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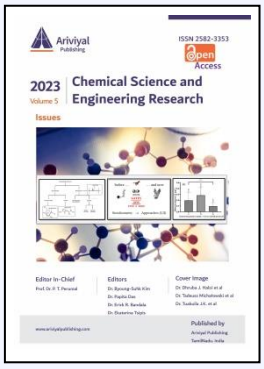

Effects of Soy Lecithin Supplementation on Liver Lipid Profile and Oxidative Stress of Rats Receiving Methionine and Choline Deficient Diet

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Abstract: Soy lecithin is a phosphatidylcholine that has shown a beneficial action in relation to hepatic steatosis. The aim of this study was to evaluate the effect of soy lecithin on NAFLD induced by a diet deficient in methionine and choline, in Wistar rats for 8 weeks. The Control group received the AIN-93 diet; the Lecithin group (Lec) received an AIN-93 diet with soy lecithin (5%); the other group received a diet deficient in methionine and choline (MCD); the last group received a MCD diet with soy lecithin (5%) (MCDL). Fasting blood glucose, plasma concentrations of total cholesterol, triglycerides, liver transaminases (ALT and AST) and liver concentrations of total cholesterol, triglycerides and total fat, malondialdehyde (MDA), reduced glutathione (GSH), vitamins A and E. We observed that the MCD diet induced a reduction in food intake, body weight gain, plasma triglyceride concentrations and hepatic vitamin A but induced an increase in the total weight of the liver and in the hepatic concentrations of triglycerides, total fat, MDA and GSH and in the values of transaminases. Supplementation with soy lecithin promoted a partial reversal in food intake, body weight gain and total liver weight. There was a reduction in plasma triglycerides and liver vitamins A and E. but there was an increase in transaminases. In conclusion, the data obtained indicate that supplementation with 5% soy lecithin promoted an improvement in the liver lipid profile and in hepatocellular damage, which are considered factors involved in the pathogenesis of NAFLD induced by the MCD diet.

Keywords: Soy lecithin; Steatosis; Oxidative Stress; Methionine; Choline

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver dysfunction in the world population. The pathophysiology of NAFLD is complex and data suggest that its etiology is related to environmental factors such as diet, exercise and/or toxins.[1] In addition, studies have associated NAFLD as a hepatic manifestation of the metabolic syndrome.[2]

NAFLD occurs in the absence of notable amounts of alcohol consumption and a wide spectrum of liver disease ranging from simple steatosis to non-alcoholic steatoepatitis NASH.[1-3]

NASH is the most serious form of NAFLD; it is characterized by the accumulation of triglycerides in hepatocytes, with progressive necro-inflammation and fibrosis. Although the simple accumulation of lipid in the liver is considered benign, NASH can result in cirrhosis, liver failure requiring transplantation and hepatocellular carcinoma.[4,5]

Nutritional models based on choline or methionine and choline deficiency are frequently used for the study of NASH in rats.[6,7]

Choline is classified as a necessary nutrient for maintaining the integrity of cell membranes, transmembrane signaling, phosphatidylcholine synthesis, neurotransmission and methyl metabolism.[8] Choline deficiency in the diet promotes hepatic steatosis due to reduced phosphatidylcholine synthesis. Thus, there is a decrease in the synthesis and secretion of very low molecular weight lipoproteins (VLDL) and consequently reduced hepatic triglyceride clearance.[9]

Rats fed a diet deficient in methionine and choline develop the highest degree of steatosis with increased oxidative stress, decreased antioxidant response and progression to steatoepatitis.[10] Some antioxidant substances can prevent the increase of free radicals and reduce fatty liver. Oliveira et al (2003) demonstrated that vitamin C reduced oxidative stress and inhibited the development of hepatic steatosis induced by a diet deficient in methionine and choline.[11]

In methionine-choline deficient rats, vitamin E treatment altered the hepatic α-tocopherol-related protein expression, which may affect α-tocopherol status in the liver, leading to reduced lipid peroxidation.[12]

Lecithin is a phosphatidylcholine belonging to the class of phospholipids that are biosynthesized in the human body and are not considered an essential nutrient but is an important component of biomembranes and lipoproteins.[13]

The oxidative stress present in rats treated with ethanol was also reversed by the administration of lecithin.[14] Lecithins of plant origin are a diverse group, with well documented actions on lipid metabolism, with the most studied lecithin to date being soy. It is worth mentioning that vegetable lecithins have a different chemical composition, depending on their source, for example soy or sunflower.[15]

Given the above, the present study aimed to evaluate the preventive effect of soy lecithin supplementation on some biochemical parameters, on hepatocellular damage and on oxidative stress triggered by the deficient diet in methionine and choline, used for the induction of NAFLD.

2. Material and Methods

Newly weaned rats (50-60 g), of the Wistar strain, from the Central Animal Farm on the Ribeirão Preto Campus - USP were used. The animals were individually environmentalized in metabolic cages at the Vivarium of the Clínica Médica, under constant temperature (23 ± 2°C) and light / dark cycle 12/12 hours (light period: 6 am to 6 pm). The animals were divided into four experimental groups and fed a diet based on the recommendations of AIN-93 (Reeves, 1997). Water and diet were offered ad libitum for 8 consecutive weeks. The control group (n = 9) received an AIN-93 diet. The second group (Lec, n = 9) received an AIN-93 diet supplemented with 5% soy lecithin. The third group (MCD, n = 5) received a diet deficient in methionine and choline. The fourth group (MCDL, n = 10) received a cholinedeficient diet and methionine supplemented with 5% soy lecithin. The experimental procedures were previously approved by the Animal Experimentation Ethics Committee (CETEA) of the Ribeirão Preto Medical School - USP.

Food intake and body weight were measured three times a week. Two days before the beheading, the animals were fasted for 14 hours to collect a drop of peripheral blood to determine blood glucose, analyze micronuclei and test the comet. All animals were beheaded between 9 am and 10 am, after 8 weeks of the experimental period for blood and liver collection. The blood collected from the trunk was used to measure AST, ALT, total cholesterol and triglycerides. The collected liver was weighed and divided into two parts. One part was immediately stored in liquid nitrogen for subsequent measurement of total fat, total cholesterol, triglycerides, MDA, GSH, vitamin A, vitamin E. The other part was stored in 10% formaldehyde solution for histopathological analysis.

Trunk blood was collected by decapitation in refrigerated tubes containing EDTA. Plasma was obtained after centrifugation for 20 minutes, 3000 rpm, at 4°C and subsequently stored at -20°C, until the time of the dosages. After beheading, the animals were subjected to bilateral incision of the skin and subcutaneous tissue of the abdomen to view the organs of the abdominal cavity. With the aid of forceps and scissors, the liver was removed and immediately weighed.

3. Laboratory Methods

Glycemia was determined in a drop of blood collected by section of the animal's tail using a glucometer (Precision QID, Abbott). Liver fat was determined by the method of Bligh and Dyer (1959).[16] From the total extraction of hepatic fat, suspended in 1 mL of isopropanol, hepatic triglycerides and cholesterol were measured by the Enzymatic-Trinder method, using the commercial kit Labtest Diagnóstica SA (Lagoa Santa, MG, Brazil), according to the manufacturer's protocol. The reactions were read on a spectrophotometer (SpectraMax, model M5).

Plasma concentrations of AST, ALT, total cholesterol and triglycerides were determined using the commercial kit Labtest Diagnóstica SA (Lagoa Santa, MG, Brazil), according to the manufacturer's protocol. The method used to determine AST and ALT was Reitman and Frankel. To determine total cholesterol and triglycerides, the Enzyme-Trinder method was used. The reactions were read on a spectrophotometer (SpectraMax, model M5). AST and ALT activities were expressed in UI/L. Plasma concentrations of total cholesterol and triglycerides were expressed in mg/dL.

Hepatic oxidative stress was assessed by determining lipid peroxidation using MDA measurement, according to the method proposed by Buege and Austi.[17] The antioxidant response was assessed using hepatic GSH concentration, according to the method of Sedlak and Lindsay.[18] The total protein used to express the values of MDA and GSH was determined by the commercial kit Lab test Diagnóstica SA (Lagoa Santa, MG, Brazil), according to the manufacturer's protocol, using the Biuret method. The determination of vitamin A and vitamin E was performed on liver samples by High performance liquid chromatography (HPLC) following methodology proposed by Arnaud et al.[19]

Liver tissue fragments fixed in 10% formaldehyde solution were embedded in paraffin, cut (thickness of 4 μm) and stained by the HE method (Harris Hematoxylin and Eosin), with the purpose of semi-quantitatively evaluating the hepatic steatosis that was classified in crosses, according to the adapted method of Oh et al.[20] The degree of steatosis was associated with the morphological location (zones 1, 2 and 3): 0 (0%); 1-25% (1: little present in zone 3); 25-50% (2: zone 3); 50-75% (3: zone 2 and 3) and 75-100% (4: zone 1, 2 and 3). The presence of inflammatory infiltrate (L: mild; M: moderate and I: intense) and Mallory's corpuscle (A: absent; CP: few corpuscles and MC: many corpuscles) were evaluated. Histopathological analysis was observed using a conventional light microscope (400 x).

From peripheral blood collected from the animal's tail, a blood smear was performed on a microscopic slide. Then, the slides were kept at room temperature for 1 hour and then fixed in 100% ethanol for further staining by the Giemsa method. For each animal, the analysis of the incidence of micronuclei was obtained by examining 2000 polychromatic erythrocytes using light microscopy under oil immersion (100 x magnification objective). The micronucleus assay was performed according to the method proposed by MacGregor et al.[21]

Table 1. Biochemical and weight gain parameters in the different experimental groups.

	CO(n=10)	LEC (n=10)	MCD(n=10)	MCD-LEC (n=10)
Weight gain (g)	401.2±20.4[a]	405.2 ± 12.2[a]	2.2 ± 2.2[b]	180.4 ± 8.1[c]
Liver weight (g)	3.0 ± 0.1[a]	2.7 ± 0.1[a]	6.9 ± 0.4[b]	4.0 ± 0.2c
Glucose (mg/dl)	66.2 ± 3.2[a]	70.3 ± 3.6[a]	73.6 ± 14.5[a]	58.3 ±3.3[a]
Liver Fat (g)	63.2 ± 6.0[a]	78.5 ± 7.2[a]	138.4 ± 15.7[b]	46.9 ± 5.8[c]
Hepatic Triglicerydes	30.0 ± 4.5[a]	33.1 ± 7.8[a]	104.6 ± 13.3[b]	18.9 ± 5.3[c]
Hepatic Cholesterol	3.7 ± 0.5[a]	5.0 ± 0.5[b]	2.5 ± 0.2[ab]	2.5 ± 0.1[ab]

CO = Control Group (CO), LEC = Group Supplemented with Soy Lecithin, MCD = Methionine and Choline Deficient Diet, MCD LEC = Methionine and Choline Deficient Diet Supplemented with Soy Lecithin.
Results expressed as mean ± standard deviation and different letters on the same line indicate $p < 0.05$

Table 2. Hepatic oxidative stress parameters in the different experimental groups.

	CO(n=10)	LEC (n=10)	MCD(n=10)	MCD-LEC (n=10)
MDA (nmol/mg protein)	0.12 ± 0.007[a]	0.12 ± 0.007[a]	0.36 ± 0.04[b]	0.12 ± 0.08[a]
GSH (nmol/mg protein)	0.1 ± 0.01[a]	0.08 ± 0.01[a]	0.62 ± 0.1[b]	0.19 ± 0.03[c]
Vitamin A (μM/g)	451.0 ± 23.5[a]	385.5 ± 33.3[a]	215.3 ± 31.8[b]	245.6 ± 32.4[b]
Vitamin E (μM/g)	414.8 ± 40.2[a]	390.1 ± 62.3[a]	354.4 ± 53.7[a]	100.7 ± 11.8[b]

CO =Control Group (CO), LEC = Group Supplemented with Soy Lecithin, MCD = Methionine and Choline Deficient Diet, MCD +LEC = Methionine and Choline Deficient Diet Supplemented with Soy Lecithin.
Results expressed as mean ± standard deviation and different letters on the same line indicate $p < 0.05$

4. Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). The differences between the means of the experimental groups were statistically assessed by applying the analysis of variance test. Analysis of variance two-way ANOVA for repeated measures, followed by the Student-Newman-Keuls post-test was applied to compare the results of weekly food intake and body weight. For the results of total body weight gain, blood glucose, AST, ALT, total liver fat, total cholesterol and hepatic plasma and triglycerides, hepatic MDA and GSH, one-way ANOVA analysis of variance was applied, followed by the post-test Student-Newman-Keuls. The level of significance adopted was 5%. Statistical analysis was performed using the SigmaStat Program, version 3.1, Software SYSTAT.

5. Results

The absence of methionine and choline in the diet promoted a lower total body weight gain (2.2 ± 2.2 g) compared to the rest of the groups (Control: 401.2 ± 20.4 g; Lec: 405.2 ± 12.2 g; MCD + Lec: 180.4 ± 8.1 g). The MCD + Lec group showed less body weight gain when compared to the Control and Lec groups. There was no difference between the Control and Lec groups. The weight and biochemical hepatic data are showed in the Table 1.

A significant increase in liver weight was observed in the group fed with MCD diet (6.9 ± 0.4 g) when compared with the groups studied (Control: 3.0 ± 0.1 g; Lec: 2.7 ± 0.1 g; MCD + Lec: 4.0 ± 0.2 g). The MCD + Lec group showed an increase ($p < 0.05$) in liver weight compared to the Control and Lec groups.

There was no difference in fasting blood glucose between the groups studied (Control: 66.2 ± 3.2 mg/dL; Lec: 70.3 ± 3.6 mg/dL; MCD: 73.6 ± 14.5 mg/dL; MCD + Lec: 58.3 ± 3.3 mg/dL), but the hepatic concentration of total cholesterol in the Lec group (5.0 ± 0.5 mg/g tissue) was higher when compared to the rest of the groups (Control: 3.7 ± 0.4 mg/g tissue; MCD: 2.5 ± 0.2 mg/g tissue; MCD + Lec: 2.5 ± 0.1 mg/g tissue). However, no difference was observed between the Control, Lec and MCD + Lec groups. We also observed an increase in the hepatic concentration of triglycerides and total fat of the animals that received a diet deficient in methionine and choline (Triglycerides: 104.6 ± 13.3 mg/g tissue; Total fat: 138.4 ± 15.7 mg/g tissue) when compared to all groups evaluated (triglycerides (mg/g tissue): Control: 30.0 ± 4.5; Lec: 33.1 ± 7.8; MCD + Lec: 18.9 ± 5.3; Total fat (mg/g tissue): Control: 63.2 ± 6.0; Lec: 78.5 ± 7.2; MCD + Lec: 46.9 ± 5.8). The Control group, Lec and MCD + Lec showed no difference between them for hepatic triglyceride values. The amount of total fat was lower in the MCD + Lec group compared to the Lec group, with no significant difference in the Control group.

Plasma total cholesterol concentration did not differ between the groups studied. For the plasma triglyceride values, the Lec, MCD and MCD + Lec groups were not different, however, they showed a reduction ($p < 0.05$) in the concentration of this lipid in relation to the Control group (Lec: 76.1 ± 9.8; MCD: 33.7 ± 4.6; MCD + Lec: 88.3 ± 10.8 mg/dL versus Control: 138.3 ± 20.2 mg/dL).

In relation to the oxidative stress parameters, showed in the Table 2, there was a significant increase in the hepatic concentration of free MDA and GSH in the MCD group (MDA: 0.36 ± 0.04 nmol/mg protein; GSH: 0.62 ± 0.1 nmol/mg protein) when compared to the other groups studied (MDA (nmol/mg protein): Control: 0.12 ± 0.007; Lec: 0.12 ± 0.007; MCD + Lec: 0.12 ± 0.008; GSH (nmol/mg protein): Control: 0.1 ± 0.01; Lec: 0.08 ± 0.01; MCD + Lec: 0.19 ± 0.03). However, the Lec and MCD + Lec groups showed no difference in relation to the Control. There was also no difference between the Lec and MCD + Lec groups.

Hepatic concentration of vitamins A and E of the MCD + Lec group (Vitamin A: 245.6 ± 34.2 μM/g tissue; Vitamin E: 100.7 ± 11.8 μM/g tissue) was lower in relation to the Control groups (Vitamin A: 451.0 ± 23.5 μM/g tissue; Vitamin E: 414.8 ± 40.2 μM/g tissue) and Lecithin (Vitamin A : 385.5 ± 33.3 μM/g tissue; Vitamin E: 390.1 ± 62.3 μM/g tissue), with no difference between the MCD group (215.3 ± 31.8 μM/g tissue) only for vitamin A data. However, for vitamin E data, the MCD + Lec group showed a reduction when compared to the MCD group (354.4 ± 53.7 μM/g tissue). The Control, Lec and MCD groups were not different for vitamin E.

The plasma concentration of AST and ALT was higher in the MCD group (AST: 85.6 ± 4.8 IU / L; ALT: 74.4 ± 2.7 IU/L) in relation to the other experimental groups (AST (UI / L): Control: 28.3 ± 2.4; LEC: 10.9 ± 1.6; MCD + Lec: 75.2 ± 2.7; ALT (UI/L): Control: 27.0 ± 1.3; LEC: 22.8 ± 0.9; MCD + Lec: 63.26 ± 2.7). The MCD + Lec group also showed an increase in the concentration of these two transaminases when compared to all groups. The plasma AST concentration was lower ($p < 0.05$) in the Lec group when compared to the Control group. However, this observation has not been verified for the ALT enzyme.

6. Discussion

The nutritional model of methionine and choline deficiency is widely used for the study of NASH in animals. This model does not fully reproduce the pathogenesis of NASH in humans, as it causes a reduction in plasma triglycerides, cachexia and does not cause insulin resistance. However, the study of this model is important to evaluate specific dietary interventions and the use of antioxidants in animal models of steatoepatitis.[10] Interestingly, a study noted that there is a very large variability in response in experimental animals receiving a diet deficient in methionine and choline.

In our study, as well as in the work of Veteläinen et al,[13] the deficiency of methionine and choline in the diet induced a lower body weight gain when compared with the other groups studied, showing a case of cachexia. Supplementation with soy lecithin in the MCD + Lec group promoted greater weight gain compared to the MCD group, however, not enough for this group to achieve body weight gain similar to the Control and Lec groups. In view of this finding, we suggest that phosphatidylcholine, present in soy lecithin, was important for the synthesis of endogenous choline, thus preventing animals from the MCD + Lec group from entering the cachexia picture.

Although studies in the literature report that hyperglycemia and insulin resistance are involved in the progression of NAFLD in NASH, in this experimental model, fasting glycemia did not show any significant difference between the groups studied, indicating absence of glucose intolerance and diabetes mellitus.[22]

Another data evaluated in this study was the total liver weight of the animals. We observed a marked increase in the weight of this organ after administration of the MCD diet for 8 weeks. However, this hepatomegaly was mitigated by the presence of soy lecithin in the diet. A similar result was verified in the study developed by Buang et al, in which steatosis and consequent hepatomegaly induced by the administration of orotic acid were attenuated by the addition of phosphatidylcholine in the diet.[23]

Trying to clarify the mechanisms involved in the emergence of NAFLD and NASH, we investigated whether some biomarkers of the lipid profile (liver and plasma) and oxidative stress were modified with the administration of a MCD or MCD diet supplemented with soy lecithin. In addition to the accumulation of triglycerides in hepatocytes, we can observe in the MCD group, an increase in total liver fat, demonstrating a possible steatosis induced by the alteration of lipid metabolism homeostasis. Some studies reports that the cause of this change in the liver is due to the increased influx of free fatty acids from peripheral adipocytes or diet.[24-26]

In addition, the decrease in its β-oxidation in this organ and/or decreased synthesis and secretion of VLDL, an important exporter of triglycerides and hepatic cholesterol to peripheral tissues also contribute to this accumulation. Supplementation with soy lecithin in the MCD diet, in turn, prevented the increase in hepatic triglyceride concentration. This effect may be associated with changes in hepatic lipid metabolism by phosphatidylcholine. This phospholipid decreases the expression of the enzyme fatty acid synthase and increases the activity of the enzyme carnityl palmitoyl transferase in the liver, causing, respectively, decreased synthesis of triglycerides and increased β-oxidation of fatty acids in the liver.[23]

In contrast, in the present study, plasma triglyceride concentration was low in methionine and choline deficiency. It is suggested that this decrease is secondary to the impairment of triglyceride export mechanisms from the liver to the periphery.

According to the literature animals with a deficit of these two nutrients, usually have lipotoxicity due to the excess of fatty acids in hepatocytes, leading to mitochondrial dysfunction and consequent increase in the production of reactive species oxygen (ROS), such as hydrogen peroxide, free hydroxyl radicals, superoxides and others. ROSs promote lipid peroxidation of the cell membrane resulting in the synthesis of MDA, and consequently, causing necroinflammatory changes in the liver parenchyma and an increase in proinflammatory cytokines, such as tumor necrosis factor α (TNF-α).[10-27]

To protect the cell against the effects of these ROS, the body contains antioxidant defense machinery. One of the participants in this machinery is the tripeptide (l-γ-glutamyl Lcisteinylglycine) GSH, which captures ROSs through enzymatic reactions.[28] In the experimental model of this work, an oxidative damage biomarker (MDA) and an antioxidant substance (GSH) were evaluated in the liver, which showed a significant increase in animals fed a diet deficient in methionine and choline. The increase in MDA shows the presence of oxidative stress caused by the accumulation of hepatic triglycerides, observed in the MCD group.

With regard to soy lecithin supplementation in the MCD diet, it seems to play an inhibitory and/or protective role in triggering oxidative stress, since there was no change in the MDA values in relation to the Control and Lec groups. On the other hand, the higher hepatic concentration of GSH in the MCD group suggests a high protective physiological response against ROSs formed in the lipid peroxidation process of this group. Interestingly, the use of soy protein, when compared to casein, leads to the expression of different genes in animals submitted to an experimental model of hepatic steatosis.[29] A specific gene, S100A4, has been placed as crucial in the development of fatty liver in this model of dietary methionine and choline deficiency.[30]

The use of betaine, another nutrient involved in the same metabolic pathway as lecithin, also improves hepatic antioxidant defense by increasing glutathione levels, in addition to other components of the system antioxidant.[31]

The presence of soy oil (source of phosphatidylcholine) in the parenteral diet seems to prevent the reduction of mRNA expression of antioxidant enzymes, such as superoxide dismutase, catalase and GSH/glutaredoxin, thus hampering the exacerbated synthesis of ROS, lipid peroxidation of the cell membrane and increased MDA.[32]

In addition to reduced glutathione, the body has other efficient protective systems composed of antioxidants from food, such as fat-soluble vitamins A (retinol) and E (tocopherol), which were evaluated in this work. Retinol has the ability to sequester peroxyl radicals and singlet oxygen (in low concentrations) normally formed under physiological conditions. The antioxidant activity of tocopherol, in turn, is exercised by its ability to act as a H donor for the peroxyl radical, blocking the spread of lipid peroxidation of polyunsaturated fatty acids in membranes and lipoproteins.[29]

In our study, we found a reduction in liver concentrations of vitamin A in the MCD and MCD + Lec groups, and of vitamin E only in the MCD + Lec group, indicating the existence of an excessive consumption of these vitamins as a mechanism to re-establish the balance between pro and antioxidant species. As seen earlier, the accumulation of triglycerides and total fat in the hepatocytes of animals with methionine and choline deficits triggers a process of oxidative system imbalance, confirmed by the increase in hepatic MDA in these animals. For this reason, the antioxidant system of vitamin A is activated. Although no changes were observed in the MDA values of the MCD group supplemented with soy lecithin, the antioxidant activity exerted by vitamin E in these animals was also activated.

In order to elucidate whether changes in the hepatic marker of oxidative damage and the accumulation of lipids in hepatocytes, seen in methionine and choline deficiency, could cause some damage to hepatocytes, we performed the measurement of two enzymes: aspartate transaminase (AST) and alanine transaminase (ALT). Significant increases in the values of AST and ALT were observed in the animals that received a diet deficient in methionine and choline in relation to the animals in the control group, indicating a probable picture of hepatitis.

7. Conclusions

The set of data obtained indicates that supplementation with 5% soy lecithin promotes an improvement in the hepatic lipid profile and in hepatocellular damage, which are considered factors involved in the pathogenesis of NAFLD induced by a diet deficient in methionine and choline. Thus, soy lecithin, a source of phosphatidylcholine, prevents the evolution of this liver disease to a severe stage, defined by the presence of an inflammatory process, fibrosis, and necrosis and hepatocellular apoptosis.

Authors' Contributions

AA Jordao and GV Portari contributed to the conception, design, statistical analysis, and drafting of the manuscript. LEC Silva and AK Aguillar contributed to the conception, data collection, and manuscript drafting. All authors confirmed the final version for submission.

Conflicts of Interest

The authors declare no conflict of interest.

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