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Research Article

Curcumin Effects on Oxidative Stress, Vascular Function and Others Parameters in Rats with Non-alcoholic Fatty Liver Diseases

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List of abbreviations in the order of appearance.

NAFL: Nonalcoholic Fatty Liver

NASH: Nonalcoholic Steatohepatitis

ROS: Reactive Oxygen Species

SOD: Superoxide Dismutase

GPx: Glutathione Peroxidase

ALT: Alanine Transaminase

AST: Aspartate Transaminase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

cGMP: cyclic Guanosine Monophosphate

Cu: Curcumin

TC: Total Cholesterol

LDL: Low-Density Lipoproteins

VLDL: Very Low-Density Lipoproteins

TG: Triglycerides

SMA: Superior Mesenteric Artery

Ach: Acetylcholine

L-NAME: N-nitro-L-arginine methyl ester

NA: phenylephrine3

Abstract

Background & Aims: Non-alcoholic fatty liver can include anything from a simple steatosis to a more severe form of liver cirrhosis. Inflammatory and profibrotic mechanisms are crucial factors in the progression of this condition. Curcumin has been shown to have antioxidant properties and possible hepatic and vasoprotective actions against this disorder.

Methods: Sprague-Dawley male rats were fed a hypercholesteraemic diet to induce non-alcoholic fatty liver and divided into two groups: fatty liver with curcumin and fatty liver without curcumin. After euthanasia, blood was collected to measure transaminases levels and lipid profile parameters in the plasma, and the livers were homogenised to measure the activity of superoxide dismutase, catalase and glutathione peroxidase enzymes. We also examined other parameters such as isolated superior mesenteric artery function, portal pressure and anatomopathological studies. The results were expressed as mean±SEM, and the differences between groups were evalued using Student´s t test and MannWhitney test with $p<0.05$ being significant.

Results: Significant differences were observed for the following parameters in the groups without curcumin and with curcumin, respectively: Glutamic pyruvate transaminase (87±11.3 vs. 60.8±9.8IU/l);superoxide dismutase activity (3.46±0.52 vs. 12.16±2.75 U/mg); and catalase activity (6414±735 vs. 9410±1791 U/mg). In addition, a greater vasodilator response to acetylcholine was found in the group given curcumin ($p<0.05$).

Conclusions: Curcumin improves antioxidant capacity by decreasing the action of free radicals and oxidative stress that cause endothelial dysfunction and hepatic 4inflammation associated to non-alcoholic fatty liver. Future clinical studies in humans are needed to evaluate the development of alternative therapeutic strategies.

Introduction

Non-alcoholic fatty liver (NAFL) is a disorder where there is an infiltration of Microvacuoles lipids in hepatocytes in patients having an alcohol consumption of less than 20-30 g per day [1]. The prevalence of this pathology in the worldwide population reaches 15-20%, and it is the most important cause of chronic liver disease [2]. Furthermore, the incidence of the principal risk factors involved in this condition (obesity and diabetes mellitus type 2) has increased [3]. NAFL is the starting point for a progressive chain of clinical symptoms that lead to non-alcoholic steatohepatitis (NASH), which has a 5-7% prevalence in the general population [4], and 3-20% of these patients develop liver cirrhosis within a period of 10 years or more [5]. NASH is characterized by hepatic steatosis, necroinflammation, apoptosis and fibrosis [6]. The excess of free fatty acids in the hepatocyte induces an intracellular beta-oxidation with an increased production of reactive oxygen species (ROS) [7] and mitochondrial dysfunction due to an overload on the antioxidant mechanisms [8].This antioxidant system is composed of enzymes that reduce ROS to water, a less harmful substance. Thus, antioxidant activity can be summarized as the sequential action of superoxide dismutase (SOD) (enzyme that catalyses the dismutation of superoxide anions, O_2^-, in peroxide and oxygen) [9], glutathione peroxidase (GPx) (enzyme that catalyses the reduction of hydro peroxides) [10] and catalase (antioxidant enzyme predominantly hepatic, renal and erythrocytic involved in the detoxification of hydrogen peroxide to water) [11]. As mitochondrial damage progresses with oxidative stress, proinflammatory cytokines that induce chemotactic signals are generated which promote the recruitment of neutrophils and others leukocytes, the subsequent development of necroinflammatory processes and an increase in Alanine Transaminase (ALT) levels [12]. The clinical manifestation of this is an increase in transaminase levels,

mainly glutamic pyruvate transaminase that is preferentially localized in the cytoplasm of Hepatocytes [13].

Oxidative stress is also related to vascular endothelial damage [14]. Vascular endothelium is a functionally complex structure in charge of maintaining equilibrium in vascular tone through the release of endothelial factors that regulate vasodilation and vasoconstriction [15]. The most important factor for the vasodilatation is nitric oxide (NO), synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) [16]. NO induces relaxation of underlying smooth muscle through the production of 3-5 cyclic guanosine monophosphate (cGMP) by stimulation of the enzyme guanylate cyclase [17]. Then with oxidative stress vascular dysfunction is induced by a reduction in the production or a reduction in the biodisponibility of NO that could be associated with a lower vasodilation response to agents that promote its production (i.e. acetylcholine) [18].On the other hand, it has been found that curcumin (*Diferuloylmethane*), a hydrophobic polyphenol derived fromtherhizome (turmeric) of the plant Curcuma Longa L., acts as a protective agent against several of the pathological mechanisms associated to NAFL [19,20]. For example, curcumin passes through the membrane of hepatocytes and impedes the intracellular generation of ROS (H_2O_2, HO_x, ROO_x) [21] and their release into the systemic circulation. Thus, these effects can reduce the endothelial damage produced by oxidative stress. However, the mechanisms responsible for this protective effect are not well known. Our objective was to evaluate the effect of curcumin on the physio pathological mechanisms involved in the progression of the disease. Then we measured plasma levels of transaminases, antioxidant capacity of hepatic tissue and vascular function in an animal model for NAFL. In our laboratory, we have developed a dietary animal model of NAFL in Sprague-Dawley rats based on previous data supporting the use of a high fat (HF) with cholesterol [22]. In mice, cholesterol can induce distinct sets of hepatic inflammatory gene expression [23] and is an important risk factor for the progression of NAFL to NASH as it sensitizes the liver to TNF and Fas-induced steatohepatitis [24]. In addition, Sprague-Dawley animals appear susceptible to steatohepatitis development when fed a high-fat diet, and this is likely associated with their susceptibility to diet-induced obesity [25].Finally, in our laboratory in the last years we have worked in a model in Sprague Dawley from NAFLD, based on previous publications that endorse the use of fat and calories rich diet, what lead to the verification that 100% of cases presented intracellular microvacuoles in > 5% of hepatocytes.

Materials and Methods

Animals: Male Sprague-Dawley rats were used at 2 months of age and maintained during 12 weeks under optimal environmental conditions. Animal care and use was in compliance with the Ethical Guidelines established by the Ethical Committee from the Faculty of Medicine of Universidad Católica del Norte.

Reagents: The cocktail of protease inhibitors was purchased from Roche (San Francisco, USA). Acetylcholine, L-NAME, sodium nitroprusside, curcumin and 8enzyme activity kits for catalase; glutathione peroxidase and SOD were purchased from Sigma (St. Louis, USA).

Study groups: The total number of animals (n=15) was divided into three groups: control group (n=3), NAFL group (n=6) and NAFL-Curcumin group (n=6).

NAFL Induction: In order to induce NAFL, treated rats were given a diet with 5% of cholesterol (36% fat, 30% protein, 24% carbohydrates, 10% humidity) for 6 weeks. Subsequently, the rats were divided into two groups (n=6/group): one group was given curcumin (30 µg/day/rat) added to the pellet (NAFL-Cu); and the second group continued with the diet described above (NAFL) for another 6 weeks. Weight measurement, water consumption and food intake: Weight variation of the rats was measured once a week and the consumption of water and food was done three times a week (model LPW-1530, Jadever, China). Weight gain was calculated in grams for each rat at the beginning of administration of curcumin and at the end of the study (final weight – initial weight). In order to avoid bias in the initial weight, the data was normalized as ([final weight-initial weight Cu]/initial weight Cu). Average water and food consumption was quantified for each cage of rats (3 rats/cage) during the entire study, which was distributed proportionately to the average weight for each rat ([average cage consumption/total weight of rats in the cage]/rat weight).

Euthanasia and sample retrieval: At 20 weeks of age, the rats were anesthetized with chloral hydrate (500 mg/kg) administered intraperitoneal. When the animals displayed are flexia, they were placed in a supine position and an incision was made in the abdominal midline, avoiding damage to the diaphragm 9.

Measurement of Portal Pressure: A barometer was calibrated with a column of water so that the pressure measurements were expressed in centimetres of H_2O (cm H_2O). After euthanasia, the portal vein was punctured after entering the abdominal cavity and the pressure from the portal vein was recorded by using a data acquisition system connected to a computer (Power lab/8SP, AD Instruments Pty Ltd, New South Wales, Australia). Subsequently, the ribs were removed and the adjacent tissue was retracted with forceps in order to have access to the thoracic and abdominal cavity where the tissue needed for experimentation was removed.

Measurement of biochemical parameters: Three to 10 ml of blood was collected in micro centrifuge tubes and sent to a clinical laboratory for analysis of alanine transaminase, aspartate transaminase (AST), bilirubin, total cholesterol (TC), and low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and triglycerides (TG).

Quantification of hepatic antioxidant capacity: The left lobule of each extracted liver from the rats was individually homogenized in 5volumes of phosphate buffered saline (PBS) buffer in the presence of protease inhibitors. Enzymatic activity for catalase, SOD and GPx was measured using a spectrophotometer (UVVIS model Specord 205, Analityk-Jena, Germany) following the instructions provided by the manufacturer. All measurements were performed in triplicated and values were expressed as U/mg of protein.

Histopathologicalnalysis of livers: The right lobule of each liver was placed in 10% formalin/PBS and sent to the Pathological Anatomy Service at the San Pablo Hospital in Coquimbo for anatamopathological analysis. The fixed tissue was sliced 10 in sections of 5 µm and stained with hematoxylin and eosin in order to visualize the cellular structure. Subsequently, the samples were stained with Masson's trichrome in order to observe the fibrotic presence in the hepatic tissue [26].Vascular function studies in isolated blood vessels: The Superior Mesenteric Artery (SMA) was removed from each rat right after euthanasia. Small resistance arteries (third branch, 370-µm internal diameter) were dissected from vascular bed and placed in ice-cold saline. Arterial segments of approximately 2 mm lengths were mounted in a four-channel small vessel myograph (610M Multimyograph,

Danish Myotechnology, Aarhus, Denmark) for measurement of isometric force. The vessel segments were incubated in Krebs Ringer bicarbonate (KRB) at 37°C and gassed with a mixture of 5% in CO_2 balanced with O_2. Following 1 h of incubation, optimal diameter was determined for each artery. This is the diameter at which the artery displayed maximal contractile responses to Krebs buffer with equimolar replacement of Na^+ with 125 mm K^+ (K-KRB). Once the optimal diameter was established, the vessels were left at rest for a further 30 minutes. All vessels were checked in each experiment with K-KRB to assess the maintenance of contractile capacity. Afterward, studies of vascular function were performed in order to evaluate vascular dysfunction by loss of vasodilation to acetylcholine.

The following experiments were done with the SMA:

a) Concentration-response curves to increasing concentrations of Ach (from 10-10 M to 10-3 M) after previous induction of contraction with phenylephrine (NA) (10-5 M);

b) Concentration-response curves to Ach after inducing contraction with phenylephrine and a previous incubation with 10-5 M of L-NAME for 30 minutes;

c) Concentration-response curves to sodium nitroprusside after inducing contraction with phenylephrine (10-5 M).11Maximal responses (Rmax) and sensitivity (EC50 or pD2) to the different dilating agents were obtained by fitting the concentration-response curves to a Boltzmann function (GraphPadPrism®). Rmax were expressed as tension (N m-1) for K^+ and for the adrenergic agonists as percentage of Rmax to K^+ (% Kmax). Sensitivity was expressed as EC50 (the concentration at which 50% of Rmax were obtained) for K^+.The Krebs buffer contained (in mM) NaCl 118.5, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, CaCl2 2.5, glucose 5.5 with a pH of 7.4. In K-KRB (125 mM K^+) all NaCl was replaced by an equimolar amount of KCl.

Statistical analysis: All data is expressed as mean±SEM. The differences between groups were analysed with Student´s t test and the Mann-Whitney test using the program GraphPadPrism®. $p<0.05$ was considered statistically significant.

Results

Weight, water consumption and food intake: No differences were found in average body weight between the groups (NAFL-Cu: 0.18±0.07 g and NAFL:0.11±0.34 g; $p>0.05$). Furthermore, there were no significant differences in water consumption (NAFL-Cu:

108.5±25.94 ml and NAFL: 105.87 ±23.89 ml) or food intake (NAFL-Cu: 54.44±14.12 g and NAFL: 49.68±6.8 g; p>0.05) between the groups (Table 1) Biochemical Parameters.

Table 1: Weight, water consumption and food intake.

	Body weight	Water consumption	Food consumption
	(Final weight- Initial weight) / Initial weight	ml	g
NAFL	0.182±0.074	328.13±61.27	137.51±10.24
NAFL-Cu	0.113±0.017	229.57±143.06	96.6±43.37

There were no differences in average body weight, water consumption and food intake between the groups.

Hepatic profile: The results for these assays are summarized in (Table 2 & (Figure 1) No significant differences were seen in AST values despite that the average values were higher in the NAFL group (125.7±11.84UI/l) as compared to the NAFL-12Cu group (112±29.32UI/l). However, the data showed significant differences for bilirubin and ALT levels, where the NAFL group exhibited a higher average values (0.37±0.018 mg/dl for total bilirubin and 87±11.3UI/l for ALT) as compared to the average values (0.21±0.074 mg/dl for total bilirubin and 61±9.8 UI/l for ALT) in the NAFL-Cu group (p<0.01) Nevertheless, calculation of the (AST/ALT) ratio demonstrated no significant differences (NAFL: 1.43±0.08 and NAFL-Cu: 1.85±0.5).

Table 2: AST, ALT, triglycerides, total cholesterol and total bilirubin levels between groups. No significant differences were seen in AST values, total cholesterol and triglycerides. However, the data showed significant differences for bilirubin and ALT levels. **: p<0.01.

	AST	ALT	Triglycerides	Total Cholesterol	Total Bilirubin
	UI/l		mg/dl		
NAFL	125.7±11.8	87±11.3	84.67±32.39	57.67±6.03	0.37±0.02
NAFL-Cu	112.0±29.3	61±9.8**	63.40±15.08	56.2±11.3	0.21±0.07**

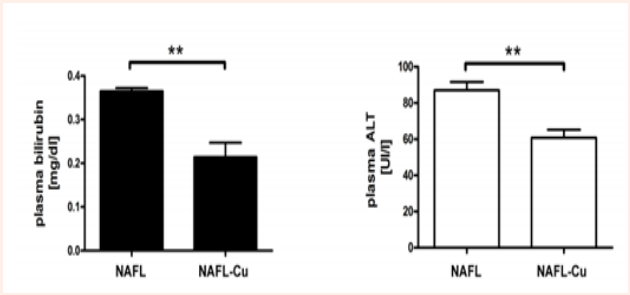

Figure 1: Levels of bilirubin and ALT. Analysis of bilirubin and ALT levels revealed significant differences between treated groups, with higher values seen in the NAFL group. **: p<0.01.

Lipid profile: The average values for triglycerides and total cholesterol were higher in the NAFL group (TG: 84.67±32.4 mg/dl; TC: 57.7±6.03) compared with the NAFL-Cu group (TG: 63.4±15.1mg/dl;TC:56.2±11.03),showing not statistically significant differences between groups (Table 2).

Oxidative stress: Evaluation of the antioxidant activity in the livers of rats with NAFL in the presence and absence of curcumin is shown in (Figure 2). The data demonstrates a significant increase in the antioxidant activity of the enzymes SOD and catalase in NAFL-Cu rats as compared to the NAFL group (SOD activity NAFL-Cu: 12.24±2.33 U/mg prot, SOD activity NAFL: 3.25±0.65 U/mg prot, p<0.0001, figure 2A; catalase activity NAFL-Cu: 9743±1606 U/mg prot, catalase activity NAFL: 6218±814.8 U/mg prot, p<0.01, (Figure 2B). The same tendency was found with the enzyme GPx, although the differences were not significant (GPx activity NAFL-Cu: 173.5±24.42 U/mg prot; GPx activity NAFL: 128.4±17.22 U/mg prot, p=0.08, (Figure 2C).

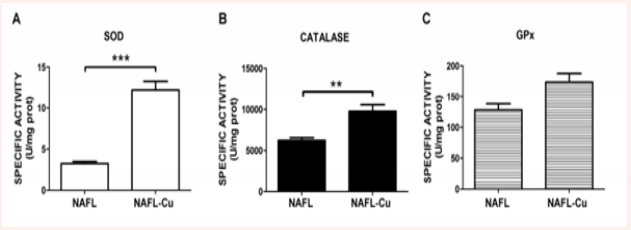

Figure 2: Enzymatic Activity. (A) Difference of specific activity for (A) SOD, (B) Catalase, and (C) GPx according to the study group. *: p<0.05; **: p<0.01; *** p<0.0001.

Histopathological analysis of livers: The samples of hepatic tissue from all of the rats showed fat vacuoles in more than 20% of the hepatocytes. However, none of the samples demonstrated inflammatory infiltration, necrosis or fibrosis. 13 Vascular function in isolated vessels Evaluation of vascular function in the SMA is shown in (Figures 3 and 4).

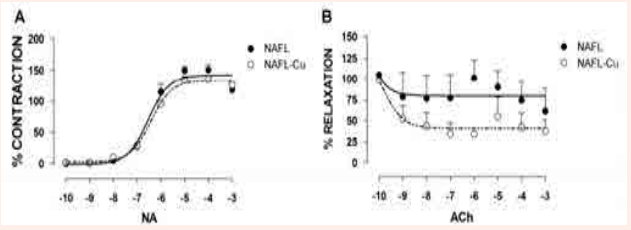

Figure 3: Vascular response of superior mesenteric arteries to phenylephrine and Ach. (A) The graph shows the percentage of contraction of the superior mesenteric arteries. The diameter obtained after induction of contraction with phenylephrine was considered as 100%. (B) Dilator response to increasing concentrations of acetylcholine, after contraction with phenylephrine.

Dilator response to increasing concentrations of Ach: The dilator response of SMA to Ach, after previous contraction with phenylephrine, for the NAFL group had a Rmax=20.5%±5.9; whereas the dilator response of the SMA in the NAFL-Cu group had a R max=59.2%±3.3; 38.7% more than the NAFL group (p<0.05, (Figure 3B).

Response to Ach after incubation with L-NAME: Vasodilation of the SMA in response to increasing concentrations of Ach after incubation with L-NAME exhibited the following results: Rmax=30.3%±4.2 for the NAFL group, and R max=38.6%±3.7 for the NAFL-Cu group. No significant differences were found between the groups (Figure 4A).

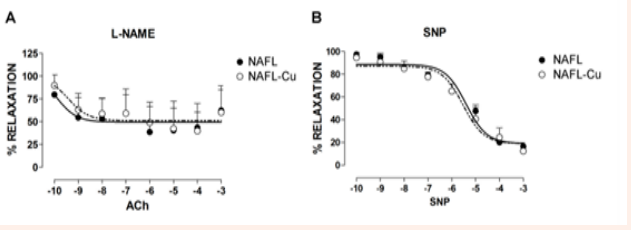

Figure 4: Vascular response of superior mesenteric arteries to L-NAME and nitroprusside. (A) Acetylcholine response after incubation with L-NAME. (B) Acetylcholine response after incubation with nitroprusside.

Response to nitroprusside: Vasodilation of the SMA in response to increasing concentrations of nitroprusside showed Rmax values of 69.3% ± 6.36 for the NAFL group, and 68.0% ± 5.04 for the NAFL-Cu group, with no significant differences between the groups (Figure 4B).

Portal Pressure: The average pressure was 7.38±2.48 cm of H_2O for the NAFL group and 9.3±1.27 cm of H_2O for the NAFL-Cu group.

Discussion

NAFL is a pathology increasingly diagnosed worldwide and is the most common liver disease in western countries [27, 28]. It covers a wide spectrum of conditions, ranging from simple fatty liver to non-alcoholic steatohepatitis with or without fibrosis; cirrhosis and its associated complications [29, 30].14There are studies that evidence that some of the mechanisms that can stimulate this pathological progression are an increase in oxidative stress and a reduction in the intrahepatic antioxidant capacity. Consequently, the appearance of necroinflammatory phenomena gives way to the progression of the disease. Therefore, the deterrence of one of these pathophysiological mechanisms could result in a lower probability of the disease reaching a cirrhotic stage. Our study presents a model of NAFL in which increased oxidative stress and endothelial damage is recognized. Our results, obtained using rats submitted to rich fat diet, support a condition of NAFL with presence of vacuolar lipids in hepatic cells and without evidence of inflammation, fibrosis and necrosis. This model allowed us to evaluate the role of curcumin in the restitution of antioxidant activity and vascular function. Due to the high oxidative stress in NAFL, there is a notable increase in the levels of free radicals in the blood. Several studies report that curcumin prevents lipid peroxidation by binding to phosphatidylcholine micelles and inhibiting fatty acid deoxygenation induced by lipoxygenase [32]. Indeed, it has been shown that the more frequent metabolite of curcumin, tetrahydrocurcumin, performs this process in liver microsomes and erythrocyte membranes increasing the levels of endogenous antioxidants, giving a protective effect against the pathological and physiological processes like aging [33]. Our results showed that the NAFL-Cu group exhibited levels of antioxidant enzyme activity close to normal values and significantly higher than those from the NAFL group. This could be explained by curcumin induction of antioxidant enzymes expression [34] or by the larger availability of GPx, SOD and catalase enzymes to carry out redox reactions, due to 15 the reduced production of free radicals by the action of curcumin [35]. This capacity of curcumin is the same one responsible for the results obtained in ALT, the more specific and predicative liver enzyme for cytolytic liver damage [36]. Our results do not shown any statistically significant differences in the fatty acid metabolism in rats treated in the presence or absence of curcumin. However, their values were lower on the NAFL-Cu group. The lack of statistical significance could perhaps be due to the small sample number of rats for each group. Another possible reason could be that in this model the lipid scavenger effect of curcumin is not enough to fully offset the increased daily intake of TG. Therefore, we suggest that future studies should evaluate the effect of curcumin on another NAFL model having a lower dietary intake of TG and TC to examine if there exist a significant effect of curcumin in improving the lipid profile. In order to have an increase in portal pressure, the liver has to reach advanced stages of inflammation followed by per sinusoidal fibrosis, regeneration bridges, intrahepatic endothelial dysfunction, and therefore, an increase in intrahepatic vascular resistance that leads to a augmentation in the portal pressure [37]. While hepatocyte damage with accumulation of fat vacuoles it was not sufficient to produce an increase in portal pressure, therefore, we were unable to evaluate the influence of curcumin on portal hypertension. The data from our vascular experiments demonstrated that NAFL produces modifications at endothelial level affecting the vasodilation response mediated by acetylcholine of the mesenteric arteries. Interestingly, our results emphasize the capacity of curcumin to revert this pathological endothelial phenomenon. Experiments measuring the vasodilator response after incubation with L-NAME or16nitroprusside showed no significant differences. This is because the pathways that promote and inhibit, respectively, these two compounds depend on NO. The pathway that has NO as a product that acts on smooth muscle only depends on endothelial cells when they are stimulated by Ach through its muscarinic receptors [38]. Therefore, when the synthesis of NO was blocked or stimulated, the vessels from both groups were affected in the same way. We postulate that, together with the possible destruction of endothelial cellular membranes, many cell receptors are lost including muscarinic that are responsible for vascular dilation pathways, and the mesenteric vessels of rats with NAFL do not have the capacity to auto regulate the splanchnic blood flow. Curcumin, through its antioxidant capacity, stabilizes membranes [39], allowing endothelial cells to maintain muscarinic receptor dependent pathways and the synthesis of endothelial-dependent NO. It is possible that these effects found in SMA could also be replicated at the level of intrahepatic circulation. If intrahepatic action exists, it is expected that curcumin acts by reducing the endothelial dysfunction and the consequent increase in intrahepatic resistance, which is one of the initial indicators of portal hypertension. Regarding the other measured variables (weight, food intake and water consumption), we believe that the observed changes in the biochemical

Parameters are not due to the differences in food or water intake or to the differences in body weight of the animals. Finally, the livers from all the rats exhibited fatty liver. However, no inflammatory elements related to steatohepatitis were found. This could be due to the duration of the hypercholesteraemic diet and that Sprague-Dawley rats rarely manifest hepatic phenomena. 17We can conclude that curcumin, as related to liver disease associated to a high cholesterol diet, induces an increase in hepatic antioxidant capacity and, consequently, may reduce hepatocellular damage by ROS. In addition, curcumin maintains the regulatory capacity of splanchnic arterial flows and thus could decrease inflammatory activity in livers with steatosis. The results presented in this study suggest that curcumin may play a role in future therapeutic regimen to halt the damage progression in patients with NAFL.

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