Research article

L-carnitine ameliorates bile duct ligation induced liver fibrosis via reducing the nitrosative stress in experimental animals: preclinical evidences

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A B S T R A C T

Bile duct ligation (BDL) has been extensively used in studying the mechanisms of fibrogenesis and anti-fibrotic drugs. Considering the liver regenerative capacity and the diverse results from BDL, the present study aimed to evaluate the protective effect of L-carnitine on bile duct ligation-induced liver fibrosis in experimental rats. Rats were randomly divided into seven groups (n = 6). The bile duct was ligated and serum aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin and albumin, hepatic hydroxyproline (HP), reduced glutathione (GSH), and malondialdehyde (MDA) and cytokines were measured. iNOS expression was measured by using Western blot and finally, liver tissue was processed for histopathological analysis (H&E staining)". The level of iNOS was increased in the control group, whereas a decrease in the level of iNOS was found in the L-carnitine treated group. In the present study, we found that bile duct ligation in rats showed an increase in body and liver weight, while treatment with carnitine showed normal body and liver weight. Serum AST, ALT, total bilirubin, HP, GSH, MDA, and cytokines were increased in bile duct ligated rats. In addition, L-carnitine treated rats showed a reduction in oxidative stress as well as inhibiting the release of cytokines in a dose-dependent manner and showed protection against bile duct ligation. The study concludes that L-carnitine has a protective effect against the liver fibrosis induced by bile duct ligation.

1. Introduction

Excessive accumulation of extracellular matrix (ECM) proteins owing to extraordinary chronic liver disorders, along with viral hepatitis, alcoholic or non-alcoholic steatohepatitis, is characterized by liver fibrosis [1]. The genesis of liver fibres is a complex mechanism between cells and molecular processes. While particular diseases may potentially contribute to liver fibrosis. The end product of a mismatch between ECM synthesis and degeneration can be known to be liver fibrosis. For ECM homeostasis, the equilibrium between matrix metalloproteinases (MMPs) and tissue metalloproteinase inhibitors (TIMPs) is crucial [2, 3]. Several studies have identified the functions of various biochemical markers such as aspartate transaminase, alanine transaminase, hydroxyproline, and complete bilirubin [4, 5]. These are primary biomarkers that are involved in liver fibrosis pathogenesis. The role of cytokines in the progression of liver fibrosis was important instead of these biomarkers, and the concentration of different cytokines was elevated during disease progression [6, 7]. Carnitine (Hydroxy-trimethylaminobutyrate), originally isolated from muscle in 1905 and named after the Latin word Carne (flesh or

meat), is a quaternary amine occurring in almost all types of animals, in addition to various microorganisms and many higher plants [8]. In the mealworm (Tenebriomolitor) and many other larvae of the same family, L-Carnitine tended to serve as a nutrient and was thus called Vitamin BT. Limited quantities of vitamin BT are necessary to ensure these insects' natural growth and development. However, since humans and other higher organisms can synthesize L-carnitine, Vitamin BT is simply a misnomer. L-carnitine, on the other hand, is classified as a vitamin-like substance rather than a vitamin [9, 10].

Several pharmacological activities with effectiveness in lung fibrosis have been documented with L-carnitine [11], uremic anemia [12], anti-inflammatory and anti-arthritic activity [13], anti-cancer activity [14], and hepatoprotective activity [15]. Bile duct ligation (BDL) induced liver fibrosis has been extensively used as an animal model for the pre-clinical activity of new therapeutic agents for studying mechanisms of fibrogenesis and anti-fibrotic drugs [16]. The current study aimed to assess the protective effect of L-carnitine in bile duct ligation-induced liver fibrosis in rats by measuring biochemical markers, cytokine levels, and antioxidant enzyme levels.

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2. Material and methods

2.1. Animals

Swiss Albino rats weighing between 180-250 g were selected for this study. Animals were housed in metabolic cages under standard conditions of room temperature (22–240 C) and relative humidity of 65% with a 12 h light/dark cycle with free access to standard rat feed (Amruta feed, Pune, India) and water. The experimental protocol was approved by the institutional animal ethical committee of the Modern College of Pharmacy, Nigdi, Maharashtra, India-411044 (Protocol approval No. IAEC/MCP/001/2020).

2.2. Drugs and chemicals

L-carnitine was procured from Sigma Aldrich, USA, ELISA kits for IL-6, TNF-α, and IL-1β, were procured from eBioscience, USA., AST, ALT, and ALP kits were procured from Erba diagnostics, India. All other reagents used were of AR grade.

2.3. Induction of liver fibrosis

Liver fibrosis was induced by ligation of the common bile duct. The animals were anaesthetized with ketamine (80 mg/kg, intraperitoneal (i.p) and chlorpromazine (0.75 mg/kg, i.p). A 1.5 cm midline abdominal incision was made and the common bile duct was located and double ligated with 4-0 silk ligatures. Sham-operated animals were subjected to the same midline incision and manipulation of the common bile duct without ligation [17].

2.4. Experimental design

Animals were randomly divided into seven groups each containing six rats.

Group 1: Normal -Rats received saline solution orally as a vehicle.
Group 2: Control - Rats subjected to BDL
Group 3: Sham control - Animals subjected to the same midline incision and manipulation of the common bile duct without ligation
Group 4: Low dose- Rats subjected to BDL + L-carnitine (100 mg/kg/day p.o.)
Group 5: Middle dose- Rats subjected to BDL + L-carnitine (300 mg/kg/day p.o.)
Group 6: High dose- Rats subjected to BDL + L-carnitine (500 mg/kg/day p.o.)
Group 7: *Perse* - L-carnitine (500 mg/kg/day p.o.)

The animals were anesthetized with urethane (1.4 mg/kg) and blood samples were taken 24 h after the last treatment. Blood was centrifuged at 5000 rpm for 10 min at 4 °C to separate serum, which was then separated and utilized for further biochemical analysis. Finally, the livers of the animals were isolated after they were sacrificed. Using ice-cold 50 mM phosphate buffer saline, a 10% tissue homogenate of the liver was prepared (pH 7.4). The homogenate was centrifuged for 10 min at 4 °C at 10000 rpm, and the supernatant was collected and utilized for further analysis [18].

2.5. Experimental parameters

2.5.1. Measurement of body and liver weight

The body weight of each animal was measured at an interval of 7 days, i.e. 0, 7, 14, 21, 28 days, and the liver weight of animals was taken at the end of the study after scarification of animals [19].

Table 1. Effect of L-Carnitine on body weight and liver weight.

Group	Body weight (gm)		Liver weight (gm)
	0 Day	35 day	
Normal	175 ± 6.8	238 ± 7.4	8.4 ± 1.2
Control	184 ± 4.7##	241 ± 5.9###	24.5 ± 4.3###
Sham	181 ± 6.7	237 ± 9.7	8.8 ± 2.0
C100	189 ± 7.5	239 ± 6.7*	21.1 ± 1.8
C300	176 ± 7.8*	234 ± 7.4*	18.5 ± 2.1**
C500	180 ± 5.7*	237 ± 7.6*	12.4 ± 1.3***
Perse	186 ± 5.1	237 ± 4.8	9.7 ± 1.1

Data were expressed as mean ± SEM, analyzed using one-way analysis of variance, *p < 0.05, **P < 0.01, ***P < 0.001 compared to disease control group and ##P < 0.01, ###P < 0.001 is compared with the sham animals.

2.5.2. Biochemical parameters

Serum biochemical parameters like AST, ALP, ALT, TB, and HP were determined as the procedure mentioned in the literature and the manufacturer's instruction manual [20].

2.6. Estimation of oxidative stress

2.6.1. Estimation of malondialdehyde of lipid peroxidation in liver tissue

The most important result of this method is that membrane lipid peroxidation as malondialdehyde (MDA) concentration in liver tissues can be measured as formerly described. Jain and colleagues (2018)The principle of lipid peroxidation depends on the pink color formation due to the reaction between MDA and thiobarbituric acid. The absorbance of pink coloration was measured spectrophotometrically at 532 nm [21].

2.6.2. Estimation of reduced glutathione (GSH)

The glutathione concentration in liver tissue homogenate was determined as previously described by Iraz et al., 2006 [22].

#### 2.6.3. Estimation of superoxide dismutase (SOD) activity

The liver homogenate (10 μl) was added to a mixture of 20 μl of 500 mM/1 of sodium carbonate, 1 ml of 0.3% Triton X-100, 10 μL of 1.0 mM/1 of EDTA, 2.5 ml of 10 mM/1 of hydroxylamine, and 89 ml of distilled water. Finally, the optical density of this reaction mixture was measured at 560 nm in kinetic mode [23].

2.6.4. Estimation of catalase

The amount of catalase was calculated using commercially available UV spectroscopic techniques. In a brief, 10 μL of liver homogenate supernatant was mixed with 0.5 mL of 10 mM hydrogen peroxide (H2O2) solution. A UV spectrophotometer was used to evaluate the decrease in optical density of this mixture at 240 nm. The rate of reduction in optical density within 3 min of adding liver homogenate was used to determine the amount of catalase activity in the homogenate [24].

2.7. Determination of iNOS level

The iNOS level in rats was determined by Western blot analysis. It was done according to the protocol mentioned earlier. In brief, tissue samples of rats were chopped and homogenized and then lysed with modified radioimmuno precipitation assay (RIPA) lysis buffer and cellular lysates were processed for western blotting. A fixed amount (60 g) of protein was loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to the nitrocellulose membrane. Then, the membrane was probed with specific antibodies according to the
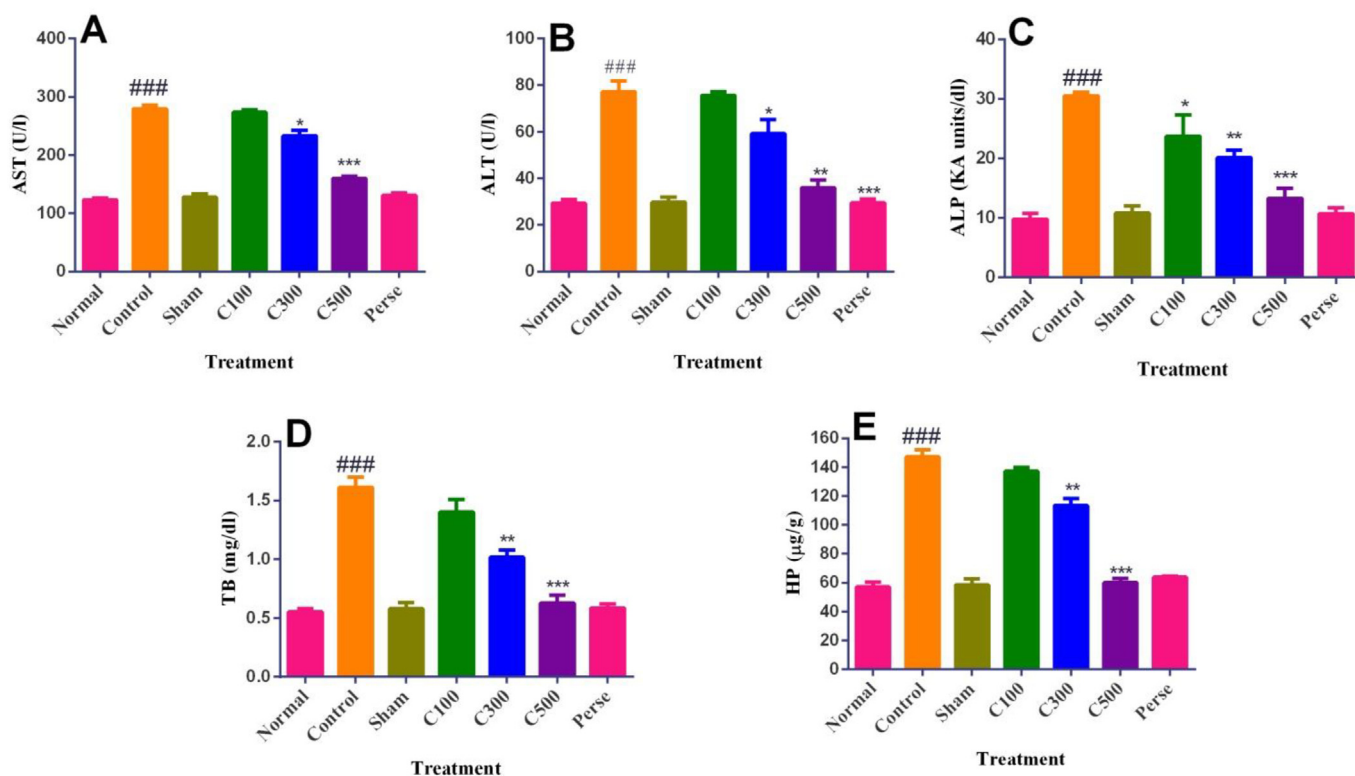
Figure 1. Effect of L-Carnitine on biochemical parameters (A: AST; B: ALT; C: ALP; D: TB; E: HP). Data were expressed as means ± SEM, n = 06. Statistical significance was determined by one-way ANOVA followed by the Dunnet test: Compared with Normal ###P < 0.01, Compared with the disease control group.

manufacturer's protocol and band intensity was analyzed by densitometry [25].

2.8. Determination of cytokine level

The level of cytokines such as IL-6, IL-1, IL-10, and TNF- in liver tissue homogenate was determined using the sandwich ELISA method according to the manufacturer's protocol. The final concentration was determined by using a standard curve. Detection limit for cytokines was 5–1000 pg/ml of sample [26].

2.9. Histopathology

At the end of the study, animals were anesthetized by using urethane (1.4 mg/kg), and the liver was dissected from each rat and stored in a 10% formalin solution and prepared paraffin blocks. Thin sections were taken from every block and stained with hematoxylin and eosin, and finally observed under a microscope for liver fibrosis, and photographs were taken by using the motic camera system [27].

2.10. MRI study

All the MRI studies were performed by a radiologist with more than 20 years of experience in conventional ultrasonography.

2.11. Statistical analysis

Data for each group were presented as the mean S.E.M. The result values were statistically analyzed by using one-way ANOVA and two-way ANOVA followed by Bonferroni's test using graph pad prism version 7.0. A difference was regarded as significant when P < 0.05, P < 0.01 and P < 0.001.

3. Result

3.1. Effect of L-Carnitine on body weight and liver weight

The bodyweight of the bile duct ligated group increased throughout the treatment periods as compared to normal groups. In the group treated with L-carnitine (500 mg/kg), they show the same pattern of increase in body weight as the normal group. L-carnitine treated with 100 mg/kg does not affect body weight as compared to the disease control group (P < 0.005). The liver weight of the animals was measured at the end of the study and we found that there was an increase in liver weight in the disease control group as compared to the normal group (P < 0.005). The L-carnitine treated with 500 mg/kg showed the most prominent effect on liver weight as compared to the disease control group shown in Table 1 (P < 0.005).

3.2. Effect of L-Carnitine on biochemical parameters

The present study showed a significant increase in the levels of serum AST, ALP, ALT, total bilirubin, and HP in the control bile duct ligation group as compared to normal rats (P < 0.001). In the sham-operated group, there is no change in the level of biochemical markers. Rats treated with a 100 mg/kg dose of L-carnitine had no effect on biochemical marker levels, but rats treated with 300 and 500 mg/kg L-carnitine showed a decrease in the levels of biochemical markers as compared to the disease control group (P < 0.005). An increase in biochemical levels in bile duct ligated rats indicated liver toxicity in rats. In the group treated with only L-carnitine, 500 mg/kg showed no effect on the level of biochemical markers shown in Figure 1.

3.3. Effect of L-Carnitine on serum cytokine

The effect of L-carnitine was determined by an increase in pro-inflammatory cytokine expression in the serum of IL-6, IL-1, and TNF-
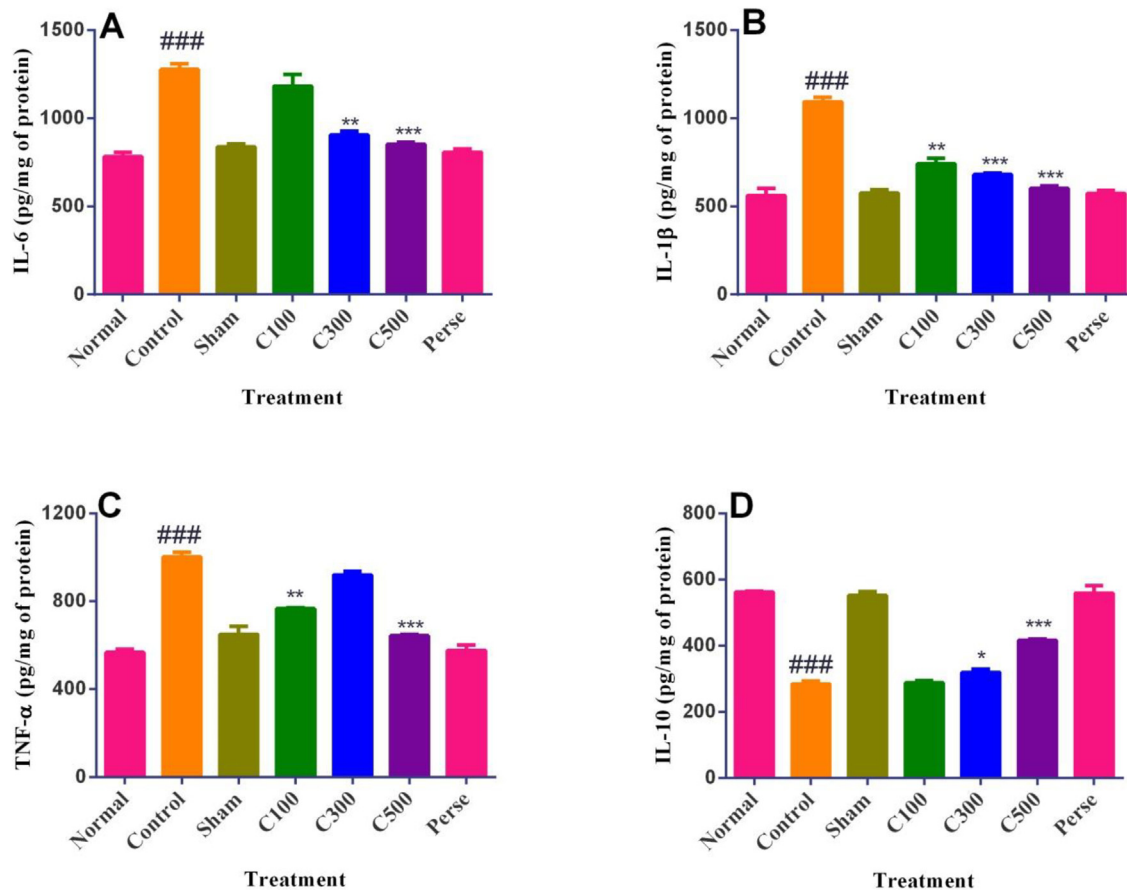
Figure 2. Effect of L-Carnitine on the release of cytokine level (A: IL-6; B: IL-1β; C: TNF-α; D: IL-10). Data were expressed as means ± SEM, n = 06. Statistical significance was determined by one-way ANOVA followed by the Dunnet test: Compared with Normal $^{###}$P < 0.01, Compared with normal; *P < 0.05; ***P < 0.001 compared to the disease control group.

α. At the end of the study, it was found that the disease control group had higher levels of pro-inflammatory cytokines IL-6, IL-1, and TNF-α than the normal group. L-carnitine (500 mg/kg) treatment for 28 days significantly reduced IL-6, IL-1, and TNF-α level almost to normal levels, indicating that L-carnitine inhibits cytokine release. Sham-operated and only the L-carnitine treated group showed the same effect as the normal group. In the case of IL-10, the level increased in the bile duct ligated group as compared to the normal group. Rats treated with L-carnitine show a dose-dependent effect as compared to the disease control group shown in Figure 2.

3.4. Effect of L-Carnitine on oxidative stress

Bile duct ligation-induced liver fibrosis rats showed a significant decrease in the levels of SOD and GSH when compared to the normal group. Treatment with L-carnitine (100, 300, and 500 mg/kg) for 28 days showed a dose-dependent increase in the levels of SOD and GSH when compared with the disease control group. Sham-operated and only the L-carnitine treated group did not affect SOD activity. Bile duct ligation-induced liver fibrosis revealed a decrease in the level of GSH when compared to the normal group. Oral treatment with L-carnitine (100, 300, and 500 mg/kg) for 28 days shows a dose-dependent decrease in the level of MDA when compared with the bile duct ligated group. L-carnitine treated at a dose of 500 mg/kg showed the most prominent effect on MDA level as compared to the disease control group. Bile duct ligation-induced liver fibrosis revealed a decrease in catalase activity when compared to the normal group. Oral treatment with L-carnitine (300 and 500 mg/kg) for 28 days shows a dose-dependently significant increase in catalase activity when compared with the disease control group shown in Figure 3.

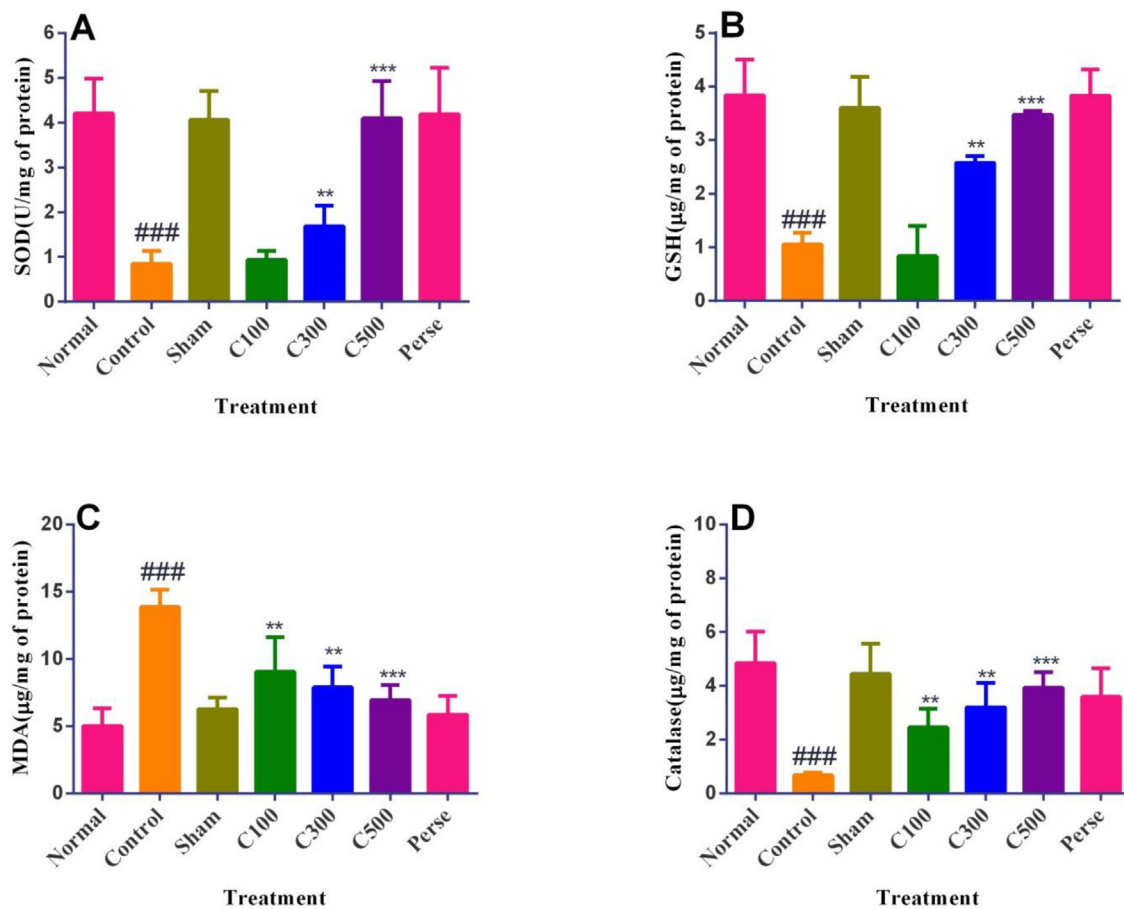### 3.5. Effect of L-Carnitine on iNOS level

Bile duct ligation-induced liver fibrosis revealed an increase in the level of iNOS as compared to the normal group. Oral administration of L-carnitine (300 and 500 mg/kg) for 28 days shows a dose-dependently significant decrease in the level of iNOS when compared with the disease control group given in Figure 4.

### 3.6. Effect of L-Carnitine on MRI study of isolated liver fibrosis

Once the animals had been euthanized, their liver parenchyma was removed for the ex-vivo ultrasound study. The explants were placed in a jar with physiological serum before the study. We then introduced the liver explant into a plastic container with physiological serum at room temperature to conduct an immersion ultrasound study. The shear wave velocity is significantly increased in the bile duct ligated group as compared to the normal control. The animals treated with L-carnitine showed normal structure and architecture, as well as a reduction in shear wave velocity, which has been noted. The MRI scans were represented in Figure 5.

3.7. Effect of L-Carnitine on histopathology on liver fibrosis

The normal and sham group's liver tissues were shown to have normal structure and architecture. The animals with BDL showed marked damage to hepatocytes as observed with edema, neutrophil infiltration, and vacuoles. Treatment with L-carnitine at the entire dose range showed less edema and fibrosis as observed in Figure 6.

Figure 3. Effect of L-Carnitine on oxidative stress (A:SOD; B: GSH; C: Lipid Peroxidation; D: Catalase). Data were expressed as means ± SEM, n = 06. Statistical significance was determined by one-way ANOVA followed by the Dunnet test: Compared with Normal $^{###}$P < 0.01, Compared with the disease control group.
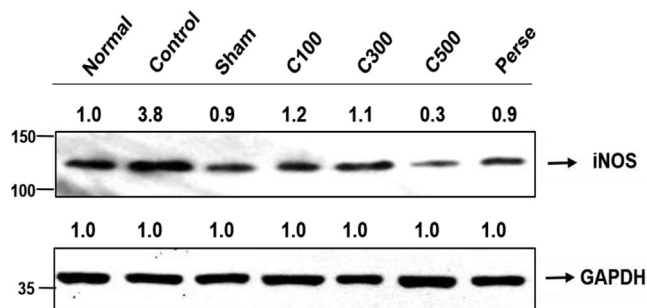
Figure 4. Effect of L-carnitine in iNOS expression. Expression of iNOS in bile duct ligation induced liver fibrosis and different treatment concentrations of L-carnitine. GAPDH served as a loading control (Original blot images provided in supplementary materials).

4. Discussion

Bile duct ligation is the most common model for inducing obstructive cholestatic damage in mice and rats (BDL). Biliary obstruction may cause hepatocellular damage, as well as bile duct growth, fibrosis, and cirrhosis. Depending on the length of the blockage, acute or chronic liver damage may develop. Various studies of the BDL model showed different findings in terms of fibrosis and/or cirrhosis outcomes. All mouse models of cholestatic liver fibrosis exhibit several features that contribute to liver injury, including direct damage to biliary epithelial cells caused by blockage, immunological responses resulting in mononuclear cell infiltration, and periductular inflammation. In line with these findings, the current research revealed that BDL surgery increased bile duct proliferation and cellular inflammatory response.

Different BDL model experiments have revealed numerous findings of their fibrosis and/or cirrhosis results.

In this study, the beneficial effect of L-Carnitine on liver fibrosis ligation of the bile duct in laboratory rats was determined. Liver fibrosis was induced via the technique provided by Lee et al., 2007, by ligation of the typical bile duct [28]. We found that the body weight of bile duct ligation rats increased; meanwhile treatment with L-carnitine also increased the body weight of the rats. In the case of liver weight, it was increased in the bile duct ligation rats and maintained in the L-carnitine treated rats. Moreover, hepatocytes can develop several secondary adaptive changes to minimize the detrimental effects of toxic biliary compounds retained as a consequence of the secretory failure [29]. The study showed that at the end of the study, the levels of serum ALT, AST, TB, and HP were increased. However, the rats treated with L-carnitine showed the normal level of all these as compared to normal. STAT3 activation in the liver is induced by several cytokines, which play important roles in initiating the acute-phase response, preventing hepatocellular damage, and supporting liver regeneration. In the present study, we found the bile duct ligated group shows an increased level of cytokine, whereas treatment with L-carnitine decreases the level of cytokine [30].

Hepatic oxidative stress was previously reported in different BDL models. Previous studies reported a status of oxidative stress, depletion of reduced GSH, and an increase in lipid peroxides induced by accumulated bile acids in BDL models, as seen in our model. The time-dependent depletion of hepatic reduced GSH may occur through the detergent action and cytotoxicity of the retained bile salts, which is partly responsible for the plasma membrane damage seen in BDL models, leading to further

A

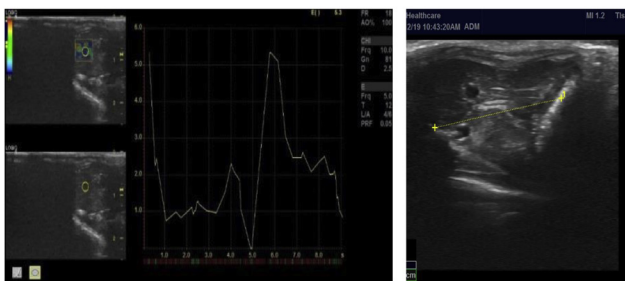Liver size: 1.6cm. Elastography score (pKa 0.9).

E

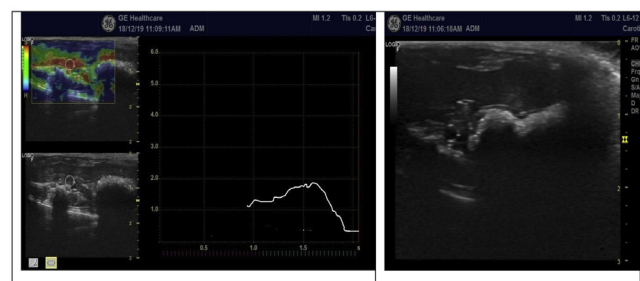Liver size: 1.5cm. Elastographic score(pKa-1.8)
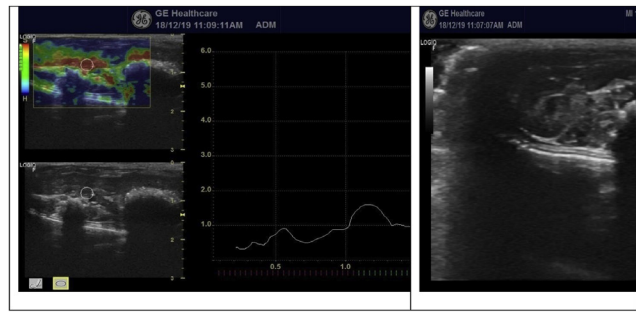
B

Liver size: 1.9cm. Elastography score (pKa-5.45)

F

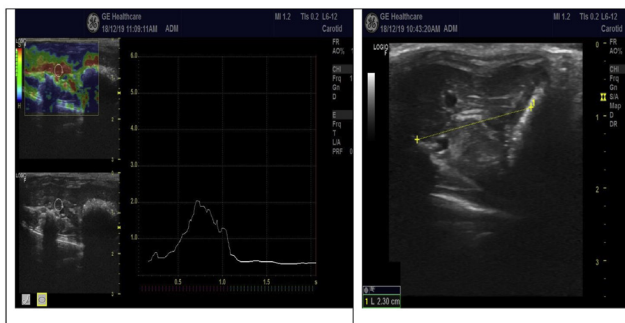Liver size: 1.4cm. Elastographic score (pKa-1.7)

C

Liver size: 1.6cm. Elastographic score (pKa-2.01)
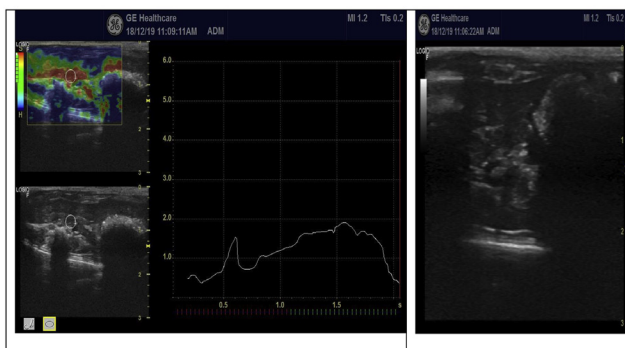

G

Liver size: 1.6cm. Elastographic score (pKa-1.7)

D

Liver size: 1.6cm. Elastographic score (pKa-1.9)

Figure 5. A: Elastography & Ultra Sonography of normal. B: Elastography and Ultrasonography of diseased control. C: Elastography and Ultrasonography of sham. D: Elastography and Ultrasonography of C100. E: Elastography and Ultra sonography of C300. F: Elastography and Ultrasonography of C500. G: Elastography and Ultrasonography of Perse.
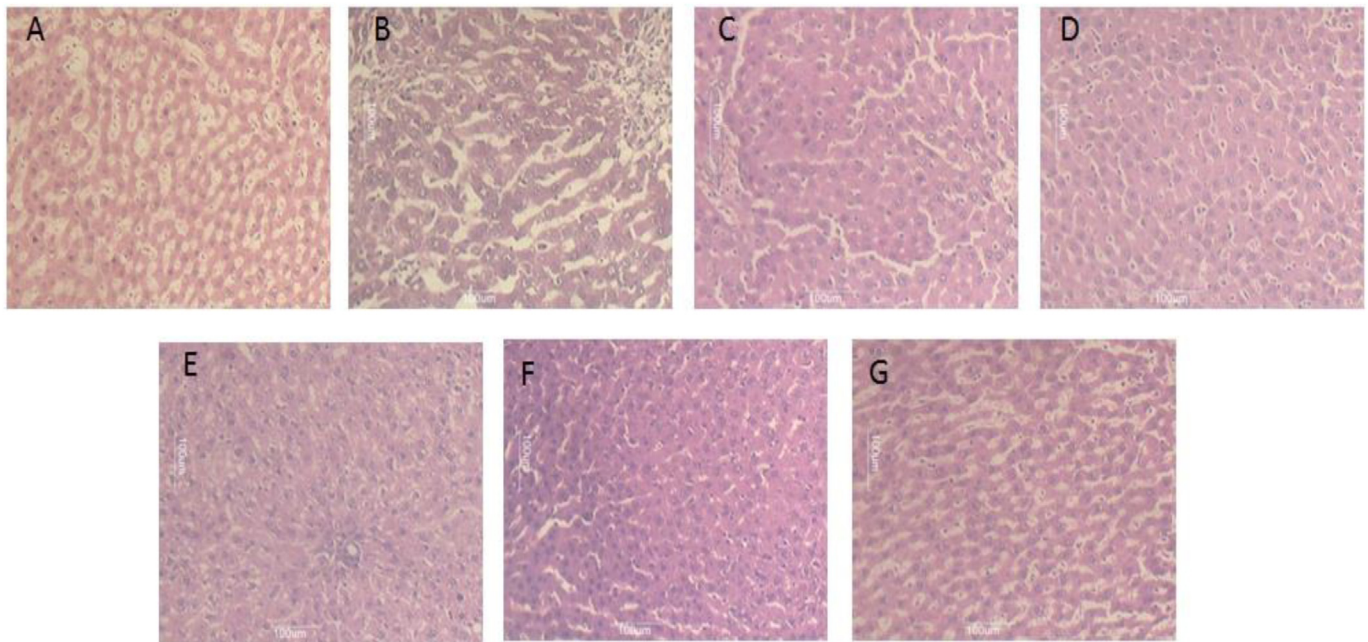
Figure 6. Effect of L-Carnitine on histopathology on liver fibrosis (A: Normal; B: Control; C: Sham; D: C100; E: C300; F: C500; G: Perse).

oxidative stress resulting in the extensive release of reactive oxygen species that is most likely attributed to a lack of adequate reactive oxygen species scavengers, leading to this depletion in hepatic reduced GSH content [31]. Rats treated with L-carnitine show the reverse effect on oxidative damage. Chronic liver disease usually begins with an inflammatory phase that develops into fibrosis following prolonged oxidative stress. Inducible nitric oxide synthase (iNOS) is increased under certain circumstances, resulting in high levels of nitric oxide synthesis (NO). The function of iNOS in the development of fibrosis is unclear [32]. In the case of iNOS level, bile duct ligated animals showed increased levels and this was observed in recently published articles [33]. L-carnitine treated animals decreased the level of iNOS. As per the above observations, it was concluded that L-carnitine shows protective effects against bile duct ligation liver fibrosis in rats by normalizing biochemical markers, maintaining the level of antioxidant enzymes and cytokines, and protecting liver tissue from damage. In further studies, we are going to conduct experiments on cell lines for investigation of the mechanism of L-carnitine in the protection of liver fibrosis.

Currently, only limited imaging tools can be employed to assess the efficacy of developing antifibrotic medication and the necessity of health care. Investigating the diagnostic performance of liver fibrosis using sonoelastography-MRI is the key driving force. The shear waveforms are higher in the animals with BDL, and the animals treated with L-carnitine showed significant reductions as compared to the BDL animals.

5. Conclusion

L-carnitine has a protective role in bile duct ligation in rats. It reduces oxidative and nitrosative stress as well as reduces the concentration of released inflammatory cytokines. Hence, we consider that further studies are needed to clarify the possible role of L-carnitine in liver toxicities.

Declarations

Author contribution statement

Vikram Nimbalkar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Neeraj Vyawahare: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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