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Dietary intake of AIN-93 standard diet induces fatty liver with altered hepatic fatty acid profile in Wistar rats

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Abstract

Background: **There are several standard diets for animals used in scientific research, usually conceived by scientific institutions. The AIN-93 diet is widely used, but there are some reports of fatty liver in Wistar rats fed this diet.**

Objective: **We aimed to evaluate the hepatic repercussions of the AIN-93 diet intake in Wistar rats.**

Methods: **Forty newly-weaned 21-day-old male Wistar rats were fed either the AIN-93 diet or a commercial diet for either 1 month or 4 months. Weight gain, serum biochemistry, hepatic histology, and hepatic fatty acid profile were analyzed.**

Results: **Hepatic steatosis was observed, especially in the group fed the AIN-93 diet. Serum blood glucose, absolute and relative liver weight and hepatic levels of oleic, palmitoleic, stearic, and palmitic fatty acids were related to the observed steatosis, while lipidogram and serum markers of liver function and injury were not.**

Conclusion: **AIN-93 diet induced acute hepatic steatosis in Wistar rats, which may compromise its use as a standard diet for experimental studies with rodents. The hepatic fatty acid profile was associated with steatosis, with possible implications for disease prognosis.**

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LA INGESTA DE LA DIETA ESTÁNDAR AIN-93 INDUCE ESTEATOSIS HEPÁTICA CON ALTERADO PERFIL DE ÁCIDOS GRASOS EN RATONES WISTAR

Resumen

Introducción: **En la investigación científica, hay varias dietas estándar para los animales, generalmente concebidas por instituciones científicas. La dieta AIN-93 es ampliamente utilizada, pero hay algunos informes de esteatosis hepática en ratones Wistar alimentadas con esta dieta.**

Objetivo: **Evaluar las repercusiones hepáticas de la ingesta de la dieta estándar AIN-93 en ratones Wistar.**

Métodos: **Cuarenta recién destetados, ratones Wistar machos, con 21 días de edad fueron alimentados con la dieta AIN-93 o una dieta comercial, durante 1 mes o 4 meses. El aumento de peso, la bioquímica sérica, la histología hepática y el perfil de ácidos grasos hepáticos fueron analizados.**

Resultados: **Se observó esteatosis hepática, especialmente en el grupo alimentado con la dieta AIN-93. Glucosa en suero, peso absoluto y relativo del hígado y los niveles hepáticos de ácidos grasos oleico, palmitoleico, esteárico y palmítico se relacionaron con la esteatosis observada, mientras el lipidograma y los marcadores sanguíneos de la función hepática, no se relacionaron.**

Conclusión: **La dieta estándar AIN-93 causó esteatosis hepática aguda en ratones Wistar, que puede comprometer su uso como una dieta estándar para los estudios experimentales con roedores. El perfil de ácidos grasos hepáticos se asoció con la esteatosis, con posibles implicaciones para el pronóstico de la enfermedad.**

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Palabras-clave: *Esteatosis hepática. Dieta AIN-93. Ratones Wistar.*

Abbreviations

g-GT: Gamma-glutamyl-transferase.
AIN: American Institute of Nutrition.
ALP: Alkaline phosphatase.
ALT: Alanine aminotransferase.
AST: Aspartate aminotransferase.
CD: Commercial diet.
FL: Fatty liver.
HDL-c: High-density lipoprotein cholesterol.
LDL-c: Low-density lipoprotein cholesterol.
VLDL-c: Very low-density lipoprotein cholesterol.

Introduction

The use of laboratory animals in biological research is particularly advantageous as it offers the opportunity to control for variables that would be either extremely difficult or impossible to control for in human subjects. Diet is an important example of one of these variables, as it influences the growth and reproductive capacity of animals and diseases processes[1]. However, it is common for researchers in the field of biological science to neglect the real impact of diet on the metabolism of laboratory animals[2].

There are several standard diets for animals used in scientific research, usually conceived by scientific institutions. Between 1977 and 1980, the American Institute of Nutrition (AIN)[3],aiming to formulate standard diets for use in animal experimentation, prepared the AIN-76 diet: a purified, open-label diet for rodents. Following this, the AIN published the AIN-76A, which was a slightly modified version of the former[4]. Since then, several issues have been reported in relation to the use of AIN-76A in rodents, including hyperlipidemia and hepatic lesions[5,6]. Hence, the AIN published a guide to formulating standard diets that cover all nutritional requirements for these animals and reduce the risk of the previously reported problems[7]. As a result, the standard diets AIN-93G (for the growth phase) and AIN-93M (for the adult phase) were proposed.

However, problems including fatty liver (FL) have still been reported in Wistar rats fed the AIN-93 diet[8-10],suggesting problems in its composition, possibly relating to the proportion of macronutrients and the amount of sulfur-containing aminoacids and choline, a widely recognized lipotropic factor.

Given the known effects of dietary composition on the risk of FL, the previously reported results, and the importance of studies that assess the impact of standard diets in animal experimentation, the objective of this study was to assess the hepatic effects of the AIN-93 diet in Wistar rats.

Methods

This study was approved by the Ethics in Research Committee of the Federal University of Alagoas, number 009428/2006-62. Experiments were conducted according to international guidelines of animal welfare.

Experimental Design

This study was conducted using a completely randomized, 2 x 2 factorial design, where factor A was 2 different diets (AIN-93 or a commercial diet) and factor B was 2 different exposure times (30 or 120 days), yielding 4 treatments that were administered 10 times each.

Diet and animals

Forty newly weaned, 21-day-old male Wistar rats obtained from the Central Vivarium of the Federal University of Alagoas. Animals were divided into 4 groups (n = 10 each), according to the diet given and exposure time: AIN-93 for 1 month (AIN-93 1m); AIN-93 for 4 months (AIN-93 4m); commercial diet for 1 month (CD 1m); or commercial diet for 4 months (CD 4m). Animals were housed in a room in which temperature (20-24ºC) and luminosity (light/dark cycle of 12 hours) were controlled and were given diet and water *ad libitum*. In the first 30 days, animals were housed in individual cages, but thereafter the AIN-93 4m and CD 4m groups were housed in communal cages with a maximum of 4 animals per cage.

Dietary intake and weight gain were recorded weekly for 1 month. Animals in the AIN-93 4m group received the AIN-93G diet during the first 2 months and the AIN-93M diet in the last 2 months. Animals of the AIN-93 1m group, in turn, received only the AIN-93G diet. Parasitological fecal analyses were conducted to assess the hygiene conditions in the vivaria[11].

Diet preparation

AIN-93 diets were manufactured at the Faculty of Nutrition of the Federal University of Alagoas, refrigerated for a maximum of fifteen days, and offered to the animals as pellets. All the ingredients were supplied by Rhoster (São Paulo, SP, Brazil), a specialized laboratory rodent nutrition store. The sucrose content of the original AIN-93 diet (10%) was replaced by cornstarch[3]. The commercial diet (Nuvital Nutrients S.A., Paraná, Brazil) was supplied by the Central Vivarium of the Federal University of Alagoas, and stored as recommended by the manufacturer. Dietary composition is detailed in Table I.

Biochemical analysis

Following the experimental period, animals were fasted overnight, anesthetized, and subjected

Table I
Detailed Dietary composition of AIN-93 G and M

Composition	*AIN-93 G*	*AIN-93 M*
Total Energy (Kcal/kg)	3.828	3.719
Protein (%)	17,8	12,8
Carbohydrates (%)	65,8	77,5
Lipids (%)	16,4	9,7
Casein (> 85% protein; g/kg)	200	140
Corn Starch (g/kg)	497,50	565,70
Dextrinized Corn Starch (90-94% tetrassacharides; g/kg)	132	155
Soybean oil (g/kg)	70	40
Microcrystalline cellulose (g/kg)	50	50
Mineral Mix AIN-93 G (g/kg)	35	-
Mineral Mix AIN-93 M (g/kg)	-	35
Vitamin Mix (g/kg)	10	10
L-cistyne (g/kg)	3	1,8
L-methionine (g/kg)	1,6	-
Choline Bitartrate (41,1% choline; g/kg)	2,5	2,5
t-butyl-hydroquinone (g/kg)	0,014	0,008

to blood collection from the retro-orbital vascular plexus, with a capillary tube for micro-hematocrit. After clot retraction, blood was centrifuged (3500 *xg*) for 10 minutes and serum was analyzed in an OlympusAU400eâ Chemistry Analyzer device (Olympus America Inc.), using specific kits. Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl-transferase (g-GT), glucose, total protein, albumin, triacylglycerols, and total cholesterol and fractions of high-density lipoprotein cholesterol (HDL-c), very low-density lipoprotein cholesterol (VLDL-c), and low-density lipoprotein cholesterol (LDL-c) were determined. VLDL-c and LDL-c levels were estimated using the Friedewald formula[12], as total cholesterol levels were <100 mg/dL[13].

Euthanasia and liver dissection

Following blood collection, the animals, still anesthetized, were euthanized via sectioning of the thoracic aorta. After euthanasia, the abdominal cavity was completely opened and the liver was withdrawn, weighed and the left lobe was sectioned in its higher diameter and stored in formaldehyde (10%) for fixation. The remainder of the liver was weighed and stored in a freezer at -70ºC.

Histological analysis of the liver

Following fixation, the liver fragments were transversally sectioned and histological analysis was carried out using the standard hematoxilin-eosin method. Whenever present, macroscopic alterations were considered in the histological analysis. FL grades were classified into 6 levels: 0, 1, 2, 3, 4, and 5, according to Ataide et al.[9] (Figure 1), by a blinded and trained pathologist.

Determination of liver fatty acid profiles

We extracted total lipids from the diets and livers and performed fatty acid (FA) methylation according to the method described by Folch et al.[14], with slight modifications. In brief, a solvent mixture of chloroform/methanol (2:1) and tert-butylhydroquinone (0.005%) as an antioxidant were added to the homogenate of the livers and of the diet. After vigorous agitation, the chloroformic phase, containing the lipidic phase, was filtered in anhydrous sodium sulfate and dried in a rotating evaporator to obtain the dried lipid extract. This was then diluted in hexane and subjected to methylation with BF_3 in methanol (14%); the reaction mixture was kept under agitation in a rotating agitator, at an ambient temperature, for 30 hours. Following this, water was added, and the hexane fraction containing the methyl esters were then dried in a rotating evaporator; 1 mL of hexane was added per 100 mg of methyl esters.

The FA methyl esters were analyzed using gas chromatography-mass spectrometry (GC-MS) using

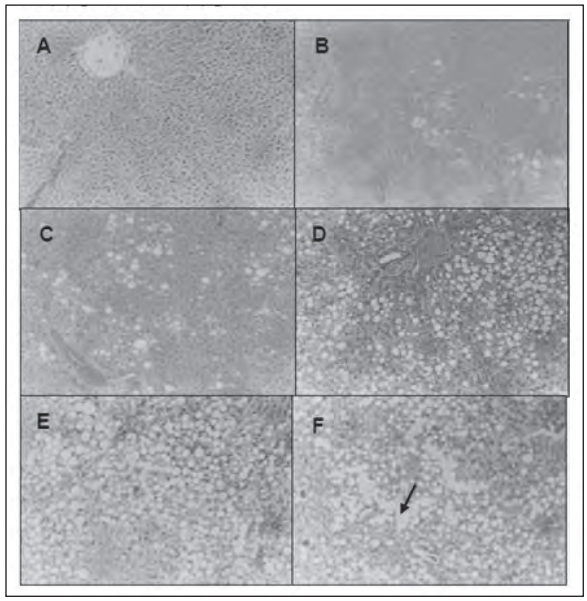

Fig. 1.—Histological assessment of hepatic steatosis in livers sections with Hematoxilin-Eosin
(A) absence of steatosis; (B) grade 1; (C) grade 2; (D) grade 3; (E) grade 4 and (F) grade 5.

a Shimadzu chromatograph (GC-17A), a SPB-5 column (30m × 0.25mm × 0.25μm), and at temperatures of 250°C and 310°C of the injector and interface, respectively, with helium as the carrier gas (1 mL/min, 50 kPa). Samples (1μL) were injected using the split control mode, with a ratio of 30:1. MS was performed using the Shimadzu equipment (GCMS-QP5050A) at 70 eV. GCMS LabSolutions v1.01 software was used to read chromatograms. The percentage of the chromatographic peak area was used for FA quantification.

Statistical Analyses

Data are presented as means and standard deviation or as absolute and relative frequency. Parametric assumptions of normality (Lilliefors' test) and homoscedasticity (Levene's test) were tested. When these assumptions were met, ANOVA was performed using Tukey's-HSD *post hoc* test; when this was not the case; the Kruskal–Wallis test was performed with Dunn's *post hoc* test, to compare continuous variables between treatments. The frequency of FL was tested using Fisher exact test. FL grades were tested using the Kruskal-Wallis and Dunn's *post hoc* test.

Additionally, according to the normality of the variables, either Pearson or Spearman's correlation were used for the FAs that are known to show some effect on FL[15-18], FL grades, absolute liver weight (ALW), relative liver weight (RLW), glucose, total proteins, ALT, AST, ALP, and γ-GT; as well as correlation between the serum lipids and the FAs that might influence it[19]. P-values of <0.05 were considered to be significant.

Results

An important histological finding, mainly in animals fed the AIN-93 diet, was the presence of FL, which occurred in 9 of 10 animals in group AIN-93 1m and in all 10 animals in group AIN-93 4m, whereas only 2 animals in group CD 4m showed this finding. None of the animals in group CD 1m showed FL.

A significant positive association between the AIN-93 diet and the frequency of FL cases ($\chi^2 = 27.022$; $P < 0.001$) was observed. The Pearson correlation coefficients showed that animals fed the AIN-93 diet tended to have a higher grade of FL than those fed the commercial diet ($r = -0.753$; $p < 0.001$). Additionally, significant differences were observed between the mean FL grades of the 4 experimental groups (Table II), with animals in the AIN-93 groups showing higher values than the others ($P < 0.001$). Animals in the AIN-93 1m group comprised 75% of the cases of FL grades 4 and 5, whereas the AIN-93 4m group comprised 25% of these FL grades. None of the animals that were fed the commercial diet had FL grades of 4 or 5. When we only considered time of exposure to the diets (1 month or 4 months) as a factor in the statistical analysis, regardless of diet type, no significant differences or correlations were observed for presence or grades of FL.

In terms of final bodyweight, older animals were heavier than younger animals regardless of diet type, as expected ($P < 0.001$; Table II). Mean absolute liver weight of the animals in the AIN-93 1m group was lower than that of the AIN-93 4m group ($P < 0.05$), but similar to the CD 4mgroup, and higher than the CD 1m group ($P < 0.01$). In turn, relative liver weight did not differ between animals exposed to diets for the same period of time. Mean relative liver weight in theAIN-93 1m group was higher than that of the AIN-93 4m group ($P < 0.01$) and the CD 4m group ($P < 0.01$). The CD 4m group had a lower relative liver weight compared to the CD 1m group ($P < 0.05$) (Table II).

Serum biochemical markers and mean hepatic FA values for all animals are shown in Table III and 4. Significant differences were found for glucose, total proteins, albumin, and ALT levels. Significant positive correlations were found between palmitic, oleic, and palmitoleic acid concentrations and FL grade ($r = 0.5$, $r = 0.76$ and $r = 0.62$, respectively; $P < 0.01$ for all). Additionally, oleic acid was positively and significant-

Table II
Frequency of fatty liver (FL), fatty liver grades, final body weight (FBW), absolut liver weight (ALW) and relative liver weight (RLW) of the animals. Values expresed as means and standard deviation

Variable	*Groups*			
	AIN-93 1m (n=10)	*AIN-93 4m (n=10)*	*CD 1m (n=10)*	*CD 4m (n=10)*
FL (%)	90.00[c,d]	100.00[c,d]	0.00[a,b]	20.00[a,b]
FL grades	2.60[c,d]	2.10[c,d]	0.00[a,b]	0.20[a,b]
Final body weight (g)	152.94±10.35[b,d]	285.42±12.68[a,c]	132.77±17.92[b,d]	261.3±8.96[a,c]
Absolute liver weight (g)	6.55±1.48[b,c]	8.07±1.28[a,c]	4.19±1.08[a,b,d]	6.98±0.52[c]
Relative liver weight	0.048±0.002[b,c]	0.030±0.002[a]	0.039±0.003[d]	0.026±0.002[a,c]

aDiffers from the AIN-93 1m group; bDiffers from the AIN-93 4m group; cDiffers from the CD1m group; dDiffers from the CD4m group.

Table III
Serum biochemical markers of the animals.
Variables were subjected to ANOVA and Tukey-HSD test. Values expresed as means and standard deviation

Biochemical variables	*Groups*			
	AIN-93 1m (n=10)	*AIN-93 4m (n=10)*	*CD 1m (n=10)*	*CD 4m (n=10)*
Glucose (mg/dL)	112.33 ± 21.56b,d	48.0± 26.41a	82.00 ± 37.34	68.75 ± 18.67a
Triglicerydes (mg/dL)	153.67 ± 20.32	109.5± 24.88	163.0± 35.19	111.25 ± 17.60
Total cholesterol (mg/dL)	85.33 ± 7.01	49.00 ± 8.58	75.00 ± 12.13	57.75 ± 6.07
LDL-c (mg/dL)	21.93 ± 4.88	7.4± 5.98	23.4± 8.46	11.5± 4.23
HDL-c (mg/dL)	32.67 ± 2.44	24.5± 2.99	19.0± 4.23	24.0± 2.11
LDL-c/HDL-c	0.67 ± 0.16	0.32 ± 0.2	1.23 ± 0.28	0.47 ± 0.14
VLDL-c (mg/dL)	30.73 ± 4.06	21.90 ± 4.98	32.6± 7.04	22.25 ± 3.52
Total proteins (g/dL)	5.67 ± 0.15b	6.55 ± 0.19a,c	5.2± 0.26b,d	6.22 ± 0.13c
Albumin (g/dL)	1.33 ± 0.07b	1.75 ± 0.09a,c	1.20 ± 0.12b,d	1.50 ± 0.06c
ALT (U/L)	40.67 ± 5.77d	62.50 ± 7.07	51.0 ± 10.0	86.75 ± 5.0a
AST (U/L)	203.33 ± 34.76	245.5± 42.57	250.0 ± 60.21	291.25 ± 30.1
AST/ALT	5.11 ± 0.6	3.89 ± 0.73	4.90 ± 1.03	3.45 ± 0.52
ALP (U/L)	462.67 ± 70.5	169.5± 86.34	207.0 ± 122.1	144.5± 61.05
γ-GT (U/L)	2.00 ± 0.26	1.50 ± 0.32	2.00 ± 0.46	1.25 ± 0.23

aDiffers from the AIN-93 1m group; bDiffers from the AIN-93 4m group; cDiffers from the CD1m group; dDiffers from the CD4m group.

Table IV
Mean percentage of hepatic fatty acids of the animals. Variables were subjected to ANOVA and Tukey-HSD test

Fatty acids	*Groups*			
	AIN-93 1m (n=10)	*AIN-93 4m (n=10)*	*CD 1m (n=10)*	*CD 4m (n=10)*
Miristic (%)	0.57 ± 0.18	0.55 ± 0.16	0.38 ± 0.12	0.34 ± 0.24
Palmitic (%)	35.20 ± 6.99	31.56 ± 7.73	26.22 ± 5.24	31.86 ± 9.34
Stearic (%)	10.26 ± 3.12c,d	11.05 ± 2.77c,d	22.61 ± 5.77a,b,d	17.17 ± 4.56a,b,c
Palmitoleic (%)	2.62 ± 1.66	3.037 ± 1.54	1.65 ± 0.98	1.61 ± 1.05
Oleic (%)	28.19 ± 3.18c,d	27.14 ± 4.53c,d	17.58 ± 4.48a,b	18.81 ± 3.46a,b
Linoleic (%)	18.742 ± 7.274	19.332 ± 6.10	15.23 ± 4.347	18.12 ± 4.43
γ-Linoleic (%)	0.154 ± 0.042	0.239 ± 0.10	0.17 ± 0.02	0.14 ± 0.02

aDiffers from the AIN-93 1m group; bDiffers from the AIN-93 4m group; cDiffers from the CD1m group; dDiffers from the CD4m group.

ly correlated with ALW ($r = 0.41$; $P < 0.05$). Finally, stearic acid concentration was significantly negatively correlated with FL grade ($r = -0.73$; $P < 0.01$) and ALW ($r = -0.57$; $P < 0.01$).

Discussion

Our results indicate that the AIN-93 pellet diet induced FL in Wistar rats, regardless of the length of time over which the animals were exposed to it. Silva et al.[8],who present similar findings, suggest that there are some issues with the composition of the AIN-93 diet, including the proportion of macronutrients and the amount of sulfur-containing aminoacids and lipotropic agents, which might contribute to the development of FL.

Medinsky et al.[6],investigating the adequacy of the AIN-76A diet for Fischer-344 rats, also reported the occurrence of FL. They assigned this finding to the high amounts of dietary sucrose. Nevertheless, the AIN-93 M and G diets used in our study were pre-

pared by substituting the recommend 10% sucrose for cornstarch; hence, the only sucrose present in the diets was that found in the vitamin mix. Despite this substitution, the animals given the AIN-93 diets showed FL, which may be attributable to other factors other than sucrose.

Samuel et al.[20] found that hepatic TAG levels tripled in rats that were fed a high-fat diet for 3 days. Gauthier et al.[21] suggested that the rat liver acts as a systemic buffer, increasing its fat content in a high-fat dietary situation. However, none of the diets used in the present study had a high fat content, so this factor might not explain the development of FL.

Dietary fiber, especially its soluble fraction present in the constituents of the commercial diet, may slow the digestion and absorption of carbohydrates. This may prevent abrupt increases in blood glucose and insulin, both of which are factors associated with FL pathogenesis[22,23]. In a previous study, rats fed an AIN diet rich in cellulose showed higher hepatic cholesterol content compared with rats fed an AIN diet containing a mix of fibers[24]. In our study, the commercial diet, which is composed of a mix of fibers, may have protected the animals in the CD groups compared with those in the AIN groups, for which the sole source of fiber was microcrystalline cellulose.

The thermal processing process used to make the pellets may have led to the formation of toxic compounds, a decrease in the bioavailability of nutrients, and the destruction of some dietary compounds[25,26]. In this context, we hypothesize that the formation of advanced glycation end-products and the possible reduction in the dietary thiamine content are both associated with hepatocyte cytotoxicity and progression of the FL[27,28]. This is partly because AIN-93 uses ingredients that are more susceptible to these kinds of modification.It is also noteworthy that, beyond the dietary advanced glycation end-products oversupply due to the provision of pellets, the accumulation of hepatic triglycerides may also lead to the formation of endogenous AGE, by forming intermediates of the lipidic peroxidation process that share common pathways with AGE production. Excessive levels of hepatic AGE can cause damage to cellular proteins and lipids, induce oxidative stress, and stimulate specific receptors related to hepatocellular lesions, inflammation, and fibrosis[29].

Regarding fatty acid composition, palmitic acid positively correlated with FL grade, as expected, although no significant differences were observed between groups. This FA promotes hepatic TAG accumulation and induces pro-inflammatory cytokines and lipoapoptosis[18,30,31]. The groups fed the AIN-93 diet had lower levels of hepatic stearic acid compared to the groups fed the commercial diet. This suggests that, in those groups, this FA might be preferentially metabolized by the stearoyl-CoA desaturase complex (SCD), yielding oleic acid[32]. This enzyme is activated by the sterol regulatory element binding protein 1c (SREBP-1c) transcription factor, which is strongly related to steatogenesis[33]. Thus, the significantly lower stearic acid levels and higher oleic acid levels in the AIN-93-fed groups, as well as the significant negative correlation coefficient between stearic acid values and FL grade, could suggest high SCD activity[34], a key enzyme to the development of FL that was not investigated here.

Additionally, *in vitro* exposure to oleic FA has been linked to increased expression of lipogenic transcription factors and a decreased expression of those factors related to FA oxidation[16,35]. Furthermore, it also increases the expression of adipose differentiation-related protein, which is known to be associated with the formation of lipid droplets and the accumulation of TAG[36]. Therefore, the significantly higher levels of oleic acid found in groups fed the AIN-93 diet and the significant positive correlation between this FA and FL grade and ALW can be explained.

Similar to other studies[37], we found that palmitoleic acid levels were significantly positively correlated with FL grade. This is in accordance with *in vitro* studies that have shown that this FA may enhance FL induced by palmitic acid. *In vivo* studies have also shown that this FA has a cytoprotector effect[17].

Our study has several limitations. First, we did not deeply investigated mechanisms related to FL induction, as insulin resistance, nevertheless, the main objective of our study was to assess if the frequency of FL differed between groups. Second, we are aware that there are quantitative methods to assess FL that are more sensible than the one used here. However, we used a semi-quantitative approach with a trained pathologist completely blinded to the experimental design, which raises the reliability of our data. Third, we did not tested the composition of the commercial diet, but rather used the information contained in the label. Nevertheless, these diets are widely used in experimental studies and reports of inadequacy are scarce.

In summary, we conclude that despite the modifications proposed to the AIN-76A diet, cases of FL still occur in rodents, suggesting that the current version (AIN-93) of the diet might not be the most suitable dietary formulation for *Wistar* rats. The mechanisms that led to FL in this case remain unknown. Diet composition, including fatty acid profile, must be considered and special attention should be given to the concentrations of stearic and oleic FAs. Upcoming studies should assess the hepatic effects of AIN-93 diet in others rodents species.

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