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Evaluation of senescence, proliferation, and cell death mechanisms in the liver of pregnant rats exposed to a high-fat diet

Avaliação dos mecanismos de senescência, proliferação e morte celular no fígado de ratas prenhes expostas a uma dieta rica em gordura

Evaluación de los mecanismos de senescencia, proliferación y muerte celular en el hígado de ratas preñadas expuestas a una dieta rica en grasas

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Abstract
This study aimed to evaluate whether the processes of proliferation, senescence, and cell death are involved in the exacerbation of liver size increase at the end of pregnancy in rats subjected to a high-fat diet. Sprague Dawley rats (n=9/group), which consumed a standard diet (control) and a high-fat diet (HFD) from weaning to the end of pregnancy. These rats were mated, consumed the diet, and at the end of 21 days of pregnancy, liver samples were collected for evaluation of immunostaining for Ki-67 (cell proliferation), cleaved caspase-3 (cell death) and Sudan black (cellular senescence). Student's t-test and Fisher's Exact test were used for comparison, considering $p<0.05$ as statistical significance. The local Ethics Committee on the Use of Animals approved the procedures. The HFD rats showed no change in the percentage of staining for lipofuscin, a lower rate of cells positive for Ki-67, and a higher rate for cleaved caspase-3 in the liver. Therefore, a high-fat diet promotes less proliferation and greater death in the hepatocytes, leading to possible functional damage to the liver.
Keywords: Liver; High-fat diet; Animals; Cell proliferation; Cell death.

Resumo
Este estudo teve como objetivo avaliar se os processos de proliferação, senescência e morte celular estão envolvidos na exacerbação do aumento do tamanho do fígado ao final da prenhez de ratas submetidas à dieta hiperlipídica. Ratas

Sprague Dawley (n=9/grupo) consumiram dieta padrão (controle) e dieta hiperlipídica (DHL) do desmame até o final da prenhez. As ratas foram acasaladas, continuaram a consumir as mesmas dietas e, ao final do 21º dia de prenhez, amostras de fígado foram coletadas para avaliação da imunomarcação para Ki-67 (proliferação celular), caspase-3 clivada (morte celular) e Sudan black (senescência celular). O teste t de Student e Teste Exato de Fisher foram utilizados para comparação, considerando $p<0,05$ como significância estatística. O Comitê de Ética em Uso de Animais local aprovou todos os procedimentos. As ratas DHL não apresentaram alteração na porcentagem de coloração para lipofuscina, menor taxa de células positivas para Ki-67 e maior taxa para caspase-3 clivada no fígado. Portanto, uma dieta rica em gordura promove menor proliferação e maior mortalidade nos hepatócitos, levando a possíveis danos funcionais ao fígado.
Palavras-chave: Fígado; Dieta hiperlipídica; Animais; Proliferação celular; Morte celular.

Resumen
Este estudio tiene como objetivo evaluar los procesos de proliferación, senescencia y muerte celular implicados en la exacerbación del aumento del tamaño del hígado al final del embarazo en ratas sometidas a una dieta hiperlipídica. Ratas Sprague Dawley (n=9/grupo) recibieron una dieta estándar (grupo control) o una dieta hiperlipídica (grupo DHL) desde el destete hasta el final del embarazo. Las ratas fueron apareadas y continuaron con las mismas dietas. Al final del día 21 del embarazo, se recolectaron muestras de hígado para la evaluación de la inmunomarcación de Ki-67 (proliferación celular), caspasa-3 clivada (muerte celular) y Sudan Black (senescencia celular). Se utilizó la prueba t de Student y Teste Exacto de Fisher para las comparaciones, considerando $p<0,05$ como significancia estadística. Todos los procedimientos fueron aprobados por el Comité de Ética en el Uso de Animales de la institución. Las ratas del grupo DHL no mostraron alteraciones en el porcentaje de tinción para lipofuscina, presentaron una menor proporción de células positivas para Ki-67 y una mayor proporción de células positivas para caspasa-3 clivada en el hígado. Por lo tanto, una dieta rica en grasas promueve una menor proliferación y una mayor muerte de los hepatocitos, lo que puede generar posibles daños funcionales en el hígado.
Palabras clave: Hígado; Dieta rica en grasas; Animales; Proliferación celular; Muerte celular.

1. Introduction

High-fat diets are characterized by a high-fat content, which has been widely used in experimental models to simulate conditions of obesity and metabolic syndrome. Chronic exposure to this type of diet can trigger systemic changes, like insulin resistance, inflammation, and oxidative stress. These disorders not only compromise maternal metabolism but also negatively affect tissue homeostasis in different organs and systems (Moraes-Souza et al., 2021). The use of rats as an experimental model allows a controlled investigation of the systemic and local effects of the high-fat diet. Additionally, this model contributes to an evaluation of cellular mechanisms in tissues of interest, such as the liver, during a critical period of plasticity and intense cellular activity. The liver is a dynamic and heterogeneous organ and is considered a critical center for the development of numerous processes, including macronutrient metabolism, growth signaling pathways, lipid and cholesterol homeostasis, and other mechanisms important for physiological control (Trefts et al., 2017).

Our research laboratory found that excessive fat intake by rats from weaning to adulthood (120 days) impairs normal cellular renewal and repair mechanisms, interfering with processes such as autophagy, apoptosis, and cellular senescence. These cellular events play a fundamental role in maintaining tissue integrity and in the hepatic adaptive response to insults (Cruz et al., 2025). These changes can lead to liver injury, including metabolic dysfunction-associated fatty liver disease (MAFLD), which is the most common cause of liver disease and responsible for increased global mortality (Eslam et al., 2020; Jiang et al., 2025). In addition, these same rats showed impairment of adipose tissue (Saullo et al., 2021).

The use of laboratory animals is relevant because ethical restrictions in humans must be considered due to the need for consecutive liver biopsies to perform more comprehensive analyses (Kwanten et al., 2014). Rodent organs, including the microarchitecture of the liver, are very similar to those of humans (Shi et al., 2015; Kruepunga et al., 2019). Therefore, evaluating the mechanisms by which an insult from a high-fat diet in the liver of pregnant rats is very important because there are no studies on this topic. One of the mechanisms that can be analyzed is the framework of cell division and death. Adequate cell proliferation is essential for tissue regeneration, while apoptosis acts as a physiological mechanism for removing damaged

cells. Cellular senescence, a state of permanent arrest of the cell cycle, can be triggered by oxidative stress or irreparable damage to DNA. The interaction between these processes reflects the balance between cell regeneration and damage and is particularly relevant in conditions of metabolic aggression, such as that imposed by high-fat diets (Moreno-Gonzalez et al., 2024).

This study aimed to evaluate whether the processes of proliferation, senescence, and cell death are involved in the exacerbation of liver size increase at the end of pregnancy in rats subjected to a high-fat diet. Understanding these changes may contribute to elucidating the cellular mechanisms involved in maternal responses to nutritional challenges during gestation. This study aimed to evaluate whether the processes of proliferation, senescence, and cell death are involved in the exacerbation of the increased size of the liver at the end of pregnancy in rats subjected to a high-fat diet. Specifically, we intend to determine the percentage of liver cells immunostained for Ki-67 and cleaved caspase-3, and the ratio of the area stained with Sudan Black to the total area of liver.

2. Methodology

2.1 Animals

The experimental procedures applied in this study are by the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and were approved by the Animal Use Ethics Committee (CEUA) of the Botucatu School of Medicine (Protocol Number: 1218/2017). Male and female Sprague Dawley rats, approximately 120 days old [250 and 220 grams (g) of body weight, respectively], were used. The animals were purchased from the UNICAMP Animal Center (CEMIB) and kept in our vivarium free of pathogens and under controlled conditions, with temperature (22±2ºC), humidity (50±10%), and light/dark cycle (12/12h). The animals were randomly allocated to polypropylene cages lined with wood shavings, with a capacity for three animals per cage. Environmental enrichment was performed by introducing crumpled paper balls into each box (Simpson & Kelly, 2011) to reduce stress. Filtered water and feed were offered ad libitum.

2.2 Study Design

This is an experimental, cross-sectional, analytical study and of qualitative nature for instance to evaluate micrographs and quantitative nature in values and percentages (Pereira et al., 2018) using simple descriptive statistics with mean values and standard deviations (Shitsuka et al., 2014; Akamine & Yamamoto, 2009) and statistical criteria (Vieira, 2021; Bekman & Costa Neto, 2009).

2.3 Experimental Sequence of the Parental Generation

Females were mated with males (3 females to 1 male) in the late afternoon, with a maximum duration of 15 days. The following morning, when sperm were found in the vaginal smear, pregnancy was confirmed and this was designated as day 0 of pregnancy. Rats that did not mate after 15 consecutive days were considered infertile and excluded from this study (Sinzato et al., 2021). During pregnancy, the rats were kept in individual boxes and the offspring, obtained through vaginal delivery, were used as the focus of this research. After birth, due to the number of functional teats and to ensure equal access to maternal milk, no more than eight pups per litter were kept until weaning (PND 22), maintaining as close as possible to a sex ratio of 1:1, i.e., 4 males and 4 females (Zambrano et al., 2005). When the litter had up to eight pups, if both sexes were present, there was no manipulation. When the litters exceeded 8 pups, males and females were randomly selected. If the litter had less than eight pups per mother, that mother and her litter were excluded from this experiment, but kept in our vivarium and used in other studies.

2.4 Treatment with High-Fat or Standard Diet

After weaning, female offspring were distributed into two experimental groups (minimum n = 5 animals/group), following a random draw. From weaning until the end of pregnancy, these rats received different diets (standard or high-fat diet) (Paula et al., 2022; Sinzato et al., 2022), being distributed as follows: 1) Control Group: fed a standard diet (SD); 2) HFD Group: fed a high-fat diet (HLD). The standard diet contained: 28.5% kcal of protein, 62.6% kcal of carbohydrates, and 8.7% kcal of fat - commercial rat chow, Purina®, Brazil) and HFD: 23.4% kcal of protein, 46.6% kcal of carbohydrates, and 30% kcal of fat) (Paula et al., 2022). HFD was prepared by hand at the Experimental Research Unit (UNIPEX) of the Botucatu School of Medicine (FMB/UNESP), using the following ingredients: soybean meal, sorghum, soybean hulls, cornstarch, refined sugar, lard, minerals (iron, biotin, vitamin B12, vitamin D, vitamin B6, vitamin E, vitamin A, selenium and potassium) and salt. These ingredients were ground, mixed, and offered as pellets. After preparation, the feed was kept refrigerated until consumption. Since the diets presented visual characteristics that allowed them to be easily distinguished, it was not possible to randomly house the animals and to blind the caretakers and/or researchers.

2.5 Oral Glucose Tolerance Test (OGTT)

At 115 days of age, after 6 hours (h) of fasting, the OGTT was performed to assess glucose tolerance. For this purpose, after six hours of fasting, a drop of blood was collected from the rat tail for glycemic determination (time 0). Immediately after, the rats received a 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 minutes (min) after administration of the glucose solution, glycemia levels were determined using a conventional glucometer (Tai, 1994; Gallego et al., 2024). The evaluation of glycemia as a function of time was estimated by calculating the area under the curve (AUC), using the trapezoidal method (Tai, 1994).

2.6. Mating of Rats

From day 120 of life, females were weighed and then mated with males of the same lineage (3 females to 1 male) in the late afternoon, with a maximum duration of 15 days. The following morning, when sperm were found in the vaginal smear, pregnancy was confirmed and this was designated as day 0 of pregnancy. Rats that did not mate after 15 consecutive days were considered infertile and excluded from this study (Sinzato et al., 2021). The rats were kept in individual boxes for monitoring throughout pregnancy.

2.7 Euthanasia and Death of Rats for Liver Sample Collection

At the end of pregnancy (21st day), the rats were anesthetized with sodium thiopental (Thiopentax® - dose of 120 mg/kg, intraperitoneal) and, after loss of reflexes, were decapitated and submitted to laparotomy for liver sample collection. The liver was weighed on a semi-analytical scale. The relative weight of the liver was calculated using the equation: (organ weight/body weight of each animal) x 100, with the result expressed in grams/100 grams of live weight (g/100g bw). Subsequently, the largest lobe (left) of each animal was sliced and stored in formaldehyde for histological analysis.

2.7.1 Histological Detection of Lipofuscin Accumulation by Sudan Black

To evaluate the accumulation of lipofuscin, indigestible residues in lysosomes that can characterize cellular senescence, fragments of liver tissue were fixed in 10% formaldehyde (P.A.) for 24 h, dehydrated in progressive concentrations of alcohol and embedded in paraffin. Histological sections of 5 μm were made in the Department of Pathology of the School of Veterinary Medicine and Animal Science of Botucatu (FMVZ_Unesp) with the aid of a microtome.

Subsequently, the slides of liver tissue underwent a deparaffinization process (15 min in xylene I, 15 min in xylene II, 5 min in absolute alcohol I, 5 min in absolute alcohol II, 5 min in absolute alcohol III, 5 min in 95% alcohol and 5 min in 85% alcohol). After deparaffinization, they were washed four times in distilled water and stained in a humid chamber with 500 µL of 0.3% Sudan Black (Êxodo Científica®, Brazil) for 30 min. They were then washed in distilled water to remove excess dye, dried for 30 min at room temperature, and counterstained with Harris hematoxylin for 3 min. After staining, the slides were mounted using a coverslip and resin, and the analysis was performed blindly under an optical microscope. The images were captured using a computerized imaging system (KS-300 Software, version 3.0, Zeiss®, Germany), which receives an image from a digital camera (CCD-IRIS/RGB, Sony®, Japan) coupled to the microscope (DMR, Leica®, Germany). The analyses were performed according to the methodology of Yzydorczyk et al. (2017) and the area marked for lipofuscin staining was quantified using ImageJ software (National Institute of Health, USA). The results were presented as the ratio of the lipofuscin area to the total area.

2.7.2 Immunostaining for Ki-67

To assess cell proliferation, the liver tissue slides underwent a deparaffinization process (15 min in xylene I, 15 min in xylene II, 5 min in absolute alcohol I, 5 min in absolute alcohol II, 5 min in absolute alcohol III, 5 min in 95% alcohol and 5 min in 85% alcohol). After deparaffinization, they were washed four times in distilled water and subsequent antigen retrieval was induced in a pressure cooker (Elite Bistro®) with citrate buffer (pH 6.0) for 30 minutes. After cooling for one hour, the slides were washed again with distilled water four times and a protein blocker (Dako®) was added for 30 minutes. The antibody for Ki-67 (Elabscience Biotechnology Inc®, USA, code #22027) was diluted 1:100 and incubated overnight in the refrigerator. After incubation of the primary antibody, the secondary antibody (Histofine®) was added for 30 minutes at 27°C. For the peroxidase reaction, the chromogen DAB (3,3-diaminobenzidine) was used for 3 minutes at room temperature. The slides were counterstained with Harris hematoxylin for 1 minute. After staining, the slides were mounted using a coverslip and liquid resin. The images will be captured by a digital camera (CCD-IRIS/RGB, Sony®, Japan), coupled to the microscope (DMR, Leica®, Germany), and analyzed blindly using the ImageJ® software (NIH, USA).

2.7.3 Immunostaining for Cleaved Caspase-3

To assess cell death, the liver tissue slides underwent a deparaffinization process (15 min in xylene I, 15 min in xylene II, 5 min in absolute alcohol I, 5 min in absolute alcohol II, 5 min in absolute alcohol III, 5 min in 95% alcohol and 5 min in 85% alcohol). After deparaffinization, they were washed four times in distilled water and subsequent antigen retrieval was induced in a pressure cooker (Elite Bistro®) with citrate buffer (pH 6.0) for 30 minutes. After cooling for 30 minutes, the slides were washed again with distilled water four times, and a protein block (Dako®) was added for 20 minutes in an oven at 27ºC. The Cleaved Caspase-3 antibody (Cell Signaling Technology®, USA, D175) was diluted 1:200 and incubated overnight under refrigeration. After incubation of the primary antibody, the secondary antibody (Histofine®) was added for 30 minutes at 27°C. For the peroxidase reaction, the chromogen DAB (3,3-diaminobenzidine) was used for 3 minutes at room temperature. The slides were counterstained with Harris hematoxylin for 1 minute. After staining, the slides were mounted using a coverslip and liquid resin. The images will be captured by a digital camera (CCD-IRIS/RGB, Sony®, Japan), coupled to the microscope (DMR, Leica®, Germany), and analyzed blindly using the ImageJ® software (NIH, USA).

2.8 Statistical Analysis

All analyses were performed with the assistance of the Biostatistics specialist from the Research Support Office

(EAP) of the Botucatu School of Medicine, Unesp. Data were presented as mean ± standard deviation and percentage. Comparisons between groups were performed using the Student's t-test and Fisher's exact test for proportion data. The minimum confidence limit of 95% ($p<0.05$) was used. All analyses were performed using the SAS for Windows program, v.9.4.

3. Results

General Characteristics of the Experimental Groups

At 115 days of life (adulthood), the rats in the high-fat diet (HFD) group showed a high area under the curve (AUC), obtained from the glycemic data during the 120 min of the OGTT, and an increase in body weight compared to the control rats (Table 1).

No statistically significant differences were observed in liver weight, and in serum concentrations of total cholesterol, triglycerides, VLDL-cholesterol, ALT, AST, de Ritis index, and TyG index between the study groups (data obtained in another study from our laboratory - Cruz et al., 2025 – Table 1).

Table 1 - Body weight, liver weight, and biochemical parameters of blood samples at 120 days of life of rats that consumed a standard diet (Control) and a high-fat diet (HFD) from weaning until the end of pregnancy.

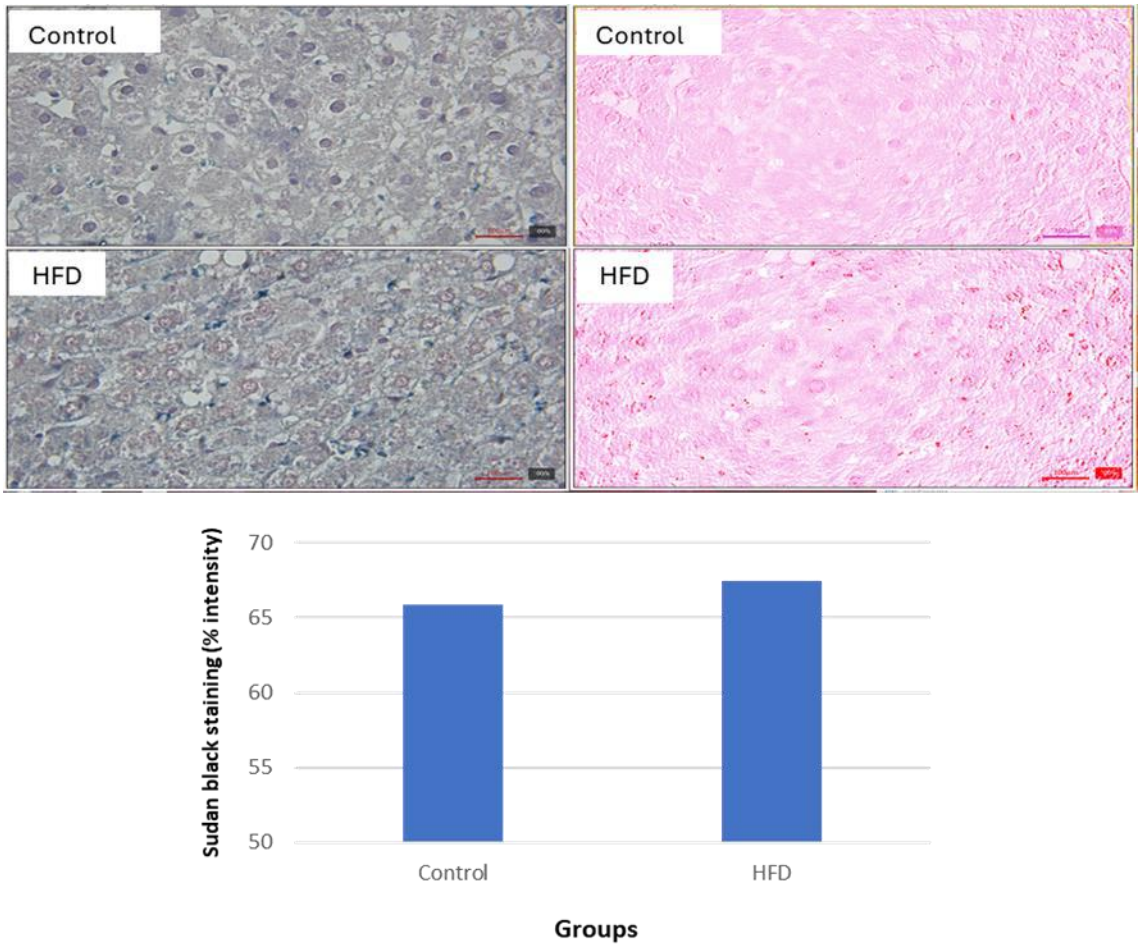
Variables	**Groups**	
	***Control* (n= 9)**	***HFD* (n=9)**
Fasting glycemia (total blood) (mg/dL)	108.0 ± 9.2	118.6 ± 11.7
Area under the curve (mg/dL*120 min)	13217.1 ± 618.0	16651.9 ± 1291.8*
Serum total cholesterol (mg/dL)	58.1 ± 4.5	55.2 ± 4.0
Serum triglyceride (mg/dL)	27.6 ± 13.9	32.2 ± 10.2
Serum VLDL-cholesterol (mg/dL)	5.5 ± 2.8	6.4 ± 2.0
TyG index	7.2 ± 0.5	7.5 ± 0.3
AST (U/L)	24.8 ± 3.9	23.2 ± 5.4
ALT (U/L)	12.6 ± 1.8	11.9 ± 2.0
Ritis index (AST/ALT)	2.0 ± 0.4	2.0 ± 0.6
Liver relative weight (g/100g)	3.2 ± 0.4	3.0 ± 0.2
Body weight (gram - g)	254.3 ± 15.2	266.3 ± 11.9*

Legend: VLDL, very low-density lipoprotein; TyG, fasting triglyceride-glucose product; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
Values expressed as mean ± standard deviation.
*p<0.05 - compared to the control group (Student's t-test).
Source: Original researcher data (2025).

Assessment of the Presence of Lipofuscin in Liver Cells

Lipofuscin is a pigment that accumulates lipids and intracellular oxidized proteins. The presence of this pigment was analyzed at the end of pregnancy using Sudan black staining. It was found that the percentage of area stained for lipofuscin did not differ in liver cells in the HFD group compared to the control group (Figure 1).

Figure 1 - Sudan black staining in the liver of rats and representative photomicrographs of histological sections of the experimental groups.

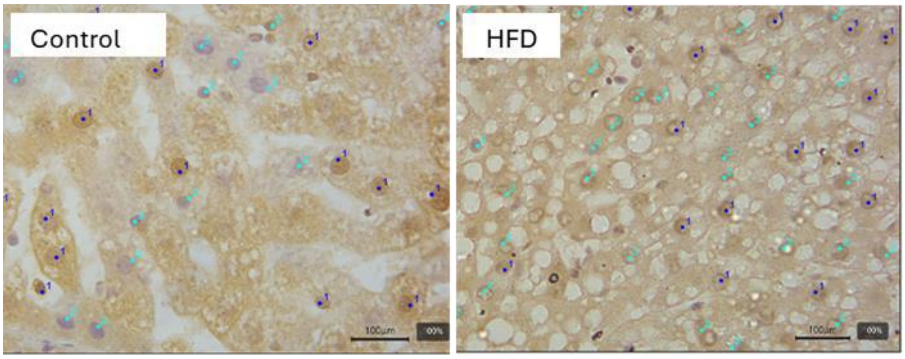


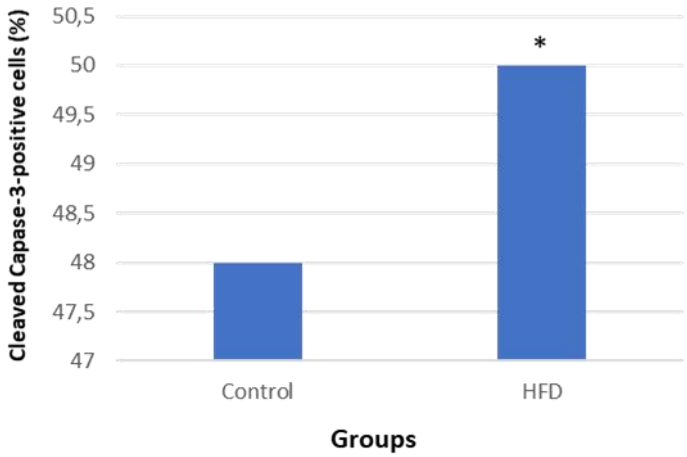
The bars represent the mean intensity of staining for sudan black (lipofuscin) per microscopy field (n=5 rats/10 photos/group). The images correspond to the respective groups: A) Control Group; B) HFD Group (rats that consumed a high-fat diet) from weaning until the end of pregnancy. Magnification 40X.
Data presented as percentage.
$p>0.05$ – no statistically significant difference (Fisher´s Exact Test).
Source: Original researcher data (2025).

Analysis of Cell Proliferation in Liver

Regarding cell proliferation, animals that received a high-fat diet from weaning to the end of pregnancy had a lower percentage of cells stained for Ki-67 compared to the control group (Figure 2).

Figure 2 - Immunostaining for Ki-67 in the liver of rats and representative photomicrographs of histological sections of the experimental groups.



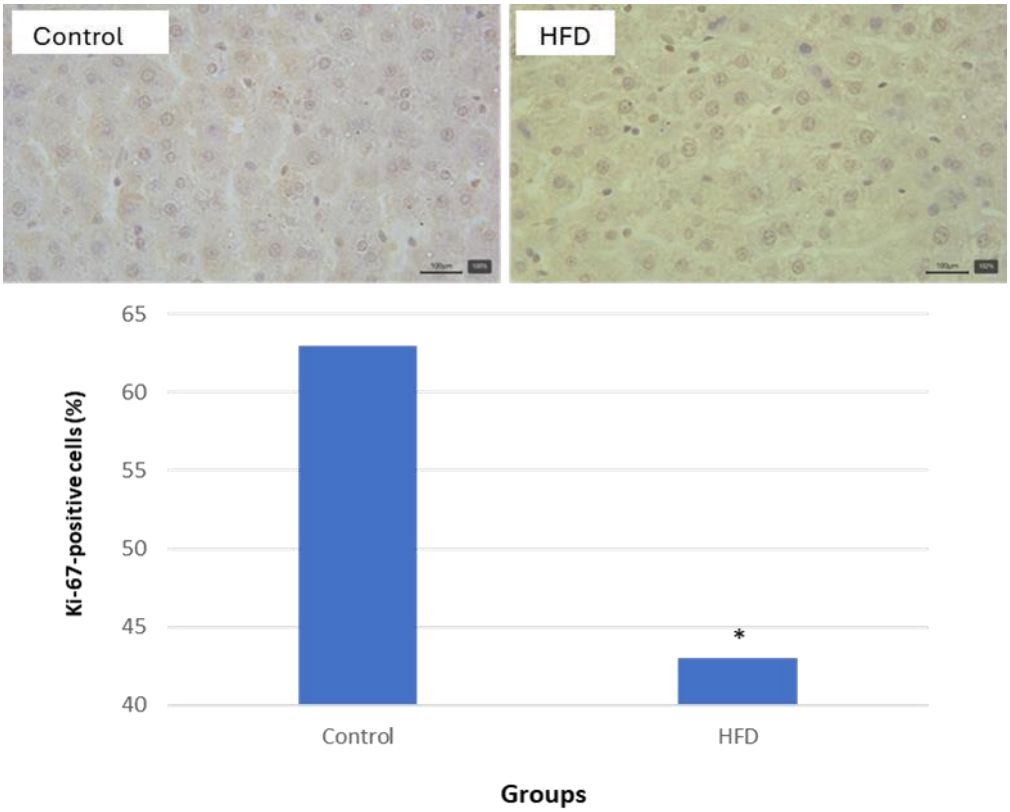


The bars represent the mean staining for Ki-67 (in percentage of stained cells) per microscopy field (n=5 rats/10 photos/group). The images correspond to the respective groups: A) Control Group; B) HFD Group (rats fed a high-fat diet) from weaning until the end of pregnancy. Immunostaining with anti-Ki-67 antibody for analysis of cell proliferation. Magnification 40X.
Data presented as percentage.
*p<0.05 - compared to the control group (Fisher´s Exact Test).
Source: Original researcher data (2025).

Assessment of the Presence of Apoptosis in Liver Cells

Figure 3 shows that at the end of pregnancy, females in the HFD group had a higher percentage of liver cells immunostained for cleaved Caspase-3 compared to rats fed a standard diet (Control).

Figure 3 - Immunostaining for cleaved caspase-3 in the liver of rats and representative photomicrographs of histological sections of the experimental groups.



Source: Original researcher data (2025).

4. Discussion

Our findings showed that prolonged exposure to a high-fat diet, from weaning to the end of pregnancy, caused significant changes in the liver of rats, as evidenced by histological and immunohistochemical analyses. Among the markers evaluated, there was no change in the percentage of lipofuscin staining, a reduction in Ki-67 expression, and an increase in cleaved caspase-3 staining. Animals that consumed the high-fat diet presented altered glucose homeostasis and diabetes in adulthood, which reinforces the importance of a healthy diet.

Lipofuscin is a marker associated with the accumulation of cellular degradation residues, often related to chronic oxidative stress and cellular aging. The absence of changes in its expression suggests that, during the period evaluated, there was no substantial accumulation of chronic oxidative damage, which may indicate an early or compensated stage of liver injury (Xu et al., 2020). On the other hand, reduced Ki-67 staining, a nuclear antigen expressed in proliferating cells, indicates an impairment of the regenerative capacity of liver tissue. Liver regeneration is an essential process for maintaining hepatic homeostasis in the face of cell damage and wear, especially in a situation of metabolic stress, such as that induced by high-fat diets. Furthermore, decreased cell proliferation may compromise the liver's ability to respond to pregnancy, since maternal hepatocytes had an increased proliferation rate as pregnancy progresses in order to meet the mother's needs and promote fetal growth (Bustamante et al., 2010).

Additionally, increased expression of cleaved caspase-3, a classic marker of apoptosis, reveals an intensification of the programmed cell death process, since high exposure to fat can impair mitochondrial function and cause increased mitochondrial membrane permeability and caspase activation (Bruce et al., 2009). These changes lead to significant cell loss, which, associated with the low proliferation rate, can result in a reduction in functional liver mass. These hepatic changes, together, lead to a state of imbalance in cell turnover in the liver, with a predominance of cell death over regeneration. Functionally, this scenario can progressively compromise the integrity and functioning of the organ, favoring the development of metabolic liver diseases, such as MAFLD, nonalcoholic steatohepatitis (NASH), and, in more advanced stages, cirrhosis and hepatocellular carcinoma (Eslam et al., 2020). The study presents positive points, such as the analysis of the balance between the rates of cell proliferation and death, and the importance of a balanced diet during crucial periods of development, such as preconception and gestation. However, there are gaps that need to be filled, such as the relationship between these rates and the analysis of inflammatory markers and oxidative stress during pregnancy to highlight the relationship between the cellular mechanisms and repercussions from the hyperlipidic insult.

5. Conclusion

Therefore, our results indicate that the chronic hyperlipidic diet during the critical period of pregnancy not only negatively impacts the maternal hepatic cellular balance, but may also represent a risk factor for the development of future liver dysfunctions, with possible systemic repercussions for the female and, potentially, for the offspring.

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Conflict of interest

The authors declare no conflict of interest.

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