ORIGINAL ARTICLE

ANTIOXIDANT ROLE OF L-CARNITINE IN AN EXPERIMENTAL MODEL OF OXIDATIVE STRESS INDUCED BY INCREASED FRUCTOSE CONSUMPTION

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ABSTRACT

Objectives: To evaluate the role of L-carnitine (LC) on fructose-induced oxidative stress in Holtzman rats**. Materials and methods:** An experimental study was carried out during 56 days, in patients assigned to 4 groups: control, control+LC, fructose and fructose+LC. Patients in the fructose group received treatment during 56 days, and those in the LC groups were treated during the last 28 days. Fructose was given on demand and LC was administered orally at a dose of 500 g/kg/24 h. Lipid peroxidation (MDA), superoxide dismutase activity, free LC and mitochondrial and post-mitochondrial proteins were measured in liver tissue. Glycemia, insulin and the homeostasis model assessment of insulin resistance (HOMA-IR) were measured in blood plasma. We measured insulin concentration and studied the histology of pancreatic tissue. **Results:** LC treatment showed a decrease ($p < 0.05$) of MDA when compared to the control group (21.73 ± 5.36 nmol/g tissue vs. 64.46 ± 7.87 nmol/g tissue). Mitochondrial and post-mitochondrial proteins increased ($p < 0.05$) in comparison to the control group; pancreatic insulin also increased when compared to the control (341.8 ± 42.3 µUI/ml vs. 70.1 ± 9.6 µUI/ml, $p<0.05$). The role of LC against fructose-induced oxidative stress did not show any decrease of MDA, but decreased ($p < 0.05$) SOD Cu/Zn activity (9.39 ± 1.5 USOD/mg protein vs. 13.52 ± 1.5 USOD/mg protein). We observed that LC improves HOMA-IR in blood plasma. Histological analysis of the pancreas showed that the presence of LC increased the number and size of the islets of Langerhans. **Conclusions:** LC favors changes in the oxidative metabolism and it also contributes to glycemic homeostasis when fructose is consumed.

Keywords: L-carnitine; Oxidative stress; Fructose; Antioxidants; Insulin; Malondialdehyde Superoxide Dismutase; Glycemia (Source: MeSH NLM).

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INTRODUCTION

In Peru, over the last few years, the number of patients with chronic non-communicable diseases related to inadequate nutrition, such as cardiovascular and respiratory diseases, cancer, and type 2 diabetes mellitus, has increased. Research on experimental animals has shown that a diet rich in fructose causes chronic inflammation, which can lead to obesity, insulin resistance and metabolic syndrome. The evolution of this process can generate diabetes mellitus type 2 [1,2].

Chronic non-communicable diseases are associated with oxidative stress, as well as fructose consumption. Oxidative stress is the imbalance between the production of reactive oxygen species (ROS) and the defense mechanism, which determines the pathogenesis of several diseases [2].

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L-carnitine (L-3-hydroxy-4-N-N-trimethyl amino-butyrate) facilitates the entry of long chain fatty acids into the mitochondria, for oxidation and production of adenosine triphosphate (ATP) in different tissues [3]. L-carnitine (LC) is an essential nutrient; 75% obtained from the diet and 25% synthesized endogenously [3,4]. Several studies have shown the antioxidant effect of LC in different diseases, either as a scavenger or as a factor that increases the activity of antioxidant enzymes [3,5,6].

There are few studies on the effect of LC on oxidative stress in experimental models with high fructose diet. The aim of this research is to evaluate the effect of LC on oxidative stress associated with excessive fructose consumption in an experimental model with Holtzman strain rats.

KEY MESSAGES

Motivation for the study: The L-carnitine (LC) produced in tissues has a role in the lipid metabolism. Its antioxidant role in a model of fructose-induced oxidative stress has not been fully evaluated. Fructose is a highly-consumed sugar that is mainly present in processed foods.

Main findings: The administration of LC to Holtzman rats decreased liver lipoperoxidation and increased insulin production. LC administered during the oxidative stress model increased the activity of the mitochondrial superoxide dismutase (Mn-SOD) enzyme and significantly improved HOMA-IR.

Implications: LC shows an antioxidant role under this model. Additionally, this study was conducted in a national laboratory and contributes with new evidence.

MATERIALS AND METHOD

Population and sample

This experimental research was carried out on four groups, two of which received water on demand and food with and without LC, and the other two received fructose (40%) on demand and food with and without LC.

Animals and diet

We used 24 two-month-old male Holtzman rats with an approximate weight of 217 ± 40 g, purchased from Instituto Nacional de Salud (Lima, Peru). They were placed in polycarbonate cages with stainless metal lids throughout the study. They were kept for seven days under acclimatization and received tap water on demand as well as food based on a commercial concentrate obtained from Universidad Nacional Agraria La Molina. The experiment was carried out in the vivarium of the Faculty of Medicine of Universidad Nacional Mayor de San Marcos, at room temperature between 23 and 26 °C, and a relative humidity of 60-70% with 12 hours of light/darkness. The LC (500 mg/kg per 24 h) was orally administered by an orogastric cannula.

We formed 4 groups, each with 6 rats randomly assigned with the OpenEpi program. Acclimatization conditions were maintained.

The control group (C) received feed and tap water on demand during the whole experiment; the control + L-carnitine group (C+LC) received feed and tap water on demand during the whole experiment plus L-carnitine at 500 mg/kg/24 h from day 28; the fructose (F) group received feed and fructose (40%) on demand during the whole experiment; and the fructose + L-carnitine group (F+LC) received feed and fructose (40%) on demand during the whole experiment and L-carnitine 500 mg/kg/24 h from day 28.

On the 27th and 56th days of the experiment, all the rats were fasted for the glycemia measurement. On day 57 the rats were euthanized by decapitation, after rapid and deep ether sedation. The flow chart was followed according to figure 1.

Fructose and L-carnitine preparation

The solutions were prepared daily: D-fructose >99% (Omnichem S.A.C, from Wuxi, China) and LC at 10% (Omnichem S.A.C, Ningbo, China). The tap water with fructose (40%) was based on the weight/volume formula.

Preparation of the homogenized products

The liver was washed by perfusion with 0.154 M KCl. The homogenates were prepared at 10% in saline phosphate buffer (SPB) using a Potter-Elvehjem type glass homogenizer. Three centrifugations were carried out at 4 °C (refrigerated centrifuge model MPW380R, MPW Med instruments); the first one was at 700 g for 5 minutes and the precipitate was discarded; the second one, with the supernatant, was at 9,500 g for 15 minutes. The supernatant corresponded to the post-mitochondrial fraction, and the precipitate corresponded to the mitochondrial fraction. The precipitate was washed twice with the SPB buffer at the same speed and for the same time as were needed to obtain the mitochondria. Then it was resuspended with 2 mL of the same buffer. Similarly, we prepared the pancreatic homogenate, which was the

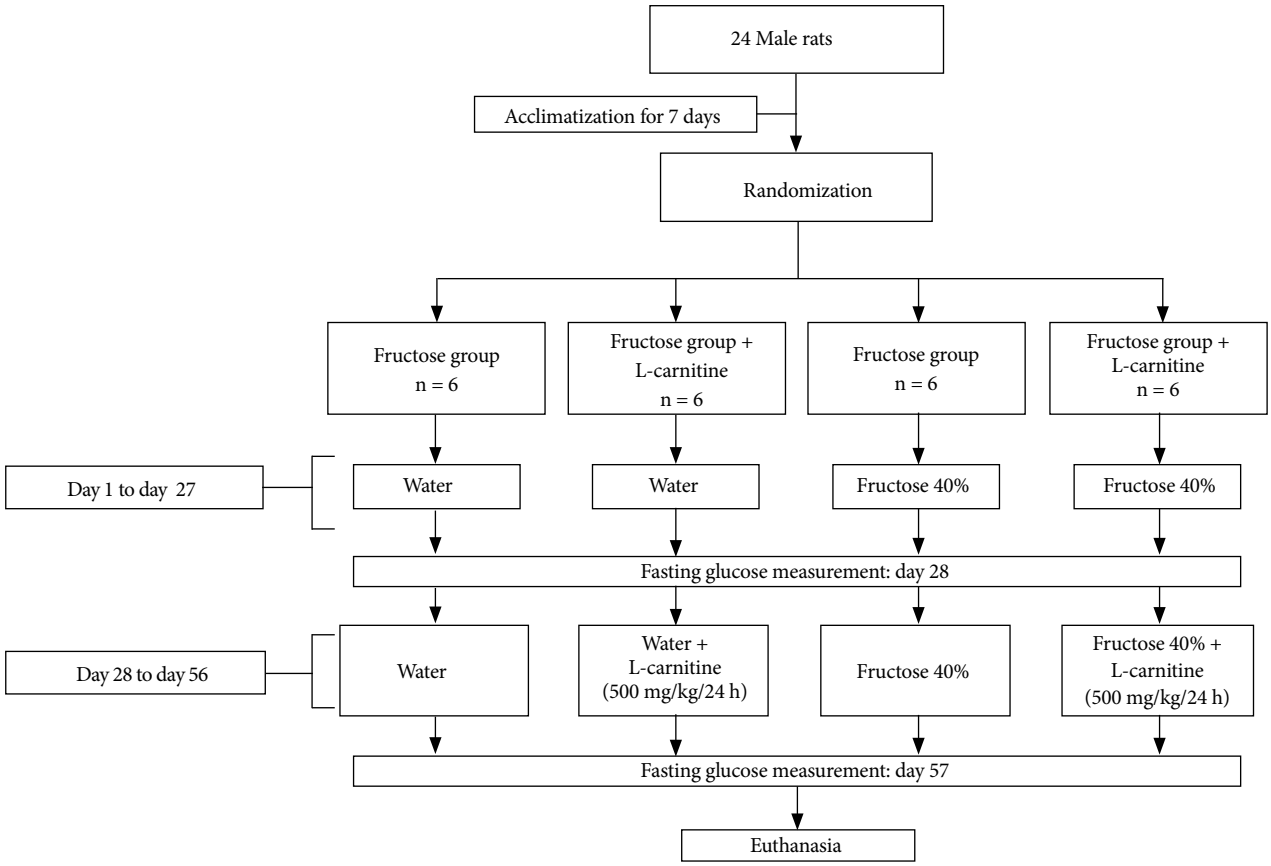

Figure 1. Treatment flow chart for rats from day 1 to day 57.

supernatant obtained after only one centrifugation at 700 g for 5 minutes.

Measurement of free carnitine, glucose, insulin, and the HOMA-IR

The blood samples were obtained from the tail vein. Glycemia was determined with a glucometer based on the conductometric method (Accu-chek Instant) on day 28 and day 57. Insulin measurement in plasma and pancreatic homogenate was performed with the ELISA kit on day 57 (Sigma-Aldrich, USA). Insulin resistance was evaluated with the insulin resistance homeostatic model assessment: HOMA-IR= [glucose (mg/dL) × insulin (mUI/mL)] /405. The measurement of free LC in the liver homogenate was performed with the ELISA kit (Sigma-Aldrich, USA).

Superoxide dismutase activity

We measured superoxide dismutase activity on the liver tissue, according to Marklund and Marklund [7]. The inhibition of pyrogallol autooxidation in alkaline medium was the same for superoxide dismutase (SOD) activity in the mitochondrial fraction (Mn-SOD) and for the post-mitochondrial fraction (Cu/Zn-SOD). The kinetics was followed for three minutes at 420 nm in a spectrophotometer (Thermo Fisher Scientific, G10S UV-Vis). To report the enzymatic activity, the definition of the SOD unit was taken as 1U SOD=Δ of absorbance 0.02/2 × min (±10%).

Measurement of lipoperoxidation

After precipitation with 20% trichloroacetic acid, we measured the action between thiobarbituric acid and the decomposition products of lipoperoxidized species, such as malondialdehyde (MDA) in the hepatic homogenization, and obtained a colored complex that was read at 535 nm. The molar extinction coefficient (ε) was $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [8].

Total protein measurement

Total proteins were quantified by the Biuret method [9]; the reading was done after five minutes at 540 nm. We used a 2% albumin solution as a standard and measured total proteins in the mitochondrial and post-mitochondrial fractions obtained from the homogenized liver [8].

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Statistical analysis

We used Shapiro Wilk's test to evaluate normality and Bartlett's test for variance homogeneity, and the parametric analysis of variance (ANOVA) and Scheffé's test as post hoc tests for multiple comparisons. Statistical significance was assumed when the value was $p < 0.05$. We used the statistical program Stata 13.

Ethical aspects

We followed the ethical standards detailed in the Guideline for Handling and Care of Laboratory Animals of Ministerio de Salud - Instituto Nacional de Salud. The chosen type of euthanasia is contemplated in Law 30407, Law of Protection and Welfare of Animals.

RESULTS

Fasting glycemia results and the HOMA-IR scores did not show significant variations. However, the HOMA-IR score increased by 28.3% because of fructose consumption, when compared to the C group. In the F+LC group, it decreased by 25.8% compared to the F group (Table 1).

Free LC, mitochondrial and post-mitochondrial total proteins showed significant group difference in liver tissue. However, the only significant difference in peer evaluation was found in the free LC, which showed an increase of 21.5% in the F+LC group compared to the C group (Table 1).

The administration of LC stimulated production of insulin in the pancreatic tissue. The increase of insulin levels in the C+LC group was highly significant ($p < 0.001$) compared to the C group; the increase was of 387% (341.8 ± 42.5 vs. 70.1 ± 9.6 µIU/mL). Fructose consumption produced a significant decrease ($p < 0.01$) in pancreatic insulin (12.6 ± 4.2 µIU/mL). LC administration plus 40% fructose consumption produced a 100% recovery rate (25.8 ± 12.7 vs. 12.6 ± 4.2 µIU/mL), but this value was not like the one obtained from group C (Figure 2).

During the macroscopic evaluation, we observed that fructose increased abdominal fat in the F group and the F+LC group. The increased fat mass included the mesentery and retroperitoneum compared to the C group and the C+LC group.

During the histological evaluation of the pancreatic tissue, we observed that the presence of LC (C+LC group) increased the number and size of the islets of Langerhans, even more than the pancreatic acini, compared to the other groups. In the F group and the F+LC group, the size of the islets of Langerhans increased in some regions near the blood vessels when compared to the C group (Figure 3).

When evaluating the antioxidant effect, we observed a significant decrease of 30.5% of Cu/Zn SOD activity in the F+LC group when compared to the C group (9.4 ± 1.5 vs. 13.5 ± 1.5 USOD/mg protein, $p < 0.05$) (Figure 4). However, the compensatory change in both fractions was notorious, while the activity decreased at the post-mitochondrial level, we observed an increase in the mitochondrial activity.

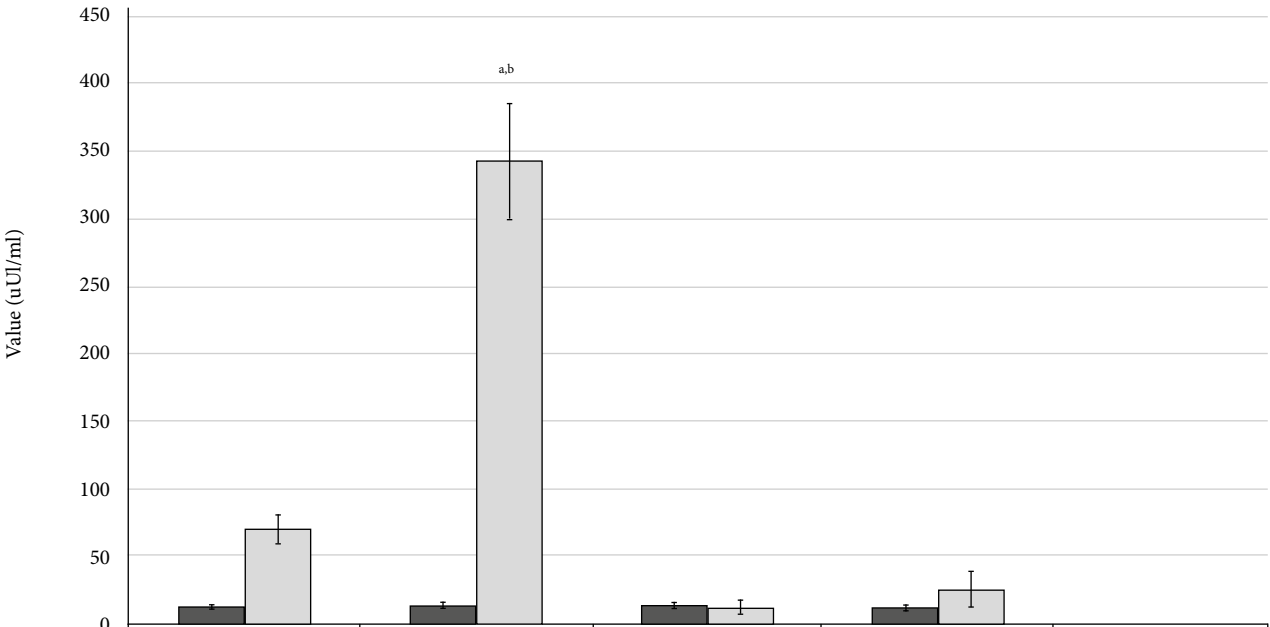
The administration of LC produced a significant decrease in MDA levels ($p < 0.01$) compared to the C group. Consumption of fructose (40%) (F group) caused a significant increase of 21% ($p = 0.03$) compared to the C group. LC administration plus fructose consumption did not show a significant decrease of the MDA levels (Figure 5).

Table 1. Serum and liver parameters.

Parameter	Control (C)	Control + L-carnitine (C+ LC)	Fructose (F)	Fructose + L-carnitine (F + LC)	p value [a]
Serum parameters					
Fasting glucose - day 28 (mg/dL)	85.1 ± 8.6	82.5 ± 3.9	85.5 ± 6.3	81.8 ± 16.4	0.912
Fasting glucose - day 57 (mg/dL)	76.5 ± 12.9	85.3 ± 5.2	78.8 ± 10.6	77.6 ± 4.33	0.689
HOMA-IR	2.4 ± 0.4	2.8 ± 0.6	3.1 ± 0.1	2.3 ± 0.30	0.131
Liver parameters					
Level of free L-carnitine (nmol/g of tissue)	553.9 ± 96.8	659.3 ± 42.3	602.8 ± 48.1	673.9 ± 64.6 [b]	0.037
Mitochondrial total proteins (mg/dL)	50.5 ± 11.1	76.3 ± 14.7 [b]	62.0 ± 12.6	65.7 ± 10.2	0.018
Post-mitochondrial total proteins (mg/dL)	27.8 ± 6.7	41.1 ± 5.8 [b]	32.2 ± 1.4	36.5 ± 6.9	0.008

Values expressed as a mean ± standard deviation

[a] ANOVA test, [b] statistically significant compared to the control group



	C	C + LC	F	F+ LC	p value [c]
Serum	13 ± 0.5	13.9 ± 1.6	13.9 ± 1.1	11.9 ± 1.5	0.157
Pancreas	70.1 ± 9.6	341.8 ± 42.5	12.6 ± 4.2	12.6 ± 4.2	< 0.001

C: control group, C+LC: control + L-carnitine group, F: fructose group, F+LC: fructose group + L-carnitine Values expressed as mean ± standard deviation
[a] statistically significant compared to the control group, [b] statistically significant compared to the fructose group, [c] ANOVA test

Figure 2. Serum and pancreas insulin values of the studied groups.

DISCUSSION

We have observed that the administration of LC plays an antioxidant role, related to the excessive consumption of fructose in rats of the Holtzman strain.

Fructose is a sugar added to processed foods and its consumption has increased in various societies. Excessive fructose intake is associated with insulin resistance, obesity, dyslipidemia, and metabolic syndrome [1,2,10,11]. L-carnitine is an endogenous aminoacid associated with lipid metabolism; it has also been reported to have antioxidant activity.

The model of fructose-induced oxidative stress was used because of the metabolic changes it produces in serum and tissue. Fructose can generate ROS *in vivo* and *in vitro,* as does glucose [1,2,10,]. In this study, fructose (40%) on demand did not modify the fasting plasma glucose levels during eight weeks. Similar results were reported by Andrade *et al.* [11] who used fructose (10%) as treatment on demand for 18 weeks. However, Mamikutty *et al.* [1] demonstrated increased glycemia using fructose at 20% and 25% in Wistar rats for eight weeks. Also, Bulboacă *et al.* [2] reported increased glycemia using fructose (10%) in Wistar rats for 12 weeks. It is important to mention that there are genetic differences that express metabolic variations according to each rat strain [12].

There is a significant difference between the absorption process of fructose and glucose. Fructose is absorbed by the GLUT 5 transporter, regardless of the absorption of glucose. After various processes, fructose can enter glycolysis, avoiding the hexokinase and phosphofructokinase-1 regulation points [10]. Entering glycolysis provides metabolites for lipogenesis and inhibits the beta-oxidation process. This process could explain the increase of visceral fat in the F group and F+LC group that we, macroscopically, observed. On the other hand, fructose is not an accurate way to measure glycemia at the pancreatic level, because beta-pancreatic cells do not have GLUT 5 transporters [1,10], so fructose metabolism is independent of insulin and would not increase glycemia [10], which would explain the results. Moreover, fructose is related to the conservation of plasma insulin, expressed as HOMA-IR, where we did not observe no significant differences in the groups. HOMA-IR, as a parameter of insulin resistance, showed an increase of 28.3% in the F group compared to the C group; this moderate increase would suggest that the use of fructose for a longer time could generate insulin resistance, as described in other studies [1,2,10,13]. Furthermore, a

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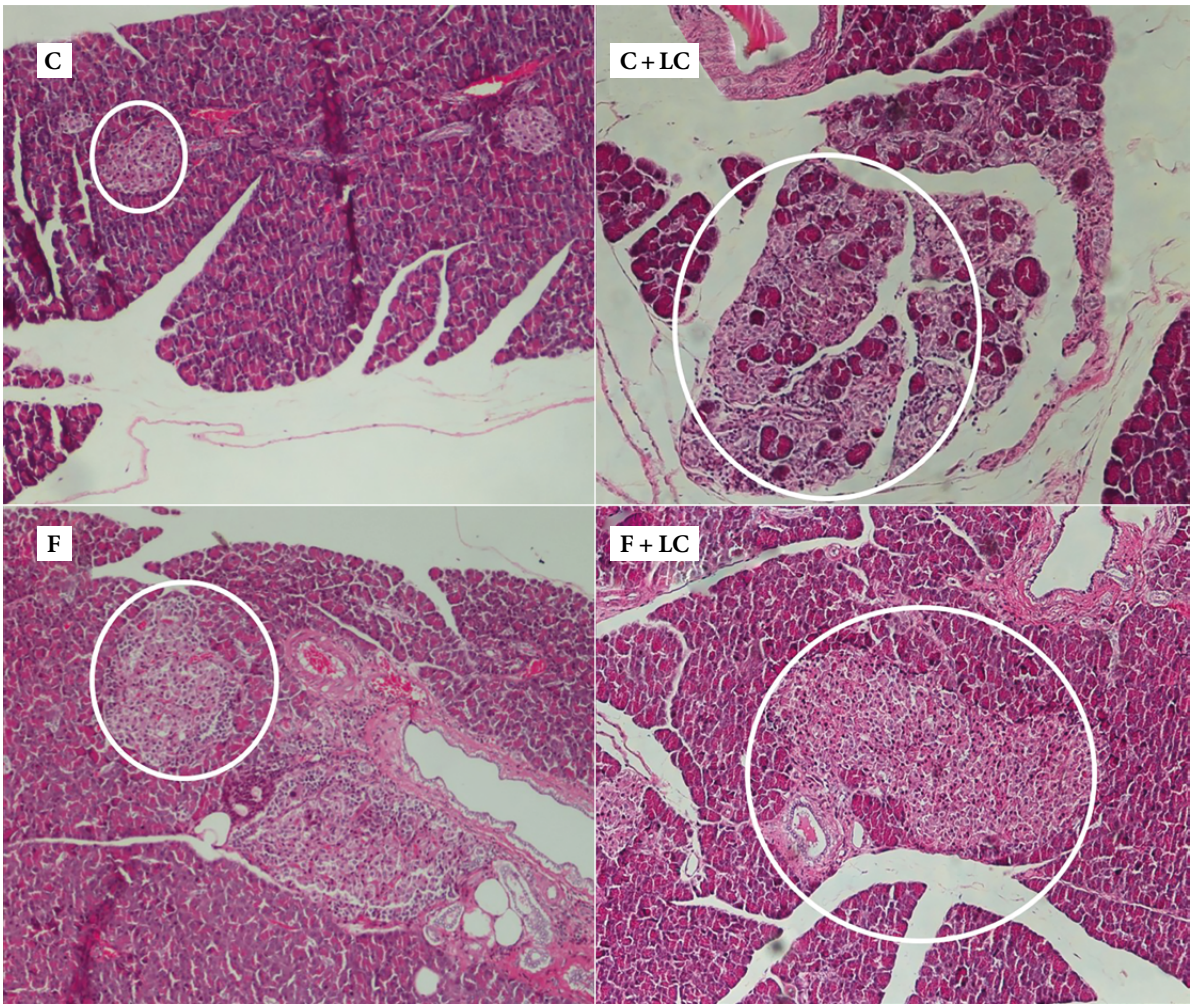


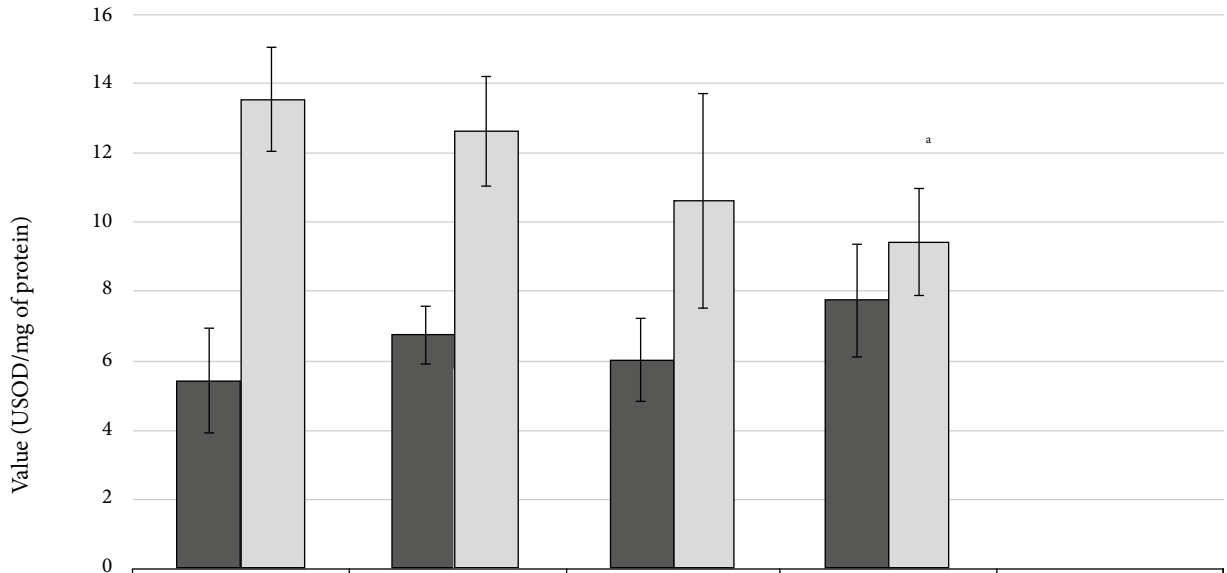
C: control group, C+LC: control + L-carnitine group, F: fructose group, F+LC: fructose + L-carnitine group
In the image for the C group, 2 to 3 islets of Langerhans can be observed in the field, without any other important aspect to describe. In the C+LC group image there is an increase in the number and size of the islets of Langerhans. In the F and F+LC groups there is an increase in size of the Langerhans islands, it is more noticeable in the last group.

Figure 3. Photomicrographs of rat pancreas cuts. Hematoxylin/eosin10×.

decrease of 25.8% was observed in the F+LC group compared to the F group. For example, Ringseir *et al.* [14] reviewed six studies on rats in which LC decreased glycemia and the HOMA-IR.

Fructose consumption produced a significant decrease in the level of insulin in the pancreas. This result, observed in the F group, can be related to the increase of the number and size of adipocytes, which causes the release of MCP-1, which leads to the recruitment of macrophages-M1 and the release of cytokines such as TNF-α, IL1 and IL6, which cause a state of chronic inflammation [15]. Likewise, TNF-α binds to its death receptor, activating the extrinsic pathway and then the intrinsic pathway of apoptosis to finally produce the death of the beta-pancreatic cells [16]. Maiztegui *et al.* [17] used 10% fructose on free demand for three weeks and showed the reduction of the number of beta-pancreatic cells due to increased apoptosis. In contrast, during the histological evaluation we observed an increase of the size of the islets of Langerhans in the F group, probably due to compensatory effect of the stimulation of islets' alpha, delta, F and G cells.

By using this model of stress induced by fructose at 40% consumed on free demand, we observed that the administration of LC (F+LC group) induced a recovery of 100% of tissue insulin when compared to the consumption of only fructose (group F), this result, although not significant for this study, is important because it is evidence of the role of LC in the pancreatic tissue. On the other hand, the C+LC group had a different behavior, we observed a 387% increase in the level of insulin, and a greater number and size of the islets of Langerhans (there were even more islets by regions)



	C	C + LC	F	F + LC	p value [b]
Mn-SOD	5.4 ± 1.5	6.7 ± 0.8	6.0 ± 1.2	7.7 ± 1.6	0.085
Cu/Zn-SOD	13.5 ± 1.5	12.6 ± 1.6	10.6 ± 3.1	9.4 ± 1.5	0.022

C: control group, C+LC: control + L-carnitine group, F: fructose group, F+LC: fructose + L-carnitine group. Values expressed in mean ± standard deviation.
Mn-SOD: superoxide dismutase in the mitochondrial fraction
Cu/Zn-SOD: superoxide dismutase in the post-mitochondrial fraction
[a] Statistically significant compared to the control group, [b] ANOVA test

Figure 4. Superoxide dismutase (SOD) activity in liver tissue of the studied groups.

than pancreatic acini compared to C group.

Several studies show that the administration of LC inhibits apoptosis. Bonomini *et al.* [18] reviewed different studies and suggested that LC could possibly inhibit caspase 3. Agarwal *et al.* [6] reported a similar result after analyzing several studies, they found that LC inhibits caspases 3, 7 and 8 and regulates tumor suppressor proteins, which favors oocyte survival. Likewise, Cao *et al.* [3] conducted an in vitro study and found that the use of LC favors the decrease of the Bax/Bcl-2 ratio and the production of ROS. In metabolic terms, according to the study by Jiang *et al.* [19], the presence of LC favors the expression of CPT1 mediated by PPARγ, which increases the process of beta-oxidation. The results of our study lead us to believe that LC could inhibit apoptosis of beta-pancreatic cells, which significantly increased the level of pancreatic insulin in the C+LC group, while in the F + LC group it did not increase as much due to previous damage by fructose. Therefore, the administration of LC (C+LC group) demonstrated the ability to significantly ($p < 0.01$) stimulate insulin production at the tissue level (Figure 2) without affecting the plasma levels of the hormone.

In the liver, free LC levels increased significantly by 21.5% when it was given as a treatment to F group compared to group C. In addition, we observed that the administration of LC did not produce a significant increase with their peers, probably because LC can act as a scavenger. According to Gülçin [20] the *in vitro* LC acts as a scavenger of superoxide anion and hydrogen peroxide and favors the chelation of the ferrous ion, due to its carbonyl group, which can stabilize free radicals in alpha carbon by conjugation. It can also be stated that the levels of free LC are stable in physiological situations. However, this changes under physiopathological conditions, such as the consumption of fructose through various mechanisms as reported by Chang *et al.* [4], who stated that the increase of ROS could reduce the expression and function of OCTN-2 (carnitine transporter in the plasma membrane of tissues).

In different studies, long-term fructose consumption increased the production of ROS [2,21]. NADH and $FADH_2$ are produced when fructose enters glycolysis and the Krebs cycle. These two molecules then go to the electron transport chain in the mitochondria, where there is a large production of superoxide anion. If fatty acids are formed, they can be metabolized by beta-oxidation, which produces ROS and

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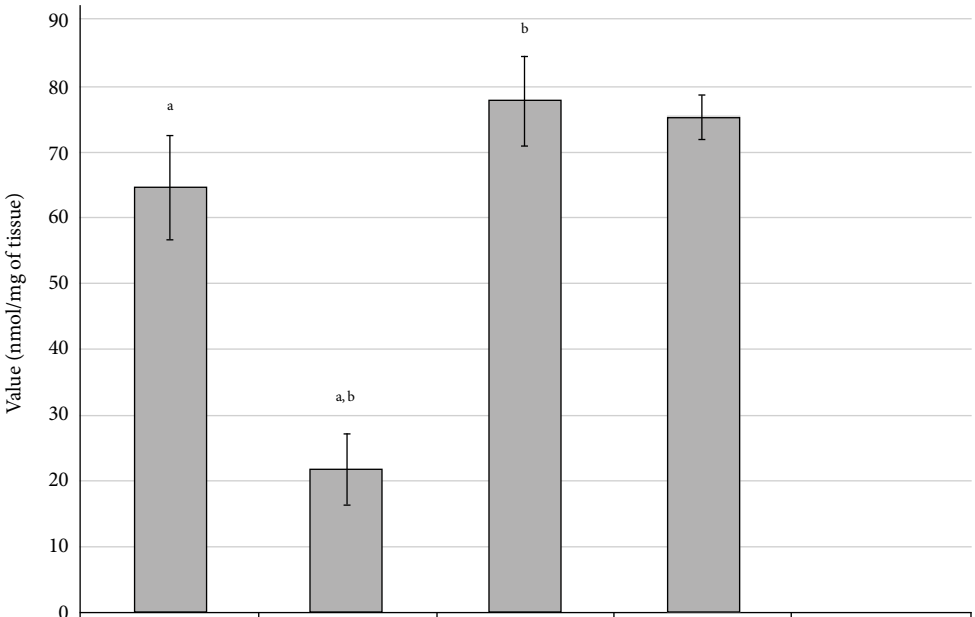

	C	C + CL	F	F + LC	p value
MDA	64.5 ± 7.9	21.7 ± 5.4	77.9 ± 6.8	75.3 ± 3.3	< 0.001

Control group, C+LC: control + L-carnitine group, F: fructose group, F+LC: fructose + L-carnitine group. Values expressed in mean ± standard deviation.

[a] Statistically significant compared to group F, [b] statistically significant compared to group C

C group compared to the C+LC group (p value < 0.001), C group compared to the F group (p value = 0.030), F group compared to the C+CL group (p value < 0.001), C group + LC compared to the F+LC group (p value < 0.001)

Figure 5. The level of malondialdehyde (MDA) in the liver tissue of the groups studied.

acetyl-CoA, which can generate more NADH and $FADH_2$ [22]. In this sense, Furukawa *et al.* [16] reported greater activity of the NAPDH-oxidase in the adipocytes of obese people and a decrease in the expression of antioxidant enzymes, which can easily generate oxidative stress.

The higher production of ROS compromises the antioxidant defense mechanisms; at the enzymatic level, SOD is the first one that acts against the univalent reduction of oxygen. As mentioned, LC exerts its main role in the mitochondria, this can explain the 25% increase in mitochondrial enzyme activity on the C group. The LC role in the mitochondria can also explain the slight increase of LC levels (C + LC group). Also, we observed a coupled behavior between cytosolic and mitochondrial isoenzymes. When compared to the C group, the F+LC group showed a 30.5% decrease of the Cu/Zn-SOD activity, while the Mn-SOD activity increased 42%. According to Suzuki *et al.* [23], excess ROS may lead to inhibition of the Cu/Zn-SOD enzyme and an increase of Mn-SOD, which is probably an adaptive response to ROS production. It should be noted that Mn-SOD is probably the most important enzyme for survival in an oxidative environment [24].

In this oxidative environment, derived from mitochondrial activity, LC administration favors the production of large amounts of acetyl-CoA, which generates acetyl groups for protein or histone acetylation processes, and produces post-translational or epigenetic changes [25]. In their research, Kerner *et al.* [26] observed that acetyl-CoA treatment increased Mn-SOD acetylation. It can be assumed that acetylation could favor increased the activity of this enzyme. On the other hand, we observed in the C+LC group a significant increase of mitochondrial and post-mitochondrial total protein levels, a similar effect was observed in the F group, although it was not significant. These results allow us to presume that the LC would not only act as an activity regulator, but could also be related to protein synthesis, which includes antioxidant enzymes.

The assessment of lipoperoxidation shows the damage made to the membrane by peroxidative reactions of polyunsaturated fatty acids (PUFA); the level of MDA is considered a marker of oxidative stress. The antioxidant properties of LC displayed in liver tissue were also observed in the MDA levels, which significantly decreased ($p < 0.01$) in the absen-

ce of oxidative stress factors such as fructose, which corroborates the scavenger role discussed above [3,4,6,20]. Consumption of fructose at 40% on free demand produced an increase in lipoperoxidation by 21%, and the administration of LC could not reverse this change. The consumption of fructose generated a large amount of ROS, so probably a longer treatment time could reduce oxidative stress, expressed as MDA, as has been shown in other studies [5,27,28]. Lipoperoxidation could be diminished by mechanisms that increase the expression of Mn-SOD and Cu/Zn-SOD, which is mediated by the increase of the mRNA expression of PPARα, as reported by Liu *et al.* [29].

Several studies show that the presence of PPAR activates the expression of Mn-SOD and Cu/Zn-SOD genes through the transcriptional pathway [5,29,30]. Then, based on the results of this study, we could propose that a longer treatment with LC would reduce the levels of ROS, which would avoid the lipoperoxidation and its harmful effects at the cellular level.

The availability of resources was one of the limitations of our study, therefore we could not assess the basal concentrations of MDA and SOD and why a longer treatment was not used.

In conclusion, we observed that fructose does not affect glycemia, but it favors lipogenesis and an oxidative environment; in this scenario, administration of LC favors metabolic changes that corroborate its antioxidant function.

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