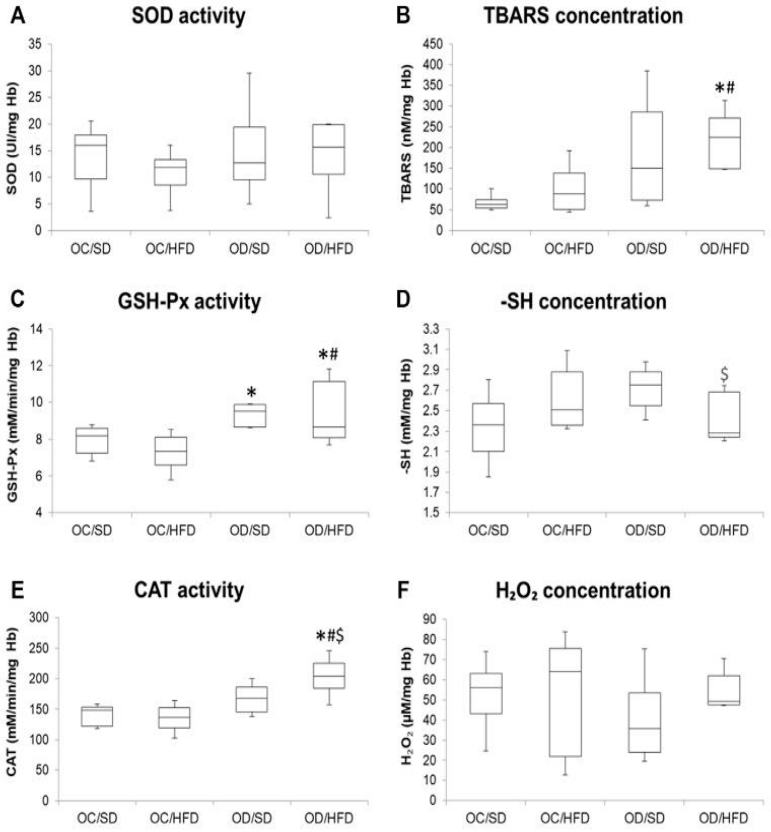


FIGURE 3 | (A) SOD, superoxide dismutase activity; **(B)** TBARS, tiobarbituric acid reagent species concentration; **(C)** GSH-Px, glutathione peroxidase activity; **(D)** reduced thiol groups concentration (-SH) **(E)** CAT, catalase activity; and **(F)** hydrogen peroxide concentration (H_2O_2) in washed erythrocytes at full-term pregnancy from OC, control offspring; and OD, diabetic offspring that received a SD, standard diet or HFD, high-fat diet; from weaning. Values are expressed as mean ± SD, standard deviation; $n = 10$ rats/group. *$p < 0.05$—compared to the OC/SD group; #$p < 0.05$—compared to the OC/HFD group; $$p < 0.05$—compared to the OD/SD group (Tukey Multiple Comparison Test).

maternal washed erythrocytes. There was an increase in TBARS concentration in OD/HFD dams compared to the OC/SD and OC/HFD dams. GSH-Px activities were increased in OD/SD dams compared to control dams and in OD/HFD dams compared to OC/SD and OC/HFD dams. CAT activity was increased in the OD/HFD group compared to the other groups.

Considering maternal liver samples, there were no differences in H_2O_2 concentrations, GSH-Px, and CAT activities among groups. MDA concentration was decreased in OD/HFD compared to OC/HFD rats. There was an increased SOD activity in OC/HFD dams compared to OC/SD dams and a decreased SOD activity in the OD/HFD rats compared to OC/HFD rats. Increased -SH levels were observed in the OC/HFD and OD/HFD rats when compared to the control group.

3.4 Adiponectin and Leptin Concentrations From Maternal and Fetal Serum and Placental Homogenate

Figure 5 shows, respectively, leptin and adiponectin concentrations in maternal (A and B) and fetal (E and F) serum and placental homogenates (C and D) of rats at term pregnancy. As there was no difference between male and female rats, we chose to present the data together. The adiponectin levels showed no differences in the different tissues among the groups. The leptin concentrations of the maternal serum and placental homogenate presented no difference among groups. There was an increase in serum leptin concentration in the fetuses from OD/SD and OD/HFD dams compared to the OC/SD rats.

3.5 Maternal Reproductive Performance, Sex Ratio, and Fetal Weight Classification

Table 1 shows the fertility rate, percentage of non-pregnant rats, reproductive performance, relative fat weight, placental and fetal weight, and placental efficiency of the rats. There were no significant differences in the number of corpora lutea, postimplantation loss percentage, and maternal weight gain among groups. The OC/HFD and OD/SD dams showed reduced litter and fetal weight compared to the OC/SD group. The relative fat weight was increased in the OC/HFD

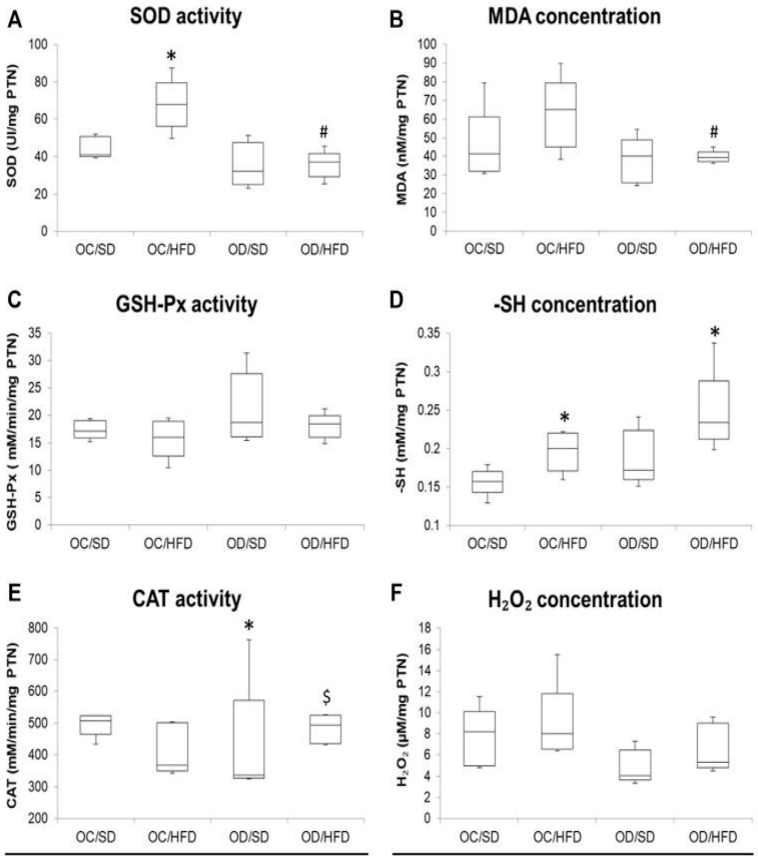


FIGURE 4 | (A) SOD, superoxide dismutase activity; **(B)** MDA, malondialdehyde concentration; **(C)** GSH-Px, glutathione peroxidase activity; **(D)** -SH, reduced thiol groups concentration; **(E)** CAT, catalase activity, and **(F)** H_2O_2, hydrogen peroxide concentration in maternal liver at full-term pregnancy from OC, control offspring; and OD, diabetic offspring that received a SD, standard diet or HFD, high-fat diet; from weaning. Values are expressed as mean ± SD, standard deviation; $n = 10$ rats/group. *$p < 0.05$—compared to the OC/SD group; #$p < 0.05$—compared to the OC/HFD group; $$p < 0.05$—compared to the OD/SD group (Tukey Multiple Comparison Test).

rats than that in the OC/SD dams. The OD/SD group showed lower fetal weight and placental efficiency, and a greater placental weight compared to OC/SD rats. The placental weight and percentage of preimplantation losses were also decreased in OC/HFD and OD/SD in relation to OC/SD. The OD/HFD presented a decrease in the number of implantations, live fetuses, litter, and weight, and increased preimplantation loss percentage compared to the OC/SD, OC/HFD, and OD/SD groups. In addition, the OD/HFD rats showed lower placental weight compared to the OC/SD and OD/SD (**Table 1**).

The fetal weight classification showed an increased number of fetuses classified as small for gestational age (SGA) and a decreased number of fetuses classified as adequate for gestational age (AGA) in all groups when compared to the OC/SD (**Figure 6**). When we compared the fetal weight classification by sex, we observed an increased ratio of SGA and a decreased ratio of AGA in the female pups from OC/HFD and OD/HFD when compared to male pups from the same group. There was no difference in the male and female ratio between the groups and among groups (**Figure 6**).

4 DISCUSSION

Clinical and experimental studies have been performed to evaluate diabetes-induced repercussions (Metzger et al., 2009; Tam et al., 2017; Lowe et al., 2019; Bueno et al., 2020) and/or malnutrition on descendants (Zhang et al., 2008; Mdaki et al., 2016; Tellechea et al., 2017; Hsu & Tain, 2019; Castro-Rodríguez et al., 2020). However, studies addressing the influence of postnatal malnutrition on female offspring that were programmed by intrauterine hyperglycemia are still lacking. Therefore, this study used a rat model with maternal diabetes to reproduce the

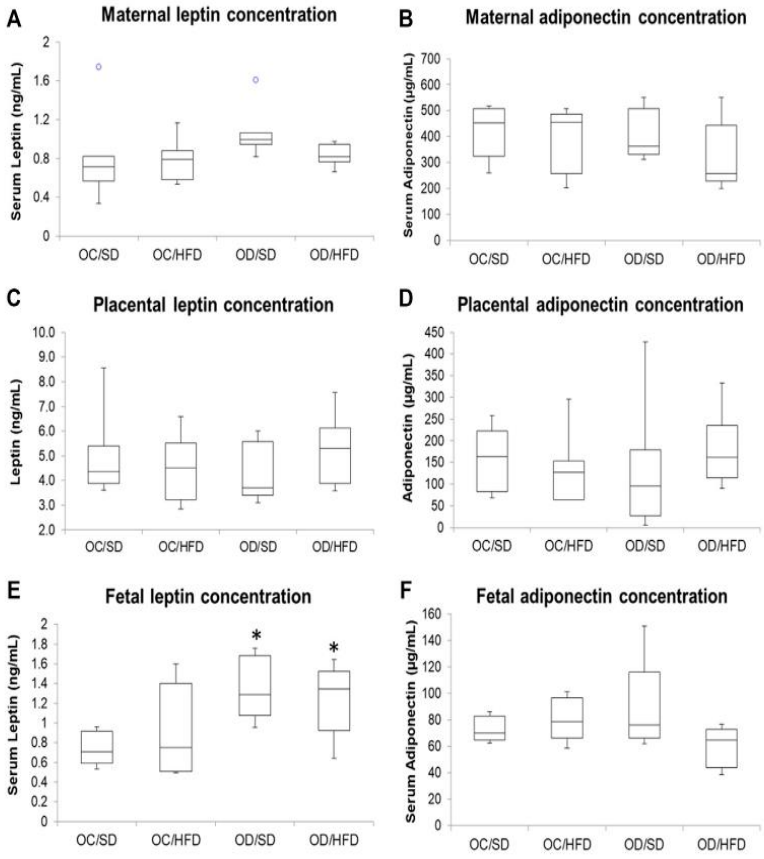


FIGURE 5 | (A) Maternal serum leptin; **(B)** maternal serum adiponectin; **(C)** placental leptin; **(D)** placental adiponectin; **(E)** fetal serum leptin; and **(F)** fetal serum adiponectin concentrations from control offspring (OC) and diabetic offspring (OD) that received a standard diet (SD) or high-fat diet (HFD) from weaning. Values are expressed as mean ± standard deviation (SD). n = 10 rats/group. *$p < 0.05$—compared to the OC/SD group (Tukey Multiple Comparison Test).° Purple dots represent the outliers of the respective groups.

transgenerational effects of hyperglycemia on female offspring (F1 generation) fed with a high-fat diet from weaning to full-term pregnancy (~ PND 150). We found that maternal-diabetes fetal programming associated with high-fat diet consumption, was responsible for increased infertility rates, increased lipoperoxidation in maternal erythrocytes, glucose intolerance, decreased number of implantation sites and live fetuses, decreased litter, fetal and placental weight and increased preimplantation losses in the F1 generation, and increased fetal leptin serum levels and fetal growth restriction in the F2 generation.

The increased infertility rate in OD/HFD rats, in our study, may be related to the beginning of oxidative stress status verified in maternal erythrocytes, characterized by higher antioxidative enzyme activities (CAT and GSH-Px) in maternal erythrocytes of OD/HFD rats and an increased TBARS indicating lipoperoxidation. Oxidative stress is related to hyperglycemia, and it is also one of the main causes of alterations in the reproductive systems of women (Agarwal et al., 2012). Previous studies have reported that the balance between reactive oxygen species (ROS) and antioxidants greatly influences different phases of the reproductive activities in female mammalian animals (Al-Gubory et al., 2010; Wang et al., 2017), and oxidative stress conditions may compromise the reproduction and fertility (Agarwal & Allamaneni, 2004; Agarwal et al., 2012). The beginning of oxidative stress status in maternal erythrocytes can be a signal that organs, like the pancreas and ovaries, also present with an unbalanced redox system, and initial changes in this redox status are seen in the red blood cells of OD/HFD rats in our study might be related to the lower fertility and implantation rates as well. Unlike the liver, which presented the high activity of antioxidative enzymes and no alterations in MDA levels in our study, redox status during pregnancy varies among different organs and depends on the local regulation. The pancreas has a lower antioxidant defense and, consequently, a greater susceptibility of beta (β)-cells to oxidative stress (Lenzen et al.,

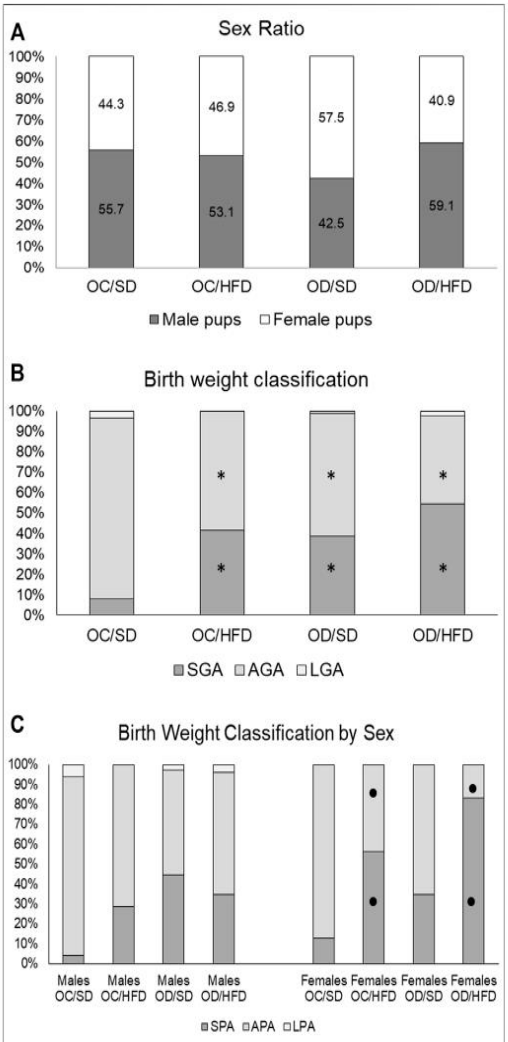


FIGURE 6 | (A) Sex ratio, **(B)** birth weight classification, and **(C)** birth weight classification by sex from OC, control offspring; and OD, diabetic offspring that received SD, standard diet or HFD, high-fat diet from weaning. Values are expressed in percentages. Legend: SPA—small fetuses for gestational age, APA—adequate fetuses for gestational age, and LPA—large fetuses for gestational age. *$p < 0.05$—compared to OC/SD group (Chi-square test) and •$p < 0.05$—between male and female pups from the same group (Chi-square test).

1996). Thus, the maladaptation of the endocrine pancreas to pregnancy can affect maternal glucose and insulin concentrations as seen in OD/HFD rats and harm reproductive organs. Exposure to adverse intrauterine conditions can lead to permanent changes in the structure and function of major physiological systems in developing fetuses (Yao et al., 2021). In rodents, hyperglycemia interferes with the development of fetal female gonads by reducing the weight, diameter, and volume of the ovaries and reducing the number of ovarian follicles and follicular diameter in these offspring (Khaksar et al., 2013). Similarly, overnutrition correlates with poor oocyte development in humans (Larsen, 2015), and a high-fat diet impairs oocyte quality and embryo development in mice (Bermejo-Alvarez et al., 2012). However, to understand the possible mechanisms involved in decreased fertility rates observed in OD/HFD female rats, a study focused on morphologic and molecular alterations in the ovaries of these offspring is underway in our research group.

Despite low fertility rates, when considering only the female rats that reached full-term pregnancy, the indirect parameter evaluated to study the delivered oocyte number-—the number of corpora lutea—presented no difference among experimental groups, showing no ovulation changes in this period. Nevertheless, OD/HFD rats showed a decrease in the number of implantations and live fetuses and higher rates of embryonic losses before implantation. Studies with early embryos (from two cells to blastocyst) in culture with high mean glucose concentration (Fraser et al., 2007) or *in vivo* study using hyperglycemic mother rats to evaluate their pre-embryos (Bueno et al., 2014) showed damages caused by hyperglycemic exposure during early embryonic development. This led to reduced cell numbers and increased apoptosis rates, which caused developmental delay and affected the success of embryonic implantation. Then, the lower number of implantations and live fetuses confirmed in the OD/HFD group might be related to embryonic complications in the early development, leading to higher percentages of preimplantation losses, which contributed to lower litter weight, compromising especially the female fetus growth.

Our results about glycemic metabolism surprisingly showed that the OD/HFD pregnant group presented no difference in insulin sensitivity, but the OD/SD rats had a decreased insulin sensitivity, being both groups performed by the quantitative insulin sensitivity check index (QUICKI). According to Katz et al. (2000), QUICKI was developed as an alternative method for glucose clamps. This method presented the best overall linear correlation with the gold standard clamp measurement, and it was more precise than the homeostasis model assessment of insulin resistance (HOMA-IR). Nevertheless, the authors disclose that QUICKI has limitations as the difficulty in applying it to type 1 diabetic individuals (without endogenous insulin secretion) or in patients with type 1 or type 2 decompensated diabetes (Katz et al., 2000). Considering the diabetic offspring, the OD/SD and OD/HFD rats had higher fasting glucose levels and AUC, confirming intolerance glucose status and higher insulin concentrations, confirming insulin resistance. Another study from our research group with control virgin female pups (OC) and diabetic offspring (OD) that received a standard diet (SD) or high-fat diet (HFD) from weaning until 120 days of life showed the presence of glucose intolerance in the OC/HFD, OD/SD, and OD/HFD rats, an increase in insulin synthesis, and the presence of insulin resistance before pregnancy on OC/HFD and OD/SD rats (Paula et al., 2021). The apparent conflicting result might indicate that these animals possibly

maintained an adequate adaptive pancreatic response in pregnancy, ameliorating the glucose intolerance presented before pregnancy. Normal pregnancy is associated with increased insulin resistance as a metabolic adaptation to the nutritional demands of the placenta and fetus. Insulin resistance is normally compensated by an adaptive increase in pancreatic β-cell mass together with enhanced glucose-stimulated insulin release (Moyce and Dolinsky, 2018). Studies have shown an increase in the number of cells and/or size of the pancreatic islet to compensate for the physiological demands of pregnancy (Fujimoto and Polonsky, 2009). The increased β-cell mass depends on a combination of the proliferation of existing β-cells, hypertrophic expansion, differentiation of resident progenitor β-cells, or islet cell transdifferentiation accompanied by a temporary decrease in apoptosis (Butler et al., 2010; Ernst et al., 2011; Bonner-Weir et al., 2012; Moyce and Dolinsky, 2018; Szlapinski and Hill, 2021). Thus, an overview of our results suggests the loss of an adequate adaptation of β-cells in OD/HFD rats during pregnancy, suggesting that QUICKI was not a good index in these cases.

The crosstalk among the maternal pancreas, placenta, and peripheral tissues is essential, and adiponectin and leptin are hormones involved in this signaling. The intrauterine exposure to hyperglycemia and placental methylation of leptin and adiponectin might lead to the development of leptin and insulin resistance and alter appetite circuits leading to postnatal hyperphagia, decreased satiety, and subsequent development of metabolic syndrome (Greco et al., 2019). However, our results showed no alterations in leptin concentration and food consumption related to total energy intake, showing no alterations in these circuits. The data about body weight gain and energy intake are controversial in several studies using different types of high-fat diets. According to the high-fat diet characteristics, overeating is induced, which may work together to promote the storage of dietary fat (Schack-Nielsen et al., 2010; Hull et al., 2011; Donovan et al., 2013). Besides, only female pups coming from control dams fed with a high-fat diet showed higher relative fat weight at term pregnancy, but it had no influence on adiponectin and leptin levels in maternal serum in the OC/HFD, OD/SD, and OD/HFD, corroborating Desai et al. (2014). There were no reported changes in the leptin and adiponectin concentrations measured in the placental tissue. Despite these results, the fetuses from OD/SD and OD/HFD rats had increased leptin serum levels, and these data do not corroborate the literature. Clinical studies have been addressing that leptin levels in the umbilical cord and newborns are positively correlated to birth weight and adiposity (Misra & Trudeau, 2011; Misra et al., 2013; Josefson et al., 2014; Ökdemir et al., 2018; Stefaniak et al., 2019). However, our results showed lower birth weight (SGA fetuses) and hyperleptinemia in the second generation of diabetic rats, which was not what we expected. These conflicting data observed in our rat model can be related to the low-fat percentage at birth in these newborns. Leptin plays an important role in controlling satiety and is an indicator of body fat mass. It is important to consider that this peptide may be involved with fetal growth in the third trimester when human fetal fat is deposited (Desai et al., 2014). Thus, in humans, the newborn fat depot occurs in the intrauterine milieu, however, in rats, this takes place after birth (Herrera and Amusquivar, 2000), which might have influenced the lower birth weight in our laboratory animals in this experiment. Additionally, higher serum leptin levels at birth should predispose to increased blood pressure in adult rats since leptin is well known to be a relevant marker and mediator of vascular dysfunction and hypertension (Gonzalez et al., 2013; Schinzari et al., 2013).

The classification of birth weight showed a significant increase in the proportion of small fetuses for gestational age and a decrease in the proportion of fetuses with adequate weight for gestational age in all groups when compared to control. In humans, high maternal body mass index (BMI) is associated with fetal overgrowth and macrosomia, and an increased risk of premature birth, instead maternal underweight increases the risk of low birth weight and small fetuses for gestational age (Liu et al., 2019). Regardless of this controversial finding, adequate placental function is directly responsible for fetal growth. Our study verified impaired placental efficiency in OD/HFD rats. The placenta plays a role underlying fetal programming and, therefore, is in part related to the origin of the development of health and disease (DOHaD). Both maternal glucose and nutrients from the high-fat diet are transmitted from the maternal to the fetal compartment through the placenta. This dynamic process might lead to changes in long-term health and disease outcomes in the first generation. In addition, the second generation might also be affected and may undergo reprogramming during the embryonic period in the primordial germ cells, which contributes to genetic and epigenetic information in the second generation (Dunn et al., 2011).

Our study showed that fetal restriction was predominant in female fetuses from both groups of dams fed a high-fat diet. The sex of the embryo affects the size of both the fetus and the placenta and the ability of the placenta to respond to adverse stimuli (Clifton, 2010; Mao et al., 2010; Gabory et al., 2013). In our study, this suggests that a high-fat diet interferes differently with male and female placental tissue, and thus, the differences in how male and female placentas cope with stressful conditions indicate that this tissue should also be taken into account if we want to understand how it contributes to health and disease later in life.

In conclusion, our findings show that fetal programming caused by maternal diabetes or lifelong high-fat diet consumption, in isolation, leads to similar repercussions in pregnant rats. In addition, the association of both conditions was responsible for glucose intolerance and oxidative stress in the first generation and increased fetal leptin levels in the second generation. Thus, our findings show both the F1 and F2 generations were negatively affected after the maternal hyperglycemic intrauterine environment and exposure to a high-fat diet from weaning until the end of pregnancy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for the Use of Animals of Botucatu Medical School, which approved all the methods adopted in this study (Protocol CEUA Number: 1218/2017).

AUTHOR CONTRIBUTIONS

All authors who have reviewed the manuscript agree with its contents and consent to its publication and that no other person has satisfied the criteria for authorship. We further confirm that all of us have approved the order of authors listed in the manuscript. YS, VP, and RM-S performed experiments; YS and DD drafted the manuscript and analyzed the data; JC performed statistical analysis; JC, YS, FG, GV, and DD interpreted the statistical data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.818621/full#supplementary-material

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1. **Paula VG,** Cruz LL, Sene LB, Gratão TB, Soares TS, Moraes-Souza RQ, Damasceno DC, Volpato GT. Maternal-fetal repercussions of *Phyllanthus niruri* L. treatment during rat pregnancy. J Ethnopharmacol. 2020 May 23;254:112728. doi: 10.1016/j.jep.2020.112728. Epub **2020** Mar 4.PMID: 32145330.

2. Cruz LL, Ferreira Silva BS, Araujo GG, Leal-Silva T, **Paula VG**, Souza MR, Soares TS, Moraes-Souza RQ, Monteiro GC, Lima GPP, Damasceno DC, Volpato GT. Phytochemical and antidiabetic analysis of *Curatella americana* L. aqueous extract on the rat pregnancy. J Ethnopharmacol. **2022** Apr 12;293:115287. doi: 10.1016/j.jep.2022.115287. Online ahead of print.PMID: 35421527.

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Divulgação e eventos científicos durante o Doutorado relacionadas ao projeto de pesquisa de doutorado

Apresentação de trabalhos como autora principal:

1. **Paula, V.G.**; Sinzato Y.K.; Cruz, L.L.; Galego-Souza, F.Q.; Aquino, A.M; Scarano; W.R., Corrente, J.E; Volpato, G.T; Damasceno, D.C. Mitochondrial function and apoptosis on ovaries of adult rats exposed to maternal hyperglycemia and submitted to a high-fat diet after weaning. **I Congresso DOHaD Brasil**, 2022.
2. **Paula, V.G**.; Sinzato Y.K; Souza, R.Q.M. ; Cruz, L.L ; Kloppel, E. ; Galego-Souza, F.Q.; Barco, V.S.; Saullo, C.M.; Vesentini, G.; Volpato, G.T.; Damasceno, D.C. Repercussion of Maternal Diabetes and Post-Weaning High-Fat Diet Consumption in Laboratory Animals. In: **18th Annual World Congress on Insulin Resistance Diabetes & Cardiovascular Disease**, 2021, Los Angeles. Part of special issue: Abstracts from the 18th Annual World Congress on Insulin Resistance Diabetes & Cardiovascular Disease, 2021. v. 116.
3. **Paula, V.G.**; Cruz, L.L.; Sinzato Y.K.; Colombelli, K.T.; Cruz, L.A.J.; Volpato, G.T.; Damasceno, D.C. Diabetes or protein restriction diet-induced fetal programming impair reproductive outcomes and fetal viability in rats. **International Symposium on Reproductive Health**. In: Clin. Exp. Obstet. Gynecol. 2021 v. 48(3), 733-777.
4. **Paula, V.G.**; Sinzato Y.K.; Kloppel, E.; Souza, R.Q.M.; Volpato, G.T.; Damasceno, D.C. Diabetes-induced fetal programming and post weaning consumption of high fat diet in rats – preliminary results. **4th Meeting of Latin American DOHaD Chapter, 4th International Symposium of DOHaD and Stress**. 2020.
5. **Paula, V. G**.; Sinzato Y.K; Souza, R. Q. M.; Soares, T. S.; Damasceno D.C.; Volpato, G. T. Avaliação glicêmica e imunológica de ratas expostas à hiperglicemia intraútero e dieta hiperlipídica após o desmame. **XI Encontro de Pós-graduação da Faculdade de Medicina de Botucatu UNESP.** 2020.
6. **Paula, V.G**.; Sinzato Y.K.; Volpato, G.T.; Souza, R.Q.M.; Soares, T.S. ; Kloppel, E.; Miranda, C.A.; Damasceno, D.C. Long-term Effects of Diabetes-induced Fetal Programming and Postnatal High-fat Diet Exposure on Glycemia. In: **6th International Symposium on Metabolic Programming and Microbiome 3rd Meeting of Ibero-American DOHaD chapter**, 2018, Cancún. Book of Abstracts, 2018. v. 1.
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Certificado de menção honrosa pela apresentação de trabalho no I Congresso DOHaD Brasil, na XXXVI Reunião Anual da FESBE e no LVII Congresso Anual da SBFIS



XXXVI REUNIÃO ANUAL DA FESBE
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05 A 09 DE AGOSTO DE 2022

Tema Central: "Ciência e Tecnologia, Caminhos para a Independência"

MENÇÃO HONROSA

Certificamos que **Verônyca Gonçalves Paula** recebeu menção honrosa pela apresentação oral do trabalho intitulado: "*Mitochondrial function and apoptosis in ovaries of adult rats exposed to maternal hyperglycemia and submitted to a high-fat diet after weaning*" de coautoria de: Yuri Karen Sinzato, Larissa Lopes Cruz, Franciane Quintanilha Gallego, Ariana Musa de Aquino, Wellerson Rodrigo Scarano, José Eduardo Corrente, Gustavo Tadeu Volpato e Débora Cristina Damasceno, no **I Congresso DOHaD Brasil**, na **XXXVI Reunião Anual da FESBE** e no **LVII Congresso Anual da SBFIS** de forma virtual de 05 a 09 de agosto de 2022.

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