EXPERIMENTAL NON-ALCOHOLIC FATTY LIVER DISEASE INDUCED BY HIGH-CHOLESTEROL DIET

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in adults. It is often associated with metabolic syndrome, obesity, type 2 diabetes, and cardiovascular disease (CVD), especially atherosclerotic CVD, which is closely related to cholesterol levels. The recommended daily cholesterol intake should be < 300 mg. However, the contemporary diet is marked by the high consumption of foods rich in cholesterol and saturated fats. In this context, animal models with natural disease induction, based on risk factors, mimicking contemporary dietary patterns, play a fundamental role. In

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this line, we carried out the present experiment, using 12 Wistar rats divided into 3 groups: basal control (GB), normal control (NG) and cholesterol (CG). GB was sacrificed at baseline to achieve normal baseline parameters. The NG received a standard diet and the CG received a diet rich in cholesterol, from baseline to the end of the study, when blood and liver tissue samples were collected for the preparation of slides, which were analyzed blindly, under the criteria of the scoring system for histological evaluation of NAFLD. All animals in the CG developed steatosis and balloonization in their highest scores, Moderate lobular inflammation occurred in 75% and mild in 25% of the group. Scores compatible with the presence of nonalcoholic steatohepatitis (NASH) were observed throughout the CG. It is concluded that there was the development of experimental NAFLD in Wistar rats and, based on the activity score, there was the development of NASH.

Keywords: Nonalcoholic Fatty Liver Disease. Animal Models, Atherosclerosis. Steatotic Liver Disease Associated with Metabolic Dysfunction.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is globally recognized as the most common cause of chronic liver disease in adults (Younossi et al., 2016), representing a growing and often overlooked health problem (Mitra; From; Chowdhury, 2020). It is often associated with other prevalent diseases such as obesity, dyslipidemia, and type 2 diabetes mellitus (T2DM), and has been described as a hepatic manifestation of metabolic syndrome (MS) (Younossi et al., 2019). In addition, NAFLD has been linked to increased mortality from cardiovascular diseases (CVD) (Sheka et al., 2020).

The prevalence of NAFLD has been increasing in parallel with the prevalence of obesity, diabetes, and other metabolic disorders (Younossi et al., 2019). With the increasing adoption of sedentary lifestyle habits and diets rich in saturated fats, NAFLD has been increasingly observed in young populations (Mitra; From; Chowdhury, 2020), which is worrisome, as its progression in children tends to be faster and more aggressive compared to that observed in adults (Araújo et al., 2018). In a recently published meta-analysis, the worldwide prevalence of NAFLD was estimated at 30.1% (Younossi et al., 2023). However, the global prevalence of the disease is on the rise, and it is estimated that by 2040, about half of the adult population will have NAFLD (Le et al., 2022).

As for histology, NAFLD can be divided into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFL is characterized by the presence of steatosis ≥5% of hepatocytes without evidence of hepatocellular injury evidenced by balloonization. NASH is characterized by the presence of inflammation and ballooning of hepatocytes with or without fibrosis, in addition to the presence of steatosis (Chalasani et al., 2018). Recently, NAFLD was renamed as metabolic dysfunction-associated steatotic liver disease (MASLD) and NASH as metabolic dysfunction-associated steatohepatitis (MASH), with the new definition characterized by the presence of at least one cardiometabolic risk factor, absence of alcohol consumption ≥ 20 grams/day for women or ≥ 30 grams/day for men, and other causes of hepatic steatosis. As reported by a study that analyzed the NAFLD database, 99.8% of patients met the MASLD criteria (Younossi et al., 2024).

NAFLD is an acquired metabolic disease, induced by metabolic stress (Younossi et al., 2016). Its pathophysiology is complex, multifactorial, and involves interactions between environmental, metabolic, demographic, and genetic factors (Caviglia et al., 2017), which are directly associated with a westernized lifestyle (Mitra; From; Chowdhury, 2020). Insulin resistance (IR) is widely accepted as the underlying cause of lipid accumulation in hepatocytes (Savage; Petersen; Shulman, 2007), which triggers oxidative stress and lipid peroxidation which, together with the inflammatory response, culminates in injury, cell

death, and fibrotic replacement. However, in many aspects the complex pathophysiological mechanism involved remains without adequate elucidation (Sturzeneker; Precoma; Noronha, 2022; Sanyal, 2018).

On March 14, 2024, resmetirom (Rezdiffra) became the first drug approved by the U.S. Food and Drug Administration (FDA), a regulatory body in the United States, for the treatment of adults with NASH, non-cirrhotic with moderate to advanced fibrosis, but associated with lifestyle changes. Therefore, the treatment of NAFLD/MASLD across its spectrum remains a challenge. Resmetirom is a selective thyroid hormone receptor beta (THR-β) agonist that, in the liver, stimulates lipid metabolism and prevents lipotoxicity-induced liver injury. Its release occurred after the publication of the results of the study: A Phase 3, Randomized, Controlled Trial of Resmetirom in NASH with Liver Fibrosis (Harrison et al., 2024).

NAFLD is frequent, significant, and sometimes independently associated with CVD, especially atherosclerotic disease. This association has been demonstrated in different ways and has reinforced the hypothesis that NAFLD is at least a risk marker for CVD. This strong relationship, despite being widely reported, remains unelucidated. The presence of CVD risk factors, commonly observed in patients with NAFLD, makes it difficult to establish the real role of this disease in the atherogenic process. However, common characteristics related to the pathophysiology of both point to a significant number of potential links between them (Francque; Van Der Graaff; Kwanten, 2016; Stahl et al., 2019). In this context, cholesterol, especially the low density lipoprotein (LDL) cholesterol fraction, is extremely important.

The contemporary diet is marked by the high consumption of foods of animal origin, important sources of cholesterol and saturated fats, as well as refined carbohydrates. Although high cholesterol levels are commonly associated with affluent Western nations, Asian countries have emerged as the new global epicenter of dyslipidemia when analyzing non-HDL cholesterol (Mitra; From; Chowdhury, 2020). This reflects the influence of globalization on the adoption of high-calorie diets, rich in refined sugars and processed foods, in addition to the reduction in the frequency of physical activity (Younossi et al., 2019). The recommended human intake of dietary cholesterol is less than 300 mg/day (Mach et al., 2020). According to the National Health and Nutrition Examination Surveys (NHANES) from 2013 to 2014, in the United States, 39% of adults ≥ 20 years of age, 46% men and 28% women, ingested > 300 mg/day of cholesterol (Xu; Mcclure; Appel, 2018). On average, meat (including poultry, mixed dishes, red meat, processed meat) and seafood

contributed 42% of total cholesterol intake, eggs 25%, and other food groups the remaining percentage (Carson et al., 2020).

Research using animal models of NAFLD, particularly rodents, has been crucial to clarify the mechanisms and test therapeutic interventions in controlled environments (Van Herck; Vonghia; Francque, 2017). However, the methodologies employed vary considerably, reflecting the absence of a standard model. The ideal animal model for NAFLD is, in theory, one in which the disease is induced by natural methods, based on its risk factors, mimicking contemporary dietary patterns and being able to present the entire basic histopathological spectrum of the condition: steatosis, lobular inflammation, and ballooning degeneration of hepatocytes, with scores that characterize steatohepatitis (Santhekadur; Kumar; Sanyal, 2018). In this sense, we carried out the present experiment with the objective of developing an animal model of NAFLD that corresponds to such expectations.

OBJECTIVE

To develop an animal model of NAFLD in Wistar rats, using a diet rich in cholesterol to replicate, in an approximate way, the eating habits known to be associated with the development of the disease in humans.

METHOD

SAMPLE

A total of 12 8-month-old male Wistar rats were divided into three groups with four animals: basal control group (GB), normal control group (NG) and cholesterol group (CG). The GB group was sacrificed at baseline to obtain normal, baseline parameters of serum variables, body weight, and liver histology. The NG was fed with standard diet for laboratory rats, with no potential to induce metabolic disorders, and the CG received the diet developed by the research team, called cholesterol diet. Both groups received their respective food and water *ad libitum* from baseline to euthanasia, which occurred at the end of the 16th week.

PROCEDURES

Experimentation environment

The animals were transferred from the vivarium to the experimentation laboratory of UEPG, where they remained for an adaptation period of seven days before the beginning of the experiment. During this phase, they had free access to standard food for laboratory rats

and water. In the macro-environment, the 12-hour lighting cycles, continuous air exchange and controlled temperature between 19 and 23ºC were respected. In the micro-environment, the animals were housed in metal cages, sanitized daily, with four animals per cage, identified by a color mark on the tail, defined for each group, to facilitate individual monitoring. The study was approved by the pertinent ethics committee and followed the established guidelines adopted by the Department of Medicine (DEMED) of UEPG for the handling of animals in experiments.

Preparation of cholesterol feed

To prepare the cholesterol diet, 30 grams of powdered cholesterol, 130 grams of refined sugar, 100 grams of refined wheat flour, 140 grams of lard, 600 grams of standard feed for laboratory rats and 300 grams of water were used, with the approximate composition in 100 grams of the finished food: sucrose 10 grams, Carbohydrates 44.81 grams, proteins 10.7 grams, cholesterol 3.13 grams, total fats without cholesterol 14.9 grams and residual water. All ingredients were properly weighed on a precision scale, mixed to obtain homogeneous dough and baked in a preheated oven for 15 minutes. After cooling, the product was cut to an appropriate size for adaptation to the feeder.

Blood sampling

In euthanasia, all animals, under anesthesia, were submitted to blood samples after a 12-hour fasting period, in order to analyze the serum levels of glucose, aminotransferases, total cholesterol and triglycerides.

Liver resection and weighing

Liver resection was performed in all rats, under anesthesia, in euthanasia. After the anesthetic procedure and trichotomy of the abdominal region, the animals were placed in the supine position and submitted to median laparotomy initiated in the xiphoid process, with exposure and removal of the liver, followed by euthanasia of the animals. The livers were weighed and segments of the lobes with the highest anatomical expression were resected and fixed in formaldehyde.

Histological analysis

Liver tissue samples previously fixed in formaldehyde and submitted to paraffin inclusion were sectioned to make slides that were stained with hematoxylin and eosin (HE) for histopathological evaluation. The slides were analyzed blindly, using the main

parameters of the scoring system for histological analysis of NAFLD (Table 1). The NAFLD activity score (EAD), a component of this scoring system, corresponds to the unweighted sum of the steatosis, ballooning, and lobular inflammation scores. In a validation study, distance learning ≤ 2 was strongly related to the absence of NASH, scores between 3 and 4 had no discriminative value, being similarly distributed among the 3 diagnoses (absence of NASH, borderline or NASH); and scores ≥ 5 were significantly related to the presence of NASH (Kleiner *et al.*, 2005).

Table 1 – Parameters of the scoring system for histological analysis of NAFLD

Histological alteration	Definition	Score
Degree of steatosis	<5%	0
	5 to 33%	1
	>33 to 66%	2
	>66%	3
Lobular inflammation	Unfocused	0
	<2 Spotlights/Field (200x)	1
	2-4 spotlights/field (200x)	2
	>4 Focuses/Field (200x)	3
Ballooning	Absent	0
	Few cells	1
	Many cells	2
Fibrosis stage	Absent	0
	Perisinusoidal or periportal	1
	Lightweight, Zone 3, Perisinusoidal	1A
	Moderate, zone 3, perisinusoidal	1B
	Portal/periportal	1C
	Perisinusoidal and Portal/periportal	2
	Fibrosis bridges	3
	Cirrhosis	4
NAFLD Activity Score	NASH missing	0-2
	Indeterminate	3-4
	EHNA present	≥5

Source: composition of the authors

STATISTICAL ANALYSIS

The results were described by means, standard deviations or by frequencies and percentages. The distribution of normality was evaluated by the Shapiro-Wilk test and homogeneity by the Levene test. The groups were compared for quantitative variables with normal distribution, using the one-factor analysis of variance (ANOVA) model, and the comparison was 2 to 2 performed using Tukey's post-hoc test. Regarding quantitative variables without normal distribution, the comparison between the groups was performed using the analysis of variance model (ANOVA) to a non-parametric factor (Kruskal-Wallis), and the comparison 2 to 2 was performed using the Dwass-Steel-Critchlow-Fligner test. The data were analyzed using the Jamovi computer program, version 2.3, and values of $p<0.05$ were considered significant.

RESULTS

No significant differences were observed in relation to animal weight, liver weight, and liver weight/animal weight between the groups, both at baseline and at euthanasia (Table 2).

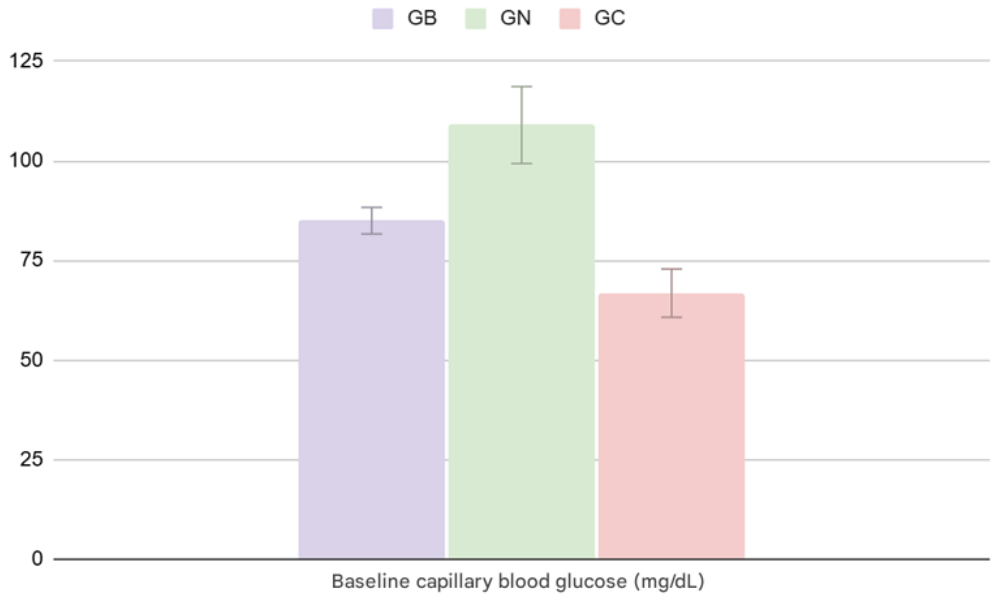
Table 2 – Mean weights in grams, baseline and euthanasia.

	Baseline			Euthanasia		
	GN	GB	GC	GN	GB	GC
Animal weight	502	456	428	540	-	471
Hepatic weight	-	16.3	-	17.5	-	17.3
Liver/animal weight ratio	-	0.0360	-	0.0326	-	0.0362

NG: normal control group; GB: basal control group; CG: cholesterol group.
Source: composition of the authors.

Capillary blood glucose levels were significantly different between the groups at baseline ($p<0.001$), with the highest and lowest mean observed, respectively, in the NG and CG (Figure 1). The *post-hoc* tests showed a significant increase in NG compared to GB ($p=0.004$) and CG ($p<0.001$), and similarly in CG compared to GB ($P=0.02$). At the end of the study, between the 15th and 16th week, capillary glucose levels were also different between the groups ($p=0.025$). The highest and lowest mean occurred again in NG and CG, but the *post-hoc* tests showed a significant difference only between NG and CG ($p=0.022$).

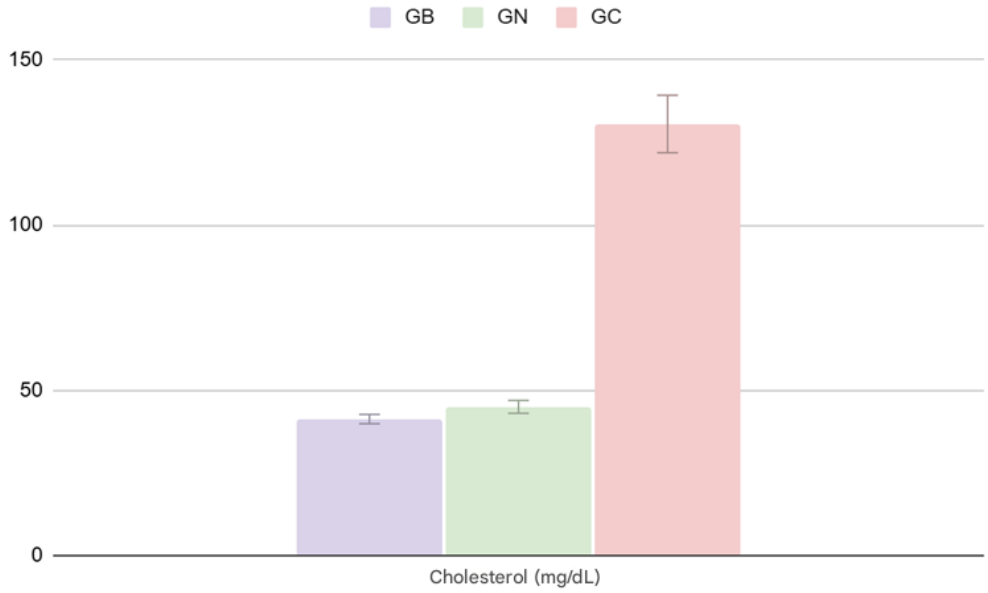
Figure 1- Capillary glucose at baseline.



Legend: GB: basal control group, NG: normal control group, CG: cholesterol group.

There was a significant difference between the groups in terms of serum total cholesterol levels ($p < 0.001$), and these levels were significantly higher in the CG compared to the control groups: GB and NG. The mean serum cholesterol in the CG was 130.6 mg/dL, in contrast to 41.3 mg/dL in the GB and 45.3 mg/dL in the NG (Figure 2). The comparison 2 to 2 showed a significant difference between the CG and the control groups, with a value of $p<0.001$ for both comparisons. However, serum triglyceride levels were similar between the 3 groups ($p = 0.650$). Regarding serum aminotransferase levels, there was no significant difference between the 3 groups regarding AST ($p=0.406$) or ALT ($p=0.982$) levels.

Figure 2: Serum total cholesterol levels.



Legend: GB: basal control group, NG: normal control group; CG: cholesterol group.

Regarding the histological analysis, all animals in the CG developed severe steatosis, in its highest score (score 3), and this histological parameter was normal (score 0) in the control groups: GB and NG (Table 3, Figure 3). Regarding lobular inflammation, only one animal in the CG (25%) showed mild inflammation (score 1), the rest of the group (75%) developed moderate lobular inflammation (score 2). Similar to steatosis, lobular inflammation did not occur in the control groups (Table 3, Figure 3).

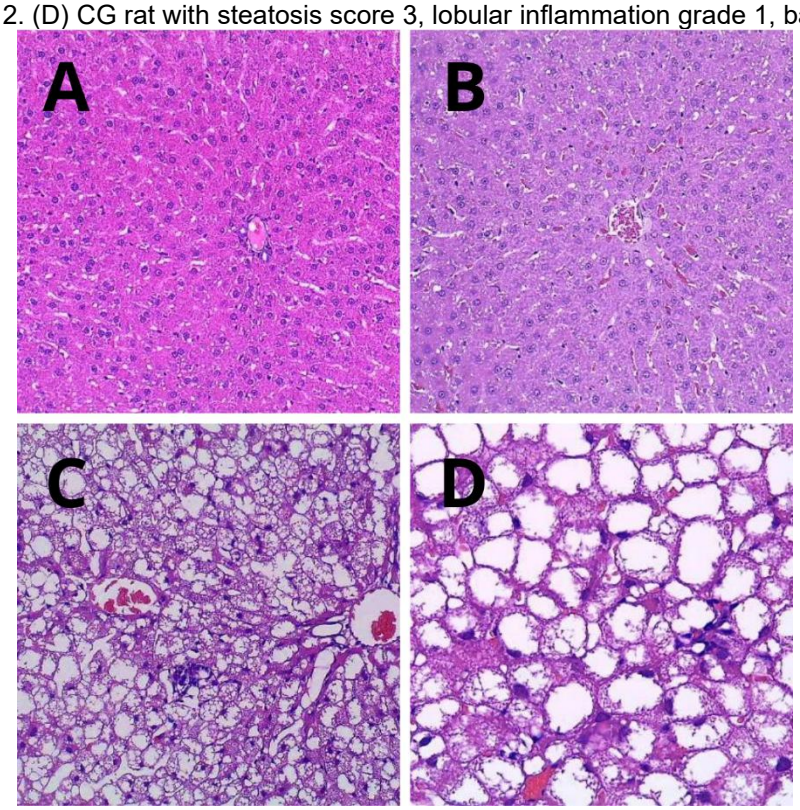
Regarding hepatocyte ballooning, all rats in the CG developed this alteration to its greatest degree (score 2). Similarly to steatosis and lobular inflammation, balloonization did not occur in the control groups (Table 3, Figure 3). Fibrosis was not observed in the CG, and similarly to the other histological alterations previously mentioned, it did not occur in the control groups. Regarding the NAFLD activity score, used to estimate the presence of

NASH, all animals in the CG had scores ≥ 5, compatible with the presence of NASH (Table 3).

Table 3 – Histological analysis of rats in the cholesterol group (CG).

Histological alteration	Definition	Score	Group			
			GC1	GC2	GC3	GC4
Degree of steatosis	<5%	0				
	5 to 33%	1				
	>33 to 66%	2				
	>66%	3	X	X	X	X
Lobular inflammation	Unfocused	0				
	<2 Spotlights/Field (200x)	1		X		
	2-4 spotlights/field (200x)	2	X		X	X
	>4 Focuses/Field (200x)	3				
Ballooning	Absent	0				
	Few cells	1				
	Many cells	2	X	X	X	X
Fibrosis stage	Absent	0	X	X	X	X
NAFLD Activity Score	NASH missing	0-2				
	Indeterminate	3-4				
	EHNA present	≥5	X	X	X	X

Figure 3: Photomicrographs of slides stained with hematoxylin and eosin, magnification of 200 X. (A) GB rat with preserved hepatic lobular architecture, without abnormalities. (B) GN rat with preserved hepatic lobular architecture, without abnormalities. (C) CG rat with steatosis score 3, lobular inflammation score 2, balloonization score 2. (D) CG rat with steatosis score 3, lobular inflammation grade 1, balloonization score 2.



Legend - GB: basal control group, NG: normal control group, CG: cholesterol group.
Source: composition of the authors.

DISCUSSION

NAFLD remained without specific pharmacological treatment for more than 40 years. In March 2024, the FDA approved the use of resmetirom for patients with NASH with well-defined histological characteristics by means of liver biopsy. Therefore, the pharmacological therapy of NAFLD is defined for a specific profile of disease presentation. Taking into account the broad histological spectrum, the complexity of the pathophysiology, as well as the association with other prevalent diseases such as CVD, this important chronic liver disease, whose global prevalence is on the rise, remains unelucidated in many aspects. Therefore, both basic and clinical research is necessary, and, in this scenario, research in animal models plays a fundamental role.

In the present study, body weight, liver weight, and liver weight/body weight ratio were similar between the groups, both at baseline and at euthanasia. Therefore, the induction method used in this model does not cause significant body or liver weight gain (Table 2). Regarding body weight, similar findings were observed in a study that used male mice of the C57BL/6J strain submitted to a high-cholesterol diet for 30 weeks (Savard et al., 2013). Similar findings were also reported in a review that evaluated animal models of NAFLD that used cholesterol-enriched diets (Santhekadur; Kumar; Sanyal, 2018). However,

significant differences between the models, such as the source of cholesterol, the animal used, as well as the time of the study make it difficult to make a proper comparison.

Regarding liver weight, in a review that addressed NAFLD induction methods, it was reported that diets rich in isolated cholesterol do not increase liver weight (Flessa et al., 2022). Discordant results have been reported in a study that evaluated the effects of a diet with 1.5% cholesterol in HCVcpTg transgenic mice for 15 months (Wong et al., 2020), in a study that used Fisher rats subjected to a choline-deficient diet (Kaji et al., 2011), and in a study that used Wistar rats submitted to a choline- and methionine-deficient diet (Hirose et al., 2007). However, the comparison with the results of the present study is limited, particularly due to the difference between the induction methods.

The presence of significantly lower capillary blood glucose levels in the CG compared to NG and GB at baseline and compared to CG in the final phase of the study suggest that there was no influence of the diet used in our study on capillary blood glucose levels. Similar findings were described in a study mentioned above (Savard et al., 2013), although the comparison is limited by the factors previously mentioned.

The significant increase in serum cholesterol levels observed in the CG, compared to the control groups, corroborates the association between the diet used and serum cholesterol levels. With the limitations previously mentioned, Savard et al. (2013) reported similar results. In a previous study carried out at our institution, Mota et al. (2022) adopting a method observed divergent results, probably because the rats were younger and because of the shorter time of exposure to the disease-inducing diet (10 weeks). Regarding serum triglyceride levels, in our study, no significant difference was observed between the groups, a result different from those reported in two studies that used a choline-deficient diet (Kurita et al., 2008; Kaji et al., 2011). However, in addition to the difference between the induction methods, the choline-deficient diet may have influenced these results.

Serum AST and ALT levels were similar between the groups, contrasting with the significant elevation of both aminotransferases described in a study that subjected Wistar rats to a choline and methionine deficient diet (MCD) (Hirose et al., 2007). However, the difference between the induction methods makes it impossible to make a reasonable comparison. In addition, only a small percentage of individuals with steatosis without an identifiable cause (2.8 to 5.4%) have elevated serum aminotransferase levels (Szczepaniak et al., 2005).

Regarding the histological analysis, all animals in the CG developed intense steatosis, at its highest score (score 3), and this histological parameter was normal (score 0) in the control groups: GB and NG (Table 3, Figure 3). Regarding lobular inflammation,

only one animal in the CG (25%) had mild or mild inflammation (score 1), the rest of the group (75%) developed moderate lobular inflammation (score 2). As with steatosis, lobular inflammation was not observed in the control groups (Table 3, Figure 3).

Regarding hepatocyte ballooning, all rats in the CG developed this histological alteration to its greatest degree (score 2). Similar to steatosis and lobular inflammation, balloonization was not observed in the control groups (Table 3, Figure 3). Fibrosis was the only basic histological alteration not observed in the CG and, similarly to the other histological alterations previously mentioned, did not occur in the control groups. Regarding the NAFLD activity score, used to estimate the presence of NASH, all animals in the CG had scores ≥ 5, compatible with the presence of NASH (Table 3).

Results similar to those found in the present study were reported by Savard et al. (2013), who observed the development of mild to moderate hepatic steatosis, mild inflammatory infiltrate, and absence of fibrosis. However, the animal, the source of cholesterol and the time of exposure to the diet were different, which limits the comparison of the results. Regarding lobular inflammation, Kurita et al. (2008) described "intense" lobular inflammation in Otsuka Long-Evans Tokushima (OLETF) rats submitted to the MCD diet for 8 weeks. This result differs from that found in our experiment in terms of the intensity of lobular inflammation developed. However, the OLEFT rat is a selected strain for spontaneously developing hyperglycemia and obesity, additional factors for the development of liver injury.

Regarding ballooning, as in the present experiment, Hirose et al. (2007) reported marked ballooning in Wistar rats fed the MCD diet for 15 weeks. The extreme difference between the induction methods makes the comparison compromised, despite the similarity between the results. Regarding fibrotic replacement, a consequence of hepatocyte injury and death, Kaji et al. (2011) observed the development of fibrosis in Fisher rats subjected to a choline-deficient diet for 12 weeks, Wong et al. (2020) in transgenic mice submitted to a high-cholesterol diet for 15 months, and Hirose et al. (2007) in Wistar rats fed an MCD diet for 15 weeks. In contrast to these results, in the present study there was no development of fibrosis. However, with the exception of the study by Hirose et al. (2007), the other studies cited used different animals and all of them used induction methods that were apparently more aggressive than the one used in our experiment. In addition, Wong et al. (2020) used transgenic mice and the study period was more than 3 times longer than that adopted in the present study.

NASH is characterized by the presence of steatosis ≥5% of hepatocytes, lobular inflammation, and ballooning of hepatocytes with or without fibrosis. Therefore, the

proposed model presented the criteria compatible with the presence of NASH. Similarly, the presence of at least one risk factor, and in this case compared to the control groups, it can be inferred that there was hypercholesterolemia in the CG, characterizes the presence of MASH. Thus, an animal model of DHGNA/MASLD and HNAE/MASH was developed in Wistar rats, using a diet compatible with current human eating habits as an inducer. We emphasize as limitations the small sample size and the death of one of the rats in the CG two weeks before euthanasia, which prevented the collection of blood samples from this animal. However, the histological alterations were identical to those presented by the other rats in the group, except for lobular inflammation, which had the lowest score in one of them (Table 3).

CONCLUSION

In the proposed model, there was the development of NAFLD/MASLD in Wistar rats, using a diet that, approximately, mimics current human eating habits, known to be associated with the development of cardiometabolic disease. Based on the NAFLD activity score, there was the development of nonalcoholic steatohepatitis/steatohepatitis associated with metabolic dysfunction.

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