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High-fat diet and fructose drink stimulate apoptotic signaling via cleaved caspase-3 protein in hepatic cells of rats

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ABSTRACT

Introduction: The intake of a high-fat, high-fructose diet during childhood may lead to obesity, hepatic steatosis, and inflammation in adulthood. **Objective:** To investigate the effects on hepatic metabolism of intake of diets with various levels of fat associated with fructose drinks during childhood. **Methods:** Male 21-days-old rats were divided into groups: Control (C, 16.3% kcal from lipids diet and water); High-fat (HF, 45% kcal from lipids diet and fructose drink); and very high-fat (VHF, 60% kcal from lipids diet and fructose drink). After 10 weeks, blood and liver were collected for biochemical, histological, lipid profile, and Western blotting analyses. **Results:** The HF and VHF animals presented higher adiposity index, hepatic accumulation of lipids, and inflammatory cells, suggesting the treatments were effective at inducing non-alcoholic fatty liver disease in its inflammatory form. The hepatic content of cleaved caspase-3 and deposition of collagen fibers were increased in the HF group. **Conclusion:** In summary, lipid-rich diets combined with fructose drinks seem to promote the increase in body lipids content and accumulation of lipids, inflammation, activation of apoptotic signaling pathways, and the initiation of a fibrotic process in the liver in adulthood.

Keywords: non-alcoholic fatty liver disease; diet, high fat; fructose; obesity; apoptosis.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is now considered the hepatic manifestation of metabolic syndrome, which is one of the most common diseases worldwide[1], including in children and teenagers[2], and encompasses a wide range of hepatic pathologies, ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH). Without intervention, these conditions can progress to cirrhosis and hepatocellular carcinoma[3-5]. While simple hepatic steatosis is characterized by the accumulation of lipids in more than 5% of the total hepatocytes, NASH is a more complex condition that is characterized by lobular inflammation and the presence of ballooning in hepatocytes with or without fibrosis, which occurs independently of the consumption of alcohol[5].

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Liver plays a key role in lipid metabolism, including the uptake and synthesis of free fatty acids (FFAs) as well as the storage and exportation of lipids. Changes in any of these processes may lead to the development of NAFLD[6]. Under normal conditions, triglyceride (TG) synthesis is stimulated to eliminate excess FFAs; TGs may then be stored as lipid droplets in hepatocytes or secreted into the blood as very low-density lipoproteins (VLDLs)[7]. The accumulation of lipids in the liver induces a progressive series of alterations in this tissue, such as lipid peroxidation, oxidative stress, mitochondrial dysfunction, and changes in the release of adipokines, impairing both liver morphology and function[8].

Although the mechanisms of NAFLD are not fully elucidated, a high intake of dietary lipids has been associated with the development of obesity, hepatic steatosis, and NASH in humans and experimental animal models[9,10]. In addition, recent investigations have shown that excessive consumption of fructose may play a key role in the development of NAFLD[2]. A variety of studies have used lipid-rich diets associated with carbohydrates as an experimental model of NAFLD/NASH to elucidate the mechanisms involved in these pathologies[11-13].

Weight loss, healthy eating, physical exercise, and pharmacological treatment (vitamin E, pioglitazone, and pentoxifylline) may improve and slow the progression of the disease but are not effective therapies for NAFLD[3,14]. Due to the high prevalence and fast evolution of the disease and the absence of effective therapies, insights into the pathways involved in the process are particularly important, mainly when the experimental model used for the study is obtained by simulating feeding habits observed in humans.

Based on the above-mentioned points, we hypothesized the intake of a high-fat diet and fructose drink during childhood that promotes obesity[15], cardiovascular damage[13], and can also cause hepatic steatosis, and inflammation in adulthood. Therefore, to test this hypothesis, we establish a well-recognized experimental model of obesity, mimicking humans, associated with NAFLD induced by lipid-rich diets combined with the consumption of fructose and we evaluated the effects of these diets on liver morphology, lipid metabolism, and the cellular pathways involved in this process.

The objective of this work was to investigate the effects on hepatic metabolism of diets with various levels of fat associated with fructose drinks during childhood.

METHODS

Animals, diets, and treatment

Male Wistar rats (*Rattus norvegicus*) weighing approximately 43 ± 2 g (21-days-old) were randomized into three groups (n=9 animals per group): Control (C), which received an AIN93G standard diet for growing rodents (containing 16.3 kcal from lipids (soy oil)) and water; high-fat (HF), that received a high-fat diet (45% kcal from lipids (soy oil + lard) and 10% fructose solution in substitution to water; and very high-fat (VHF), which was fed a very high-fat diet containing 60% kcal from lipids (soy oil + lard) and received 10% fructose solution in substitution to water. Food and water were offered *ad libitum* for seventy days (ten weeks). Diets were prepared by PragSoluções®. The control diet was produced according to the recommendations of the American Institute of Nutrition[16] for growing rats totaling 3.86 kcal/g. The obesogenic diets were based on AIN-93G but included different proportions of saturated fat (lard): high-fat diet (HF) contained 45% from lipids of the total energetic value, totaling 4.71 $kcal.g^{-1}$, and very high-fat diet (VHF) contained 60% from lipids of the total energetic value, totaling 5.27 $kcal.g^{-1}$. For details about components of the diet see[15].

Animals were housed in collective polypropylene cages with four or five animals per cage, the temperature was maintained at 22-24°C, and a light/dark cycle of 12/12 h was applied. Food and water intake and body weight were measured thrice weekly throughout the study.

Blood and tissue collection

After ten weeks of treatment and followed by a 12h fast, animals were anesthetized (CO_2 excess) and then euthanized by guillotine decapitation for collection of blood samples in glass tubes without anticoagulant. The liver was collected, weighed, photographed for macroscopic analysis, and stored at -80 °C. Epididymal, retroperitoneal, omental, and perirenal fat tissues were excised and weighed to determine the body obesity index. The relative liver weight (%) was calculated as follows: [liver weight (g) / body weight (g) x 100].

Characterization of obesity

Obesity was characterized by the measurement of body adiposity index (BAI; %) as follows: [visceral adipose tissue (VAT) / body mass x 100]. VAT (g) was considered the sum of epididymal, retroperitoneal, omental and perirenal fat[17]. The total energy intake (kcal/week) was obtained as follows: [mean food consumption x dietary metabolizable energy] (adopting values of 4 kcal for carbohydrates and protein and 9 kcal for lipids)[18].

Biochemical parameters

The levels of glucose, TGs, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP) were measured by using commercial kits (Bioclin® Quibasa Química Ltda. – Belo Horizonte/MG) and spectrophotometer (UV-mini 1240, SHIMADZU) or ELISA microplate reader (SpectraMax® 190

UV-Vis, Molecular Devices). The level of low-density lipoprotein cholesterol (LDL-c) and VLDL was calculated from data obtained for TC, HDL-c, and TG.

Histological analyses

A fresh liver tissue sample was rapidly immersed in magnesium silicate followed by Tissue-Tek® (O.C.T), isopentane, and liquid nitrogen and stored in a freezer (-80 °C) until the frozen sections were cut in a cryostat. Briefly, 10 µm thick slices were obtained and mounted on glass slides and stained with the following: a) hematoxylin and eosin (H&E) for morphological evaluation and cellular infiltration, b) Oil Red O (ORO) for quantification of lipid droplets in hepatocytes, and c) Picro Sirius Red (PSR) to visualize collagen deposition. The slides were visualized with an Axio Scope. A1 microscope (Carl Zeiss, Germany) coupled with a digital camera (H&E and ORO optical microscopy, PSR polarized light microscopy).

Hepatic lipid profile

The total lipids content in the liver was determined by gravimetric methods after extraction according to the protocol published by Folch *et al.*[19]. Data were expressed as mg of lipid/g tissue.

For the determination of triacylglycerols (TG) and cholesterol, aliquots of approximately 50 mg of the liver were homogenized in 1 mL of isopropanol and centrifuged at 2000 × g at 4 °C for 10 minutes, and 400 µL of the supernatant was used for the determination of triacylglycerols and cholesterol levels via commercial kits (Bioclin®, Belo Horizonte, MG). Data were expressed as mg/g tissue.

Western blotting

Aliquots of liver tissue were coarsely minced and immediately homogenized in 50 mmol·L^{-1} Tris-HCl buffer (pH 7.4; 4 °C) containing 150 mmol·L^{-1} NaCl, 1 mmol·L^{-1} ethylenediaminetetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 10 mmol·L^{-1} sodium orthovanadate, 100 mmol·L^{-1} sodium fluoride, 5 µg·mL^{-1} of aprotinin and 1 mmol·L^{-1} phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 10000 × g for 40 minutes at 4 °C, and the supernatant was used to determine the protein content[20]. Samples containing 100 µg of protein from each experimental group were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. PPARα (Santa Cruz Biotecnology® (sc-9000) 1:200), PPARγ (Santa Cruz Biotecnology® (sc- 7273) 1:200), AMPKα (Cell Signaling® (#2532S) 1:200), p-AMPKα (Cell Signaling® (#2535S) 1:200), SREBP-1 (Santa Cruz Biotecnology® (sc-366) 1:200), caspase-3 (Santa Cruz Biotecnology® (sc-7148) 1:200), and JNK (Santa Cruz Biotecnology® (sc-7345) 1:200) levels were detected after overnight incubation of the membrane at 4 °C with primary antibodies. β-Actin (Santa Cruz Biotecnology® (sc-130657) 1:500) was used as an internal control. The primary antibodies were detected using a peroxidase-conjugated secondary antibody (Anti-rabbit (Invitrogen® G21234) or anti-mouse (Life Technology® G21040) 1:7500) and were then visualized with chemiluminescence reagents.

Ethical approval

The experimental protocol was approved by the Ethics Committee of the Federal University of Mato Grosso (protocol n.º 23108.169089/2016-09).

Statistical analysis

The data are presented as the means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (version 5.01). Normality was analyzed by the Kolmogorov-Smirnov test. Parametric data were analyzed by one-way ANOVA followed by Tukey post hoc tests. Nonparametric data were analyzed by the Kruskal-Wallis test followed by Dunn's post hoc tests. Differences were statistically significant at $p<0.05$.

RESULTS

Body weight, energy intake, and adiposity

At the end of the treatment, the animals in the HF and VHF groups showed an increase in final body weight compared with animals in the C group. The final body weight was higher in the HF group than in the VHF group. Body mass gain was higher with the administration of the HF and VHF diets compared with the group that received the C diet. The HF group presented greater body mass gain than the VHF group (Table 1).

Food intake was lower in the VHF group than in the C group; the HF group did not alter the intake compared with the C group. The food intake was similar between HF and VHF groups. The volume (mL/week) of 10% fructose intake was similar between the HF and VHF groups and more than 100% greater than the amount of water ingested by C rats. The total caloric intake was only higher in the HF group compared with the C and VHF groups. The HF and VHF groups showed significant increases in BAI compared with the C group, and no difference was noted between the HF and VHF groups (Table 1).

Serum parameters

Statistical analysis did not demonstrate significant effects of the HF and VHF diets on fasting glycemia. However, the TG concentration was higher in both HF (104.04%) and VHF (142.34%) groups when compared with the C group (Table 1).

Serum TC levels were increased by the HF diet, while the VHF diet had no significant effect on that parameter. TC levels were 33.34% higher in the serum of the animals submitted to the HF

Table 1: The initial and final body weight, body mass gain, food and water intake, energy intake, body adiposity index, and biochemical parameters of rats submitted to 70 days of treatment with control diet (C) or diets rich in lipids combined with fructose in drinking water (HF and VHF).

Variable	Group		
	C	HF	VHF
Initial body weight (g)	44.62 ± 2.99	44.49 ± 3.15	44.58 ± 3.17
Final body weight (g)	316.30 ± 17.77[a]	462.00 ± 18.29[b]	401.20 ± 14.29[c]
Body mass gain (g)	271.70 ± 15.10[a]	417.50 ± 16.28[b]	356.60 ± 12.51[c]
Food intake (g/week) *	307.10 ± 27.00[a]	288.30 ± 20.56[ab]	222.70 ± 16.25[ac]
Water intake (mL/week)	193.30 ± 16.32[a]	374.30 ± 51.11[b]	373.10 ± 66.09[b]
Energy intake (Kcal/week) *	1146.60 ± 1006	1353.30 ± 866.50	1174.70 ± 766.10
Body adiposity index (%)	1.93 ± 0.30[a]	4.40 ± 0.13[b]	3.60 ± 0.27[b]
Glycemia (mg/dL)	140.90 ± 6.42	159.40 ± 9.48	153.90 ± 11.83
Triglycerides (mg/dL)	21.02 ± 1.29[a]	42.89 ± 3.76[b]	50.94 ± 3.32[b]
Total cholesterol (mg/dL)	95.69 ± 6.44[a]	127.60 ± 6.95[b]	118.20 ± 5.32[a]
HDL-c (mg/dL)	36.92 ± 3.29[a]	89.90 ± 4.19[b]	64.55 ± 6.048[c]
LDL-c (mg/dL)	55.57 ± 4.05	34.26 ± 7.18	37.68 ± 4.73
VLDL-c (mg/dL)	4.20 ± 0.26[a]	8.57 ± 0.75[b]	10.19 ± 0.66[b]

Data are presented as the mean ± SEM. Data submitted to analysis of variance (one-way ANOVA) or *Kruskal Wallis, and when differences were found the post hoc of Tukey HSD or *Dunn's, presented by letters [abc]; ($p<0.05$; n=9 animals per group).

diet than in C animals and were about 7.4% higher than in the VHF group. HDL-c concentrations were higher in both groups treated with the high-fat diets (HF and VHF) than in the C group, and the HF group had higher concentrations than the VHF group. Treatment with high-fat diets increased serum VLDL-c levels by 104.09% in the HF group and 142.44% in the VHF group compared with the C group. No differences in LDL-C levels were observed among groups (Table 1).

Hepatic parameters

The liver from HF and VHF groups exhibited a significant increase in both absolute and relative weight of the liver, compared with those of animals in the C group. No significant differences in absolute and relative weights were noted between the high-fat groups (Table 2). The high-fat diets modified the total lipid content (mg/g tissue) in the hepatic tissue. Both groups had an increase compared with the C group. Hepatic cholesterol levels (mg/g tissue) also showed significant changes after 70 days of treatment and were higher only in the VHF group than in the C and HF groups. No difference was observed between the HF and C groups. The TG content (mg/g tissue) was higher in the HF and VHF groups than in the C group. The livers of the animals in the VHF group presented higher TG levels than those of animals in the HF group (Table 2).

Histological analysis of hepatic tissue is presented in Figure 1. In the hepatic tissue stained with H&E, both HF and VHF groups exhibited a moderate quantity of mononuclear cell infiltration and lipid vacuoles in hepatocytes. The parameters of the hepatic tissue in the C group were within the range of normality. ORO staining revealed that the groups fed high-fat diets presented generalized deposition of micro and macrovesicular lipid droplets in hepatocytes compared with the C group, and more lipid droplets were noted in the VHF group than in the HF group. These findings were confirmed by image analysis by measuring the red areas in ImageJ software.

Significant deposition of types I and III collagen in liver samples was identified by PSR staining. Histopathological analysis revealed that the consumption of high-fat diets produced increases in periportal collagen deposition and foci of deposition of type I collagen in the sinusoids, suggesting the beginning of the fibrogenic process.

Biochemical markers of liver damage

Liver damage was evaluated by determination of the activity of the AST, ALT, GGT, and ALP enzymes. Among these enzymes, only AST presented changes after 70 days of treatment. There was a 36.40% increase in AST (U/L) serum activity in the VHF group compared with the activity in the C and HF groups; the HF group did not present a significant difference in AST compared with the C group. The ALT, GGT, and ALP enzymes did not show significant differences among the groups (Table 2).

Caspase-3, PPARα, PPARγ, AMPKα, p-AMPKα, SREBP-1 and JNK levels

The VHF diet did not induce significant changes in cleaved caspase-3 content when compared with the C group. However, the cleaved caspase-3 levels were 50.53% higher in the HF group than in the C group. No difference was observed between HF and VHF groups in cleaved caspase-3 content. The other protein content evaluated (pro-caspase-3, PPARα, PPARγ, AMPKα, p-AMPKα, and SREBP-1) the levels AMPKαp-AMPKα SREBP-1 JNK were similar among groups (Figure 2).

Table 2: Serum activity (U/L) of hepatic enzymes AST, ALT, GGT, and ALP, total (mg/g) lipid content, liver cholesterol content, and liver triacylglycerol content, absolute (g) and relative liver weight of rats submitted to 70 days of treatment with control diet (C) or diets rich in lipids combined with fructose in drinking water (HF and VHF).

Variable	Group		
	C	HF	VHF
AST (U/L)	30.85 ± 4.37[a]	30.39 ± 7.94[a]	42.07 ± 7.97[b]
ALT (U/L)	18.82 ± 7.35	18.43 ± 3.06	14.04 ± 2.29
GGT (U/L)	22.06 ± 2.08	26.95 ± 7.26	24.26 ± 3.94
ALP(U/L)	74.29 ± 17.38	82.45 ± 27.11	56.98 ± 14.36
Liver lipids (mg/g tissue)	35.45 ± 6.82[a]	56.88 ± 10.15[b]	56.61 ± 12.65[b]
Liver cholesterol (mg/g tissue)	12.04 ± 2.82[a]	28.24 ± 5.53[ab]	45.02 ± 8.48[c]
Liver triglycerides (mg/g tissue)	1.78 ± 0.71[a]	1.91 ± 0.23[b]	2.72 ± 0.79[c]
Absolute liver weight (g)	7.49 ± 1.37[a]	12.63 ± 0.59[b]	11.67 ± 1.75[b]
Relative liver weight (%)	2.36 ± 0.19[a]	2.75 ± 0.25[b]	2.89 ± 0.19[b]

Data are presented as the mean ± SEM. Data submitted to analysis of variance (one-way ANOVA) or *Kruskal Wallis, and when differences were found the post hoc of Tukey HSD or *Dunn's, presented by letters [abc]; ($p<0.05$; n=9 animals per group).

DISCUSSION

The original hypothesis that the intake of a high-fat diet and fructose drink during childhood may lead to obesity, hepatic steatosis, and inflammation in adulthood was accepted. The hyperlipidemic diets early in life led to an increase in body mass and compromised liver function in adult rats. These changes were followed by up accumulation of lipids in the liver, the infiltration of inflammatory cells, and hepatocellular damage, which are commonly observed in non-alcoholic fatty liver disease (NAFLD). NAFLD is a public health problem with a high prevalence among obese individuals. Despite the availability of multiple animal models for the study of diet-induced obesity and its comorbidities, only a limited number of studies involve dietary modifications in the initial stages of life. However, human studies suggest that infant nutrition is predictive of eating habits in adult life[21]. In the present study, rats in the initial stages of life were treated with hypercaloric-hyperlipidemic diets associated with fructose in the drinking water to induce metabolic, morphology, and cell signaling changes like NAFLD in humans.

Our results showed that animals treated with hyperlipidemic diets had higher BAIs and body mass gains than the C group, characterizing the obese profile[15,22,23]. However, the HF group presented higher food intake and body mass gain than the VHF group. Because the VHF diet had more calories per gram it could explain, at least in part, the lower food intake, since is well-known that more highly energetic foods inhibit their ingestion[22,23]. This effect would explain the absence of differences between total caloric intake in the VHF group and the HF and C groups. Nascimento *et al.*[22] showed that Wistar rats treated with a diet containing 49% lipids during fifteen weeks also did not change energy intake compared with control animals. Accordingly, there is evidence that higher fat intake is not accompanied by a proportional increase in fat oxidation in NAFLD, thus favoring TG deposition in adipose tissue and increasing body weight[24].

Typical serum changes observed in obese patients were also found in this experimental model, such as increases in TGs, VLDL, and TC levels[15,25]. The administration of fructose further intensifies these effects and TG level changes[26]. Silbernagel *et al.*[27] showed that individuals who had consumed 150 g of fructose daily for four weeks had approximately 350 mg/L TG in the serum, whereas TG levels were unchanged in individuals who consumed the same amount of glucose.

Changes in hepatic lipid levels of HF and VHF rats demonstrate that animals develop steatosis in addition to obesity at the end of treatment. The fact that VHF animals present higher hepatic TG and TC levels suggests that these parameters are related to lipid concentrations in the diet. The presence of hepatic steatosis was confirmed by tissue histology, and larger lipid droplets were observed in the livers of the animals that received higher concentrations of lipids in the diet (60%). The development of hepatic steatosis in rats fed a high-fat diet combined with fructose in drinking water is consistent with other studies in rodent species that suggest that TG and TC are important inducers in the development of experimental NAFLD/NASH[9,25,28].

A study with male Sprague-Dawley rats treated with a high-fat diet (60%) or 30% fructose in water, or the combination (60% high-fat diet + 20% fructose in drinking water) reported that the combination of fructose and a high-fat diet-induced both hepatic lipid deposition and tissue inflammation, concluding that increases hepatic inflammation and metabolic alterations compared with the administration of diets rich in lipids or fructose alone[25].

Our results indicate a 50.53% increase in hepatic intracellular levels of caspase-3 in its active form in HF animals. Caspase-3 is a classic marker of apoptosis, a physiological mechanism of cellular removal and renewal. However, when cell death presents the morphological and biochemical characteristics of apoptosis but is induced by an external and/or environmental stimulus, it can become a pathological process[28].

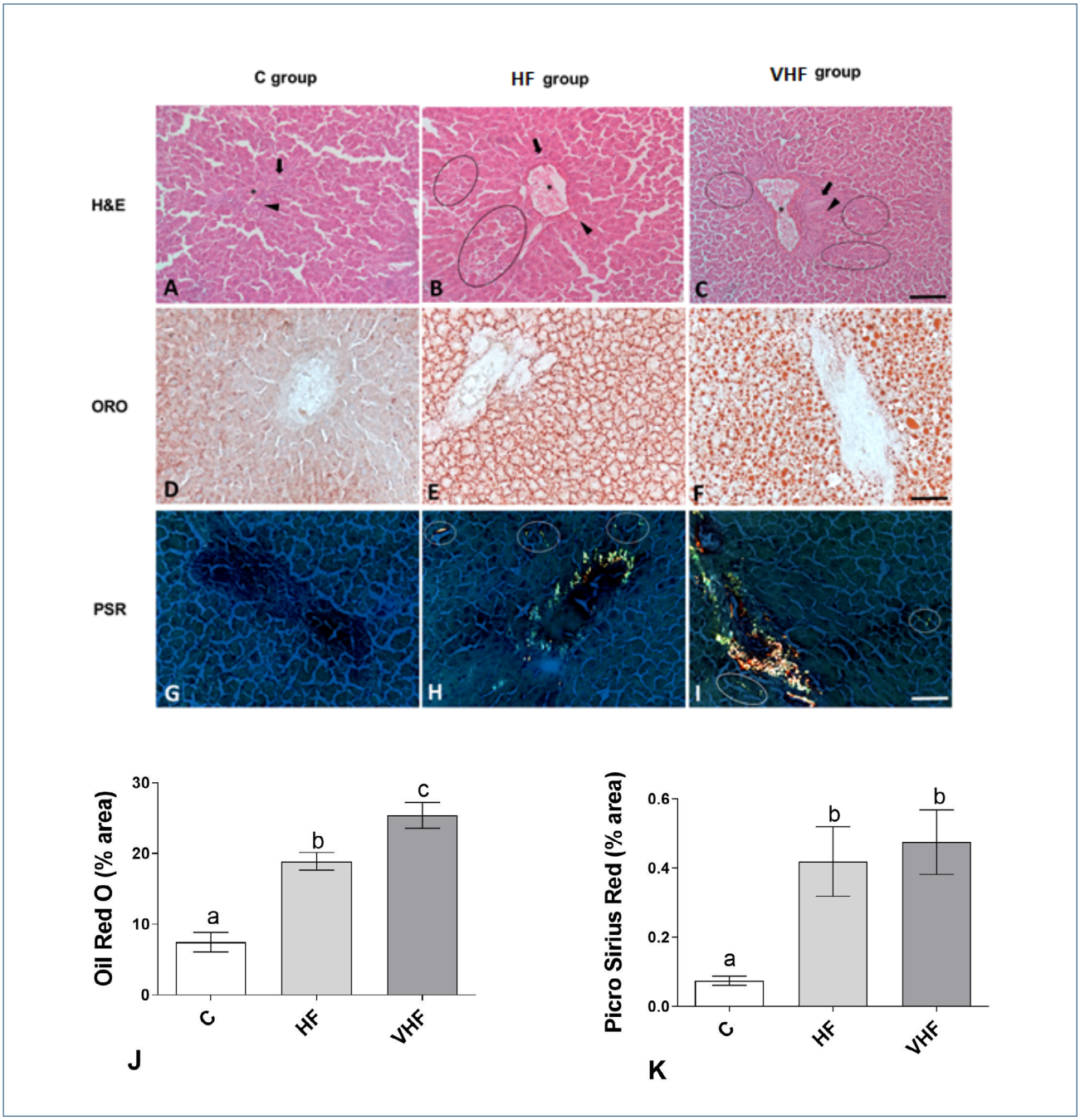

Figure 1: Histological evaluation of liver sections from rats submitted to 70 days with control diet (C), High-fat (HF), and very high-fat (VHF) diets associated with fructose drinks. Row 1: Sections stained with H&E; (A) Group C showing portal triad with typical morphological structures (Asterisk: venule, arrow: arteriole, and arrowhead: bile duct); (B) HF group and (C) VHF group showing leukocyte migration (circled areas) (Bar =100 μm). Row 2: Sections stained with ORO, (D) normal lipid deposition in group C, (E and F) lipid deposition in HF and VHF groups confirmed hepatic steatosis (Bar =50 μm). Row 3: sections stained with PSR, showing a higher collagen deposition in HF and VHF groups (H and I), which were not observed in C (G) (Bar =50 μm). In (J), we measured the intensity of the red areas of the lipid droplets in ten random photomicrographs per animal, and (K) represents the measurement of the collagen intensity in the sections stained with PSR in six photomicrographs per animal as assessed using ImageJ software. The data shown in (J) and (K) are presented as the mean ± SEM. The data were subjected to analysis of variance (one-way ANOVA), and differences identified by Tukey HSD post hoc analysis are indicated by letters ^{abc}; ($p<0.05$; n=8-9 animals per group).

Among stimuli inducing the apoptotic process, oxidative damage deserves to be highlighted. Oxidative intermediates, such as those produced during the lipid peroxidation process, can disorganize the mitochondrial membrane, and increase the cytoplasmatic levels of apoptotic effectors. One of the main sources of oxidative stress in NAFLD is the excess fatty acids resulting from the ingestion of lipid-rich diets, obesity, and insulin resistance, leading to stimulation of mitochondrial β-oxidation

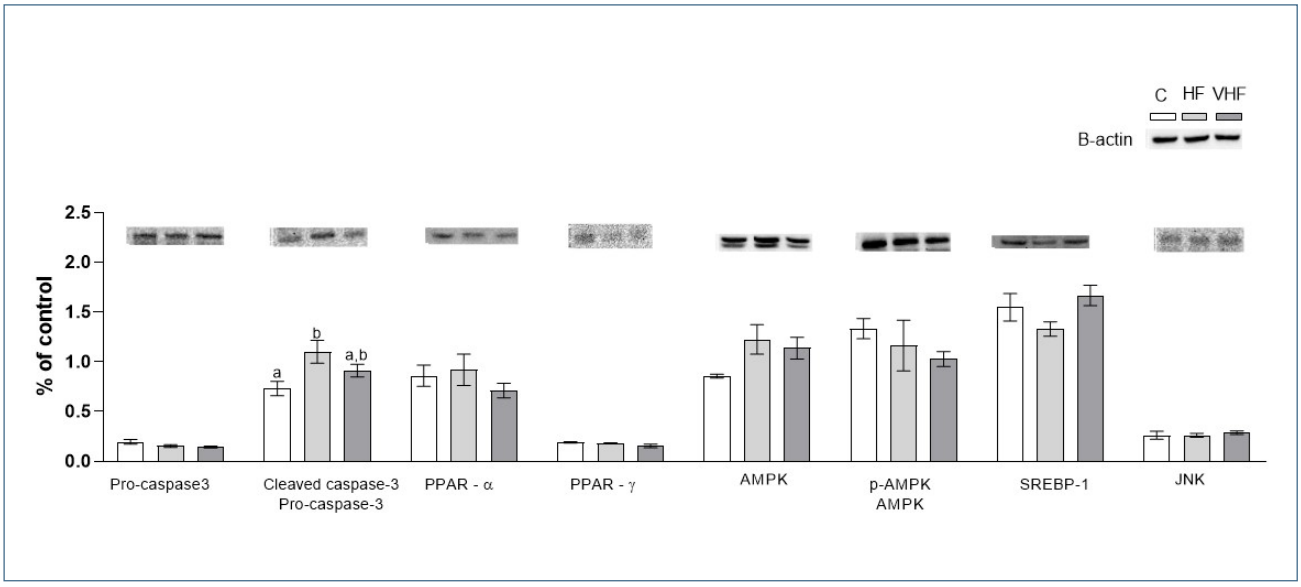

Figure 2: Content of proteins in hepatic tissue: Pro-caspase-3, cleaved caspase-3, PPAR-α, PPAR-γ, AMPKα, p-AMPKα, SREBP-1c mature and JNK from rats submitted to 70 days with control diet (C), High-fat (HF), and very high-fat (VHF) diets associated with fructose drink. The results are expressed using β-Actin as an internal control. Here, p-AMPKα was expressed as a ratio of the total AMPKα level, and cleaved caspase-3 levels were expressed as the ratio relative to the level of pro-caspase-3. Data are presented as the mean ± SEM. The data were subjected to analysis of variance (one-way ANOVA), and differences identified by Tukey HSD post hoc analysis are indicated by letters ^{ABC}; ($p<0.05$; n=5-6 animals per group).

and increase in ATP synthesis. However, even if the increase in the oxidation rate reduces the cytosolic concentration of FAs, an increase in the permeability of the internal mitochondrial membrane can occur, causing dissipation of membrane potential and lower ATP synthesis capacity which can generate hepatic oxidative damage[29]. Furthermore, hepatic oxidative damage can induce necrosis, leading to cytoplasmic and mitochondrial swelling, rupture, and release of intracellular contents with consequent activation of stellate cells and stimulation of inflammatory response causing irreversible injury and damage to neighboring cells[12,30]. Our data demonstrated an increase in serum activity of AST in VHF animals suggesting the high-fat/fructose-combined diet induced hepatic injury at the end of 70 days. AST is found in the mitochondria of hepatocytes; however, it is not a hepato-specific marker. AST alteration generally occurs in severe hepatic and mitochondrial injuries, and its serum activity remains altered for a longer period when compared to other hepatic biomarkers, such as ALT[31].

On the other hand, we did not observe changes in serum ALT, GGT, and ALP activities. Supporting our results, Cordeiro *et al.*[32] did not report significant alterations in serum ALT and ALP activity in individuals with hepatic steatosis. Other studies suggest that hepatic enzyme alterations are not necessarily found in all cases of hepatic steatosis and may be normal in more than 70% of patients with NAFLD[3,4]. Therefore, the present investigation supports literature data about the absence of a correlation between the degree of hepatic enzyme activities increased and hepatic lesion severity[33].

Histological analysis of the liver is the gold standard for accurately assessing the degree of steatosis, inflammatory changes, and fibrosis, allowing the distinction between NASH and simple steatosis[3]. It was confirmed the presence of lipid vacuoles in hepatocytes in the HF and VHF groups, is a fundamental characteristic for the diagnosis of NAFLD[34]. In addition, infiltration of inflammatory cells and collagen deposition in the sinusoids and the portal triad were observed, suggesting a hepatic inflammatory process and the initiation of the fibrogenic process. According to Ribeiro *et al.*[35] hepatocyte apoptosis and steatohepatitis severity are related. Several hepatic cells can be involved in inflammation, thereby resulting in the development and progression of steatohepatitis[36]. Among the main inflammatory cells present in the liver are macrophages and T cells, which cause hepatocyte damage by the production and release of ROS, inflammatory mediators, and promotion of fibrogenesis[37] and further contribute to the progression of NAFLD. It is important to emphasize that the hepatic stellate cells present in the liver fibrosis process are activated by oxidative stress, a situation that can be aggravated by the accumulation of lipids, lipoperoxidation, and inflammatory processes[38]. There is strong evidence that apoptotic cell death drives inflammation in NASH[39] and this is associated with an increase in caspase 3 detection in experimental models of NASH. On the other hand, necroptosis (a highly regulated necrosis pathway) requires the inhibition of caspase activity in the HF diet model[40]. All these processes, together (apoptosis/necrosis) or separately (necroptosis), can induce inflammation. Thus, our animals presented higher levels of the apoptotic cleaved caspase-3 protein,

and the development of inflammatory steatohepatitis (observed in histological analysis), which is accompanied by the onset of the fibrogenic process in animals treated with high-fat and high-fructose diets. Inflammation and fibrosis in these animals would be more pronounced after longer treatment.

Conclusion

In summary, our results suggest the introduction of hyperlipidemic diets early in life compromised liver function in adult rats. The association between excess lipids and fructose-induced the accumulation of lipids in the liver, the infiltration of inflammatory cells, and hepatocellular damage, are commonly observed in NAFLD. The significant increase in the expression of the cleaved caspase-3 protein suggests the treatment stimulates apoptotic signaling pathways into hepatic cells, thereby contributing to cellular damage and impaired hepatic functions. Further, collagen deposition in the portal triad and sinusoids suggests the initiation of a fibrogenic process after 70 days.

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