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Low-level Laser Therapy Ameliorates CCl_4-induced Liver Cirrhosis in Rats

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ABSTRACT

This study investigated the effects of low-level laser therapy (LLLT) in the liver function, structure and inflammation in a experimental model of carbon tetrachloride (CCl_4)-induced liver cirrhosis. Wistar rats were divided into Control, LLLT, CCl_4 and CCl_4+LLLT groups. CCl_4 groups received CCl_4 (0.4 g kg^{-1}; i.p.), three times a week, for 12 weeks. A 830 nm LLLT was performed with a continuous wave, 35 mW, 2.5 J cm^{-2} per point, applied to four points of the liver (right and left upper and lower extremities, in the four lobes of the liver) for 2 weeks. Liver structure and inflammation (cirrhotic areas, collagen deposition, inflammation, density of Kupffer and hepatic stellate cells) and function (aspartate aminotransferase, alkaline phosphatase, gamma glutamyltransferase, lactate dehydrogenase, total proteins and globulins) were evaluated. LLLT significantly reduced CCl_4-increased aspartate aminotransferase ($P < 0.001$), alkaline phosphatase ($P < 0.001$), gamma-glutamyl transferase ($P < 0.001$) and lactate dehydrogenase ($P < 0.01$) activity, as well as total proteins ($P < 0.05$) and globulins ($P < 0.01$). LLLT also reduced the number of cirrhotic areas, the collagen accumulation and the hepatic inflammatory infiltrate. Of note, LLLT reduced CCl_4-increased number of Kupffer cells ($P < 0.05$) and hepatic stellate cells ($P < 0.05$). We conclude that LLLT presents beneficial effects on liver function and structure in an experimental model of CCl_4-induced cirrhosis.

INTRODUCTION

The liver has an enormous functional reserve capacity and an amazing capability of regeneration after resection or when submitted to toxic substances (1). Carbon tetrachloride (CCl_4) is a widely used experimental model to induce liver cirrhosis (2–5). Between the mechanisms involved in CCl_4-induced liver cirrhosis are the activation of hepatic stellate cells, as well as Kupffer cells (2–5). Additional mechanisms include increased oxidative and nitrosative stress, proinflammatory cytokine release, matrix metalloprotease (MMP) and antiprotease release and apoptotic events (2–5). These cellular and molecular events increase with chronic administration of CCl_4 leading to biochemical, functional and structural alterations (2–5). These biochemical and functional alterations are evidenced through increased activity of liver enzymes, such as aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), alkaline phosphatase (AP), and other enzymes, *i.e.* lactate dehydrogenase (LDH), total proteins and globulins, which are also increased during hepatic cirrhosis (2–5). Increased levels of hepatic enzymes (AST, GGT, AP), as well as other biochemical markers (LDH, total proteins and globulins) are normally attributed to impaired hepatic function, *i.e.* capacity of protein synthesis, and also decreased metabolic capacity (2–5). In addition, increased levels of AST are more related to liver inflammation, while increased levels of GGT and AP to possible blockage of bile duct, a signal of biliary cirrhosis (2–5). In general, increases in the LDH levels, total proteins and globulins reflect impairment in hepatic function, while re-establishment to normal levels, especially with concomitant improvement in AST, GGT and AP levels, reflect liver regeneration (2–5). The most prominent structural alterations in CCl_4-induced liver cirrhosis are the increased deposition of components of the extracellular matrix, mainly collagen, forming cirrhotic areas, which are characterized by nodules of regeneration encircled by fibrotic tissue (2–5).

Low-level laser therapy (LLLT) has been used in several therapeutic strategies because it has shown analgesic, anti-inflammatory, bio-stimulatory and antioxidant effects (6–10). The effects of LLLT on the liver have been studied, especially after partial hepatectomy, and some beneficial effects have been reported (6–8,11). On the other hand, the effects of LLLT in CCl_4-induced liver cirrhosis have not been investigated. Thus, the present study investigated the effects of LLLT on liver function through biochemical assays for AST, AP, GGT, LDH, total proteins and globulins, as well as the liver structural/inflammatory alterations (cirrhotic areas, collagen deposition, inflammation, density of Kupffer and hepatic stellate cells) induced by CCl_4.

MATERIALS AND METHODS

Animals and experimental groups. Forty male Wistar rats (250 ± 19 g body mass) were randomized in four groups: Control, Laser, CCl_4, CCl_4+Laser. The control group (Control) did not receive any treatment; the laser group (Laser) received LLLT transdermally; the CCl_4 group (CCl_4) received CCl_4 intraperitoneally; the CCl_4+Laser group (CCl_4+Laser) received both CCl_4 and laser administration.

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CCl_4-induced hepatic cirrhosis. Intraperitoneal injection of 8% CCl_4 diluted in mineral oil (0.4 g kg^{-1}) was performed three times per week for a total of 8 weeks, as described previously (12). CCl_4 administration (three times per week) was continued during the following 2 weeks accompanying laser treatment. Therefore, the total period of CCl_4 administration was 10 weeks.

Laser treatment. After 8 weeks of CCl_4 administration, Laser and CCl_4+Laser groups were treated transdermally (laser was pressed on the skin), for 2 weeks, 5 times per week, using a 830 nm diode laser, administered at four points of the liver (right and left upper points [corresponding to right, left, caudate and quadrate hepatic lobes) and right and left lower points [corresponding to right, left, caudate and quadrate hepatic lobes]), 71 s per point. The laser power was 35 mW, which resulted in an energy dose of 2.5 J cm^{-2} per point.

Animal euthanasia, blood and tissue collection. Twenty-four hours after the last day of LLLT, all animals were anesthetized (ketamine 150 mg kg^{-1} and xylazine 50 mg kg^{-1}) and the abdominal cavity was opened and 5 mL of blood was collected from the inferior vena cava. Blood samples were centrifuged at 5°C for 15 min at 140 **g** and the serum was used for determining the hepatic function of biomarkers. Following blood collection, the liver was carefully removed, weighed, cut in slices of 0.5 cm and fixed in 10% formalin for 24 h.

Biochemical assay for hepatic functions. The serum was used for determination of hepatic functions using a Hitachi U-2001 spectrophotometer. The following analysis were performed with Gold Analisa® (MG, Brazil) commercial kits—AST, AP, GGT, total proteins, total globulins and LDH, according to the manufacturer's instructions.

Structural and inflammatory analysis using liver histology and cytochemistry. After fixation, liver fragments were processed by standard histological routine, cut in slices of 5 μm and stained with eosin–hematoxylin for structure and inflammation analysis and Masson's trichromic for collagen detection. The morphometry technique was applied for determination of the number of hepatic stellate cells and Kupffer cells, by using a reticulated ocular (Weibel reticule of 50 straight and 100 points), at $\times$1000 magnification (corresponding a 10 000 μm^2 area for 100 points) (13). Briefly, the number of points hitting the liver tissue area was counted followed by counting the number of hepatic stellate cells and Kupffer cells inside this area (13). In addition, the inflammatory infiltrate, collagen accumulation and cirrhotic areas were semiquantitatively analyzed by two specialists, blinded to the experimental protocol. The semiquantitative score was designed as follows: 0 = no presence of inflammatory cells, collagen accumulation and cirrhotic areas; 1 = scarce (monofocal) presence of inflammatory cells, collagen accumulation and cirrhotic areas; 2 = discrete (monofocal) presence of inflammatory cells, collagen accumulation and cirrhotic areas; 3 = moderate (multifocal) presence of inflammatory cells, collagen accumulation and cirrhotic areas; 4 = intense (multifocal) presence of inflammatory cells, collagen accumulation and cirrhotic areas.

Statistical analysis. All results were presented as mean and standard error (SEM) and were analyzed statistically by one-way ANOVA followed by the Newman–Keuls multiple comparison test, with the level of significance set at $P < 0.05$.

RESULTS

LLLT improves liver function after CCl_4-induced cirrhosis

Figure 1 shows the serum levels of AST (Fig. 1A), GGT (Fig. 1B), AP (Fig. 1C), LDH (Fig. 1D) in all groups studied. The results demonstrated that CCl_4 administration significantly increased the AST levels compared with the Control group ($P < 0.001$), while LLLT significantly reduced AST levels ($P < 0.01$) (Fig. 1A). Similarly, CCl_4 administration significantly increased the GGT levels compared with the Control group ($P < 0.001$), while LLLT significantly reduced GGT levels ($P < 0.05$) (Fig. 1B). We also observed that CCl_4 increased the AP levels compared with the Control group ($P < 0.001$) and that LLLT significantly reduced AP levels ($P < 0.05$) (Fig. 1C). In Fig. 1D we observe that CCl_4 significantly increased LDH levels compared with the Control group ($P < 0.01$) and that LLLT significantly reduced LDH levels ($P < 0.05$).

LLLT restores liver protein synthesis in CCl_4-induced cirrhosis

Figure 2 shows the levels of total proteins (Fig. 2A) and globulins (Fig. 2B) in all groups studied. In Fig. 2A we observe that CCl_4 significantly increased the levels of total proteins compared with the Control group ($P < 0.01$), while the LLLT-treated group (CCl_4+LLLT) significantly reduced the levels of total proteins when compared with the CCl_4 group ($P < 0.05$). However, the levels of total proteins in the CCl_4+LLLT group are still higher when compared with the Control group ($P < 0.05$). In Fig. 2B we observe a similar response, when CCl_4 administration significantly increased globulin levels compared with the Control group ($P < 0.01$), while LLLT significantly reduced globulin levels ($P < 0.01$).

LLLT abolishes CCl_4-induced formation of cirrhotic areas and inflammation

Figure 3 shows representative photomicrographs of the Control group (Fig. 3A), Laser group (Fig. 3B), CCl_4 group (Fig. 3C) and CCl_4+Laser group (Fig. 3 D) at $\times$100 magnification. Histological analysis clearly demonstrates that CCl_4 administration significantly induced hepatic cirrhosis (Fig. 3C) and that LLLT significantly reduced the formation of cirrhotic areas (nodules of regeneration encircled by fibrotic tissue) (Fig. 3D). In addition, while CCl_4 significantly induced collagen accumulation (please see collagen fibers forming encircled nodules) (Fig. 3C), LLLT significantly reduced the areas of liver fibrosis (collagen accumulation) (Fig. 3D). We also observed that CCl_4 increased the number of inflammatory cells in the liver (please see increased number of leukocytes in the fibrotic areas) (Fig. 3C), and that again, LLLT significantly reduced the leukocyte infiltrate (Fig. 3D). These histological observations were confirmed by the semiquantitative analysis technique, as demonstrated in Table 1. The semiquantitative analysis of histology revealed that CCl_4 administration significantly increased the density of the inflammatory infiltrate ($P < 0.001$), collagen deposition ($P < 0.001$) and the number of cirrhotic areas ($P < 0.001$) when compared with all other groups.

LLLT inhibits CCl_4-induced hepatic stellate and Kupffer cell accumulation

As hepatic stellate and Kupffer cells play a central role in the pathogenesis of hepatic cirrhosis (2–5,12), we investigated through a quantitative histomorphometric method the precise number of these cells in the hepatic tissue. Figure 4A shows that CCl_4 administration resulted in a significant increase in the number of hepatic stellate cells in hepatic tissue compared with the Control group ($P < 0.05$) and that LLLT significantly reduced the number of hepatic stellate cells in hepatic tissue ($P < 0.05$). Figure 4 B shows that CCl_4 administration resulted in a significant increase in the number of Kupffer cells in hepatic tissue compared with the Control group ($P < 0.01$) and that LLLT significantly reduced the number of Kupffer cells in hepatic tissue ($P < 0.05$).

DISCUSSION

The present study shows for the first time that LLLT presents potential therapeutic effects for liver cirrhosis. The study demonstrated that LLLT positively affects both functional and structural aspects of the liver in an experimental model of CCl_4-induced liver cirrhosis, demonstrated through improved values for AST, GGT, AP, LDH, total proteins, globulins and mainly by reduced inflammatory infiltrate, liver fibrosis (collagen deposition) and specially by reduced cirrhotic areas.

Hepatic cirrhosis is a devastating disease and until a few years ago, the unique viable treatment was liver transplantation (14). Although the first study claiming that liver fibrosis/cirrhosis can be reversible was published more than 40 years ago (15), only in the recent past, a growing number of clinical studies have demonstrated that in some cases, the fibrosing process can be reversible (16–19). Aiming to investigate the cellular and molecular mechanisms involved in reversion of the fibrotic process, many animals studies have been performed (20–22). These studies show that the reversion of the fibrotic process can be attributed to decreased oxidative stress, while additional mechanisms also have been demonstrated, for instance, reduction in the expression of intracellular mediators such as nuclear factor κB (NF-κB), activating protein 1, extracellular regulated kinase and mitogen-activated protein kinase (20–22). The fibrotic process in the liver is characterized by increased deposition of extracellular matrix proteins (*i.e.* collagen fibers), which is attributed to an imbalance between the activation of MMPs and tissue inhibitors of matrix metalloproteases (TIMPs) (22). In general, the studies claim that the molecular mechanisms cited above play a central role in the resolution of the fibrotic process by reducing the imbalance between MMPs and TIMPs resulting in an increased rate of degradation of extracellular matrix proteins (22). In this way, the role of hepatic stellate cells (HSC) in the liver fibrotic process has been well established (20,22). HSC express several MMPs and TIMPs and these cells present a capacity both to increase synthesis as to degradate extracellular matrix proteins (20,22). Interestingly, the present study demonstrated that LLLT was able to reduce CCl_4-induced liver fibrosis and cir-

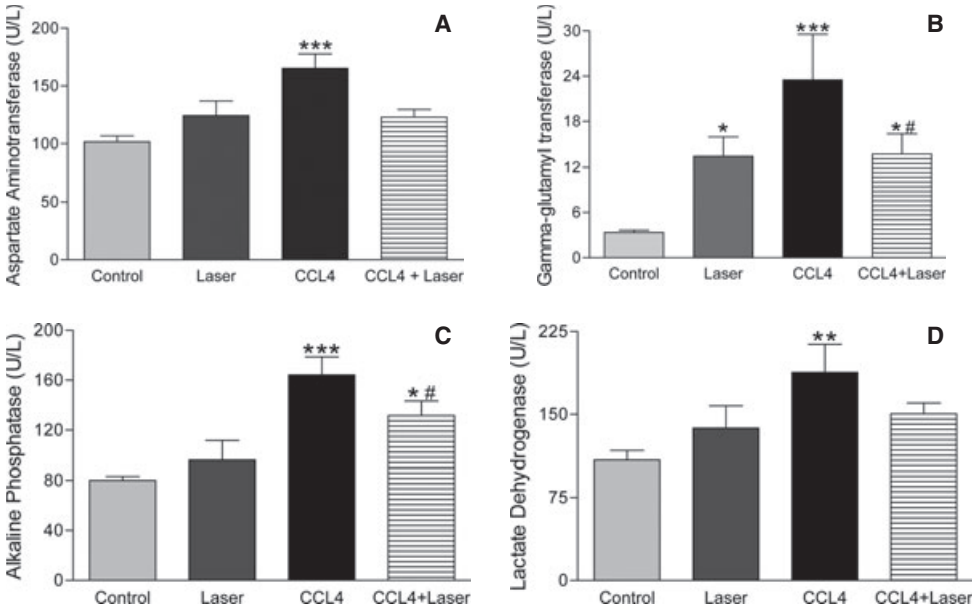

Figure 1. Effects of LLLT on hepatic function after CCl_4-induced hepatic cirrhosis for (A) aspartate aminotransferase levels (***$P < 0.001$ compared with the Control group), (B) gamma-glutamyl transferase levels (***$P < 0.001$ and *$P < 0.05$ when compared with the Control group and $^{\#}P < 0.05$ when compared with the CCl_4 group), (C) alkaline phosphatase levels (***$P < 0.001$ and *$P < 0.05$ when compared with the Control group and $^{\#}P < 0.05$ when compared with the CCl_4 group) and (D) lactate dehydrogenase levels (**$P < 0.001$ and *$P < 0.05$ when compared with the Control group) for all experimental groups.

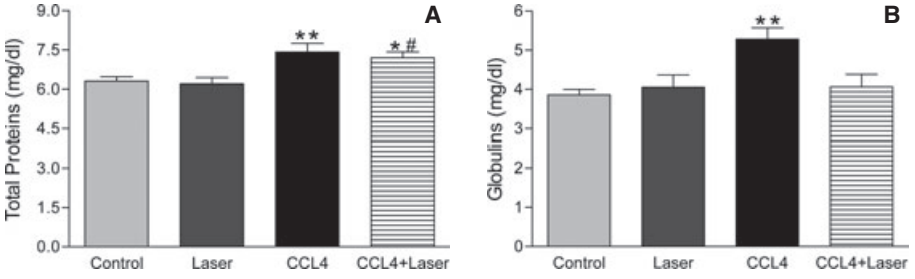

Figure 2. Effects of LLLT on (A) total protein (**$P < 0.01$ and *$P < 0.05$ when compared with the Control group and $^{\#}P < 0.05$ when compared with the CCl_4 group) and (B) globulin levels (**$P < 0.01$ when compared with the Control group) after CCl_4-induced hepatic cirrhosis for all experimental groups.

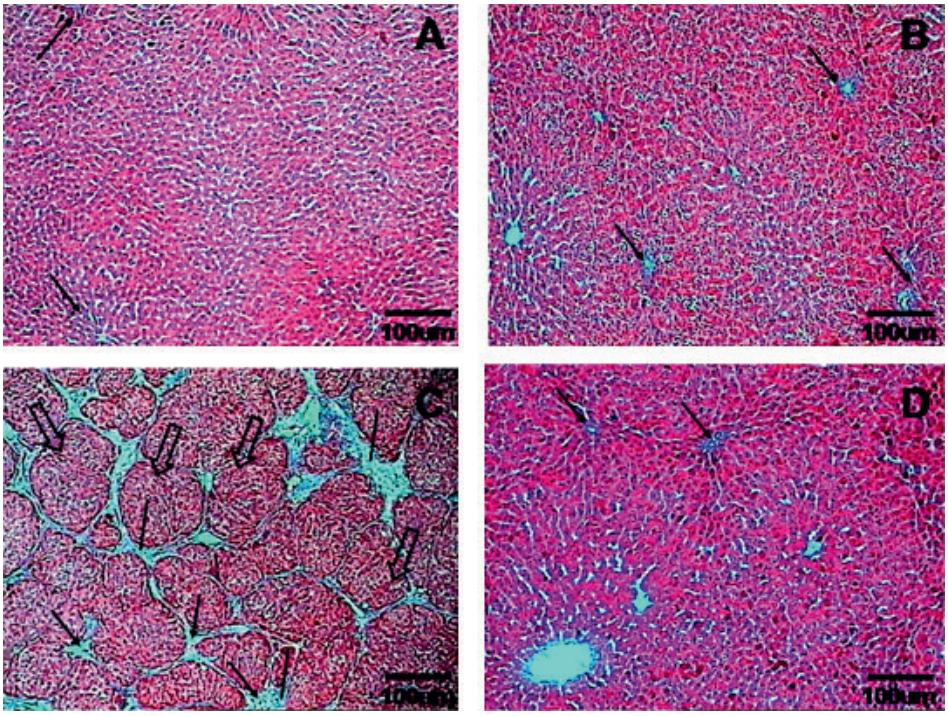

Figure 3. Representative photomicrographs of liver from Control, LLLT, CCl_4 and CCl_4+LLLT groups, stained with Masson's trichromic. Effects of LLLT on liver structure and inflammation for all experimental groups (A—Control; B—LLLT; C—CCl_4; D—CCl_4+LLLT). (A), (B) and (D) show liver with normal histopathological aspects, with normal hepatocyte distribution, normal sinusoid distribution, normal inflammatory aspects and no presence of fibrotic or cirrhotic areas. In (A), (B) and (C), we can observe only the presence of normal amount of collagen (arrows). (C) The formation of several cirrhotic areas (open arrows) is shown clearly, with intense fibrosis/collagen deposition (arrows) and intense inflammatory infiltrate (traces—see black points in fibrotic areas corresponding to nuclei of leukocytes), especially in fibrotic areas.

Table 1. Effects of LLLT on CCl_4-induced liver inflammation, collagen accumulation and formation of cirrhotic areas.

	Control	LLLT	CCl_4	CCl_4 + LLLT	*P*-value
Inflammatory infiltrate	1.0 ± 0.0	1.0 ± 0.0	3.83 ± 0.37*	1.5 ± 0.76	0.001
Collagen accumulation	1.0 ± 0.0	1.0 ± 0.0	3.57 ± 0.72*	1.42 ± 0.49	0.001
Cirrhotic areas	0.0 ± 0.0	0.0 ± 0.0	3.87 ± 0.33*	1.12 ± 1.05	0.001

LLLT = low-level laser therapy; CCl_4 = carbon tetrachloride. *$P < 0.001$ when compared with all other groups.

rhosis. In addition, the present study also showed that LLLT reduced the accumulation of HSC, suggesting a possible cellular mechanism of action of LLLT on liver cirrhosis. In this direction, we hypothesize that in the present study, LLLT could have reduced CCl_4-induced liver fibrosis and cirrhosis by decreasing MMP and TIMP activation in HSC. In addition, some studies have already reported that LLLT modulates MMP and TIMP expression (23,24).

In some studies, the activation of HSC is attributed to the inflammatory mediators released by Kupffer cells, *i.e.* proinflammatory cytokines and chemokines (4,25–29). Kupffer cells, the resident hepatic macrophages, are important cells in the reticuloendothelial system, playing an important role in the defense system of the liver (4,25–29). However, when activated, the harmful products released by Kupffer cells kill the hepatocytes and activate the HSC, initiating the fibrotic process (30). The clear role of Kupffer cells in the cirrhosis process was demonstrated in an elegant study from Muriel and Escobar when the authors killed Kupffer cells, resulting in a very efficient protection against CCl_4-induced liver fibrosis (30). Of note, the present study shows that LLLT significantly reduced CCl_4-induced Kupffer cell accumulation in the liver. As a possible consequence of LLLT-reduced Kupffer cell and hepatic stellate cell accumulation in the liver, the present study showed that LLLT reduced the areas of hepatic cirrhosis, the fibrotic areas (collagen accumulation) in the liver as well as the inflammatory infiltrate. This hypothesis is supported by previous studies, confirming that reduced accumulation of Kupffer cells and HSC results in reduced fibrosis and inflammation (4,5,20–22,25–30). These results, therefore, reinforce the possible cellular mechanism of protection of LLLT in a model of CCl_4-induced cirrhosis, fibrosis and inflammation, which seem to be mediated by inhibition of Kupffer cells and hepatic stellate cells. Furthermore, we hypothesize that LLLT could reduce proinflammatory cytokine production by Kupffer cells, as other studies have already demonstrated that LLLT can modulate the balance between pro and anti-inflammatory cytokines (31,32).

Beyond the beneficial effects of LLLT on cirrhosis, fibrosis and inflammation, the present study also shows that LLLT resulted in improved liver function/reduced hepatotoxicity. The present study showed that LLLT significantly reduced CCl_4-increased AST, GGT, AP, LDH and total protein and globulin levels, which are classical markers of hepatic function (2,20,33,34). Previous studies have investigated the effects of LLLT in models of partial hepatectomy (6–8,35). These studies reinforce the concept that LLLT may improve hepatic function

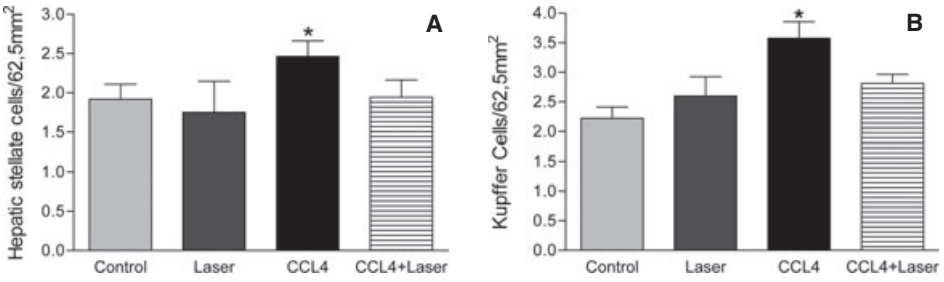

Figure 4. Effects of LLLT on the number of (A) hepatic stellate cells (*$P < 0.05$ when compared with all groups) and (B) Kupffer cells (*$P < 0.05$ when compared with all groups) for all experimental groups.

after liver injury induced by hepatectomy (6–8,35). Considering that AST is located predominantly in the mitochondria, the present results may suggest that part of the beneficial effects of LLLT on liver function could be attributed to improved mitochondrial function. In line with this concept, previous studies have shown that LLLT and LED therapy may improve mitochondrial function in hepatectomized rats (36,37). The fact that LLLT reduced the levels of liver enzymes such as AST, AP, GGT and LDH and also the total protein and globulin levels is extremely relevant and points out the possible mechanism of action of LLLT in CCl_4-induced hepatic cirrhosis. This affirmation is true considering that during the course of hepatic cirrhosis, all these markers of hepatic function are increased and reflect the inflammatory process (2–5,12,16,18). In addition, we speculate that another possible mechanism underlying the effects of LLLT in the present model of CCl_4-induced liver cirrhosis could be through LLLT reducing oxidative stress, as several studies have shown that reduction of oxidative stress results in improvement of hepatic cirrhosis (20,21,29). Corroborating our findings, some studies have already shown that LLLT also reduces oxidative stress, resulting in improvement of different diseases and injuries, including reducing fibrosis (38–40).

In conclusion, the present study shows that LLLT may be a promising strategy for the treatment of hepatic cirrhosis, considering that LLLT improved the hepatic function and structural alterations typical of hepatic cirrhosis, for instance, fibrosis and inflammation. However, further studies aiming to understand the cellular and molecular mechanisms involved in the anti-inflammatory and antifibrotic effects of LLLT should be addressed.

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