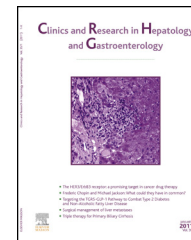




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ORIGINAL ARTICLE

Protective effects of *L*-carnitine, *N*-acetylcysteine and genistein in an experimental model of liver fibrosis

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Summary

Aim: Liver fibrosis is a reversible wound-healing response that occurs following liver injury. In this study, we aimed to investigate the possible protective effects of *L*-carnitine, *N*-acetylcysteine and genistein in liver fibrosis induced by carbon tetrachloride (CCl₄). In addition, the effects of these agents were compared in the same study.

Methods: In this study, rats were randomly allocated into 8 groups, consisting of 10 rats each, as follows: a control group, CCl₄, *L*-carnitine, *N*-acetylcysteine, genistein, CCl₄ and *L*-carnitine, CCl₄ and *N*-acetylcysteine, and CCl₄ and genistein. At the end of 6 weeks, blood and liver tissue specimens were collected. Alanine aminotransferase (ALT); aspartate aminotransferase (AST); complete blood count, tumor necrosis factor- α (TNF- α); platelet-derived growth factor-BB (PDGF-BB); interleukin-6 (IL-6); liver glutathione level; oxidant/antioxidant status; scores of hepatic steatosis, necrosis, inflammation, and fibrosis; and the expression of α -smooth muscle actin were studied.

Results: Although the ALT and AST values in the group administered CCl₄ were significantly higher than in all the other groups ($P < 0.05$), there was no significant difference between the control group and the groups administered CCl₄ combined with *L*-carnitine, *N*-acetylcysteine and genistein ($P > 0.05$). There were significant differences in the levels of TNF- α , PDGF-BB and IL-6 ($P < 0.05$) between the CCl₄ group and the groups with *L*-carnitine, *N*-acetylcysteine and genistein added to CCl₄. *N*-acetylcysteine and genistein had positive effects on the oxidant/antioxidant status and on liver necrosis and fibrosis scores.

Conclusions: In our study, *L*-carnitine, *N*-acetylcysteine and genistein showed significant protective effects in liver fibrosis induced by CCl₄.

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Introduction

Advances in hepatology have shown that cirrhosis is a dynamic, rather than static, process and that it might be reversible. Recently, it has been suggested that cirrhosis occurs in a series of critical steps that if uncontrolled, culminates in hepatic deregulation [1–3]. The treatment of the various stages of liver fibrosis and cirrhosis leads to improvements in the impaired liver structure and scarring caused by the condition. These improvements have been well documented in patients with hepatitis B [4,5] and C [6,7]. In addition, similar findings were reported in other chronic liver diseases secondary to alcohol [8], nonalcoholic fatty liver disease [9], autoimmune hepatitis [10], chronic biliary obstruction [11], hereditary hemochromatosis [12], and thalassemia [13] encouraging hepatologists to treat liver fibrosis and cirrhosis. Additionally, restoring fibrotic tissues to their previous, functional state is an active area of research, and new studies have yielded an increase in information regarding the cellular and molecular mechanisms of liver fibrogenesis.

Regardless of the etiology of fibrosis, developing antifibrotic treatments against the pathological steps in liver disease is important for therapy. Consequently, preventing, delaying and/or improving liver fibrosis have become main aims in the treatment of chronic liver diseases. To address these aims, we examined the utility of three agents, *L*-carnitine, *N*-acetylcysteine, and genistein, in improving liver fibrosis induced by carbon tetrachloride (CCl₄) in rats. In addition, we compared the efficacy of these three agents.

Animal models that are rapidly obtainable, durable and reproducible are necessary to study fibrosis and cirrhosis. One of the most validated models in the rat is the CCl₄-induced fibrosis model [14]. Highly reactive free radicals generated by the metabolism of CCl₄ are capable of covalently binding to cellular macromolecules, especially fatty acids that are present in membrane phospholipids [15]. The toxicity of CCl₄ has been attributed to covalent binding of its metabolites, lipid peroxidation, reaction with aldehydes, hypomethylation of nucleic acids, and deregulation in the homeostasis of calcium or inflammatory cytokines. This may result in necrosis, the degeneration of fats, fibrosis, cirrhosis, cell death and cancer, and is related to dose and exposure time [16].

L-carnitine is a vitamin-like dietary compound that is synthesized in the body from the essential amino acids lysine and methionine [17]. The liver is one of the main sites for endogenous carnitine synthesis [18]. *L*-carnitine is essential for the transport of long chain fatty acids into the mitochondrial matrix through the action of specialized acyl transferases [17]. Different studies have reported that *L*-carnitine prevents oxidative stress induced by CCl₄ [19,20], protects against hepatic damage induced by CCl₄ [18,21], modulates insulin resistance via its antioxidative effect on the liver [22], has a hepatoprotective effect against acute acetaminophen toxicity [23], improves liver inflammation due to insulin resistance induced by fructose [24], improves mitochondrial function by enhancing fatty acid oxidation in nonalcoholic fatty liver disease [25], and enhances liver regeneration in rats after hepatectomy [26].

N-acetylcysteine is an acetylated precursor of the amino acid *L*-cysteine and of reduced glutathione (GSH).

Historically, *N*-acetylcysteine has been used as a mucolytic agent and as an antidote for hepatotoxicity due to acetaminophen [27]. *N*-acetylcysteine is a small membrane permeable molecule that can rapidly permeate the intracellular compartments. This drug has a diversity of applications, largely because of the chemical properties of the thiol moiety present in its structure. The reduced thiol moiety can scavenge reactive oxygen species and indirectly protects the liver by being hydrolyzed into cysteine, thus serving as a precursor for GSH and increasing its levels [28].

Phytoestrogens are diphenolic molecules of plant origin that resemble estradiol in structure and function. Isoflavones, a member of the phytoestrogen family, have been the most extensively studied phytoestrogens. Genistein is an isoflavone that is primarily found in soy protein and has estrogenic and antioxidant activities [29,30]. At the molecular level, genistein inhibits the activity of ATP-utilizing enzymes. Additionally, genistein can act via an estrogen receptor-mediated mechanism, and it induces apoptosis and differentiation in cancer cells, inhibits cell proliferation and angiogenesis, modulates cell cycling, exerts antioxidant effects, and suppresses osteoclastic and lymphocytic functions [31]. Different studies have reported that genistein protects hepatic damage induced by CCl₄ [29,32], activates antioxidant profile and ameliorates fatty liver in insulin-resistant rats [33], suppresses the intestinal response to inflammation [34], and suppresses proliferative cholangitis in rats by directly affecting the bile duct [35].

In the present study, the effects of *L*-carnitine, *N*-acetylcysteine and genistein were evaluated in a rat model of liver fibrosis induced by CCl₄. Specifically, alanine aminotransferase (ALT); aspartate aminotransferase (AST); complete blood count (CBC); tumor necrosis factor- α (TNF- α); platelet-derived growth factor-BB (PDGF-BB); interleukin-6 (IL-6); liver GSH; total oxidant status (TOS); total antioxidant status (TAS); scores of hepatic steatosis, necrosis, inflammation, and fibrosis; and the expression of α -smooth muscle actin (α -SMA) were analyzed.

Materials and methods

Animals

The study included 80 male Wistar Albino rats that were 8–12 weeks old. Water and food, which was supplied in special steel containers, were provided ad libitum. During the study, the rats were housed at a constant temperature with a 12-hour light/dark cycle. The rats were not fed any special diet. The study was conducted in strict conformance with standard ethical rules concerning experimental animal studies. Approval from the Institutional Ethics Committee of Firat University was obtained, and production, housing, nutrition and the experimental study were conducted in the Experimental Animals Center of Firat University.

Chemicals

Carbon tetrachloride (99.5%, Akkimya, Istanbul, Turkey), *L*-carnitine (Carnitine ampoule, 200 mg/mL, Sigma-tau, Pomezia, Italy), *N*-acetylcysteine (Asist ampoule, 100 mg/mL, Husnu Arsan, Istanbul, Turkey), and genistein

(98% aglicon form, Bonistein™, DSM Kaiseragust, Switzerland) were obtained. Genistein was dissolved in dimethyl sulfoxide (DMSO, 20%, Merck, Hohenbrumm, Germany) [29], and CCl₄ was dissolved in olive oil at a 1:2 ratio.

Experimental design

The rats were randomized into eight equal groups ($n=10$ in each group). The study design is shown in Table 1. CCl₄ was administered twice weekly, for 6 weeks. Three days after the last CCl₄ injections, the rats were sacrificed by cervical dislocation under anesthesia with ketamine hydrochloride (60 mg/kg) and midazolam (50 mg/kg). Blood and liver tissue samples were obtained. The blood samples were centrifuged at 5000 rpm for 5 min to obtain plasma and/or serum samples. The samples were then stored at -20°C until they were analyzed. The tissue samples were cut into two pieces for the analysis of liver GSH and histopathological evaluations. One sample was fixed in 10% formalin solution and was used to prepare paraffin blocks, while the other was rapidly frozen.

Blood and liver tests

Routine biochemical analyses, including AST and ALT, were conducted using routine clinical methods with Olympus kits (Olympus Corp., Tokyo, Japan) and an Olympus AU 600 Auto-analyzer. CBC was performed using an Advia 2120 Siemens blood count analyzer (Siemens, Germany).

Serum TNF- α (Assaypro, rat TNF- α ELISA kit, St. Charles, MO, USA), PDGF-BB (Cusabio Biotech, rat PDGF-BB ELISA kit, China) and IL-6 (Boster Biological Technology, rat IL-6 ELISA Kit, Fremont, CA, USA) levels were measured using the enzyme-linked immunosorbent assay method with the appropriate commercial kits.

Liver GSH was determined using the method described by Eyer and Podhradsky [36], and the results were expressed as $\mu\text{mol}/\text{mL}$.

Serum TAS and TOS levels were measured using appropriate kits (TAS assay kit, TOS assay kit, Rel Assay Diagnostic, Gaziantep, Turkey), as described by Erel [37,38]. TOS levels were expressed as $\mu\text{mol H}_2\text{O}_2$ equivalent/L, and TAS levels were expressed as mmol Trolox equivalent/L. The oxidative stress index (OSI) was calculated using a previously described formula [38]. Prior to the calculation, the mmol values of TAS were converted to μmol as follows: $(\text{TOS}/\text{TAS}) \times 100$.

Liver histopathological and immunohistochemical analyses

Four-micrometer-thick sections obtained from the paraffin blocks were stained by hematoxylin-eosin and Masson trichrome and were examined with an Olympus BX-50 microscope. The histopathological evaluation was performed by a pathologist who was blinded to the study groups.

The percentage of the steatotic cells was determined and classified as follows: less than 5%, stage 0; 5–25%, stage I; 25–50%, stage II; and 50–75%, stage III [29].

Inflammatory cells were randomly counted in at least 10 consecutive high-power fields (HPF $\times 400$ magnifications). The mean inflammatory cell number per mm^2 was obtained by dividing the total number by 10 [29,39].

Necrosis was quantified histologically by counting the number of inflammatory foci in at least 10 consecutive HPF ($400\times$ magnification). The mean necrotic field number per mm^2 was obtained by dividing the total number by 10 [29,39].

The degree of liver fibrosis was determined using the semiquantitative method, as previously described [28,40]. According to this scoring system, fibrosis was staged as follows: 0, normal liver with no fibrosis; I, thick perivenular collagen and few collagen septa; II, thin septa with incomplete bridges across the portal regions; III, thin septa and extensive bridges; and IV, thick septa with complete bridges across portal regions and nodular appearance.

The activation of hepatic stellate cells (HSCs) was identified immunohistochemically by staining deparaffinized tissue sections with monoclonal α -SMA antibody. The cells stained with anti- α -SMA were counted randomly in at least 10 fields under $400\times$ magnification, and the mean number of HSCs per mm^2 was determined [39,41].

Statistical analyses

The data were presented as the mean \pm standard deviation (SD). The statistical analysis was performed using the SPSS 12.00 statistical analysis package program (SPSS Inc., Software Chicago, IL, USA). The differences between the groups were evaluated using the Kruskal-Wallis test, while the significance of the differences was assessed using the Mann-Whitney U-test in independent groups and Wilcoxon signed ranks test in dependent groups. The $P < 0.05$ values were considered as statistically significant.

Results

The mean \pm SD weight of the 80 rats was 232 ± 23.2 g (range: 181–293 g). There was no difference in the weights of the groups at the beginning of the study ($P > 0.05$). Nine rats (two in group II, three in group VI, one in group VII, and three in group VIII) died during the study, and the study was completed with 71 rats. When the rats' weights (mean \pm SDg, at the beginning and end of the study) were compared, groups I (230 ± 19.7 ; 285 ± 33.7), III (234 ± 20.6 ; 278 ± 32.8), IV (236 ± 32.7 ; 287 ± 49.8) and V (231 ± 23.5 ; 285 ± 43.1) showed significant increases ($P < 0.05$). However, the groups administered CCl₄, II (239 ± 27.3 ; 237 ± 37), VI (218 ± 28.1 ; 214 ± 16.1), VII (235 ± 18.7 ; 224 ± 20.2) and VIII (235 ± 13.6 ; 225 ± 16.1), did not show any significant weight gain.

Blood and liver test results

The levels of ALT and AST were significantly higher in the CCl₄-treated group compared with the other groups ($P < 0.05$). There was no difference between group II and groups VI, VII, and VIII ($P > 0.05$) (Fig. 1). The CCl₄+N-acetylcysteine-treated group had the lowest leukocyte count ($P < 0.05$). There was no increase in leukocyte count

Table 1 Study design.

Groups	Chemicals	Dose	Dose route	Dose interval
Group I (as control)	Olive oil	3 mL/kg	ip	Twice-weekly
	Serum physiological	0.2 mL	sc	Twice-weekly
Group II	CCl ₄ (with olive oil, a mixture of 1:2)	1.5 mL/kg	ip	Twice-weekly
Group III	L-carnitine	200 mg/kg	ip	Once-daily
Group IV	N-acetylcysteine	50 mg/kg	ip	Once-daily
Group V	Genistein	1 mg/kg	sc	Once-daily
Group VI	CCl ₄ (with olive oil, a mixture of 1:2)	1.5 mL/kg	ip	Twice-weekly
	L-carnitine	200 mg/kg	ip	Once-daily
Group VII	CCl ₄ (with olive oil, a mixture of 1:2)	1.5 mL/kg	ip	Twice-weekly
	N-acetylcysteine	50 mg/kg	ip	Once-daily
Group VIII	CCl ₄ (with olive oil, a mixture of 1:2)	1.5 mL/kg	ip	Twice-weekly
	Genistein	1 mg/kg	sc	Once-daily

ip: intraperitoneally, sc: subcutaneous, CCl₄: carbon tetrachloride.

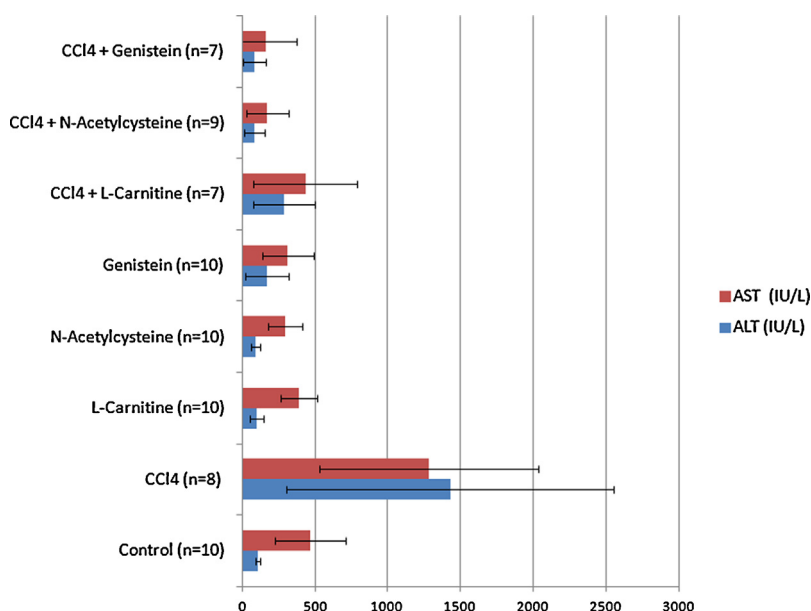


Figure 1 Comparisons of alanine aminotransferase and aspartate aminotransferase levels of the groups. ALT: alanine aminotransferase; AST: aspartate aminotransferase; CCl₄: carbon tetrachloride.

Table 2 Complete blood count results (mean ± SD) of the groups.

Groups (n)	Leukocyte count(/mm ³)	Hemoglobin(g/dL)	Thrombocyte count(/mm ³)
Control (10)	9033 ± 2944 ^g	15.1 ± 1.1	916,250 ± 1,527,235
CCl ₄ (8)	9550 ± 2056 ^{d,g}	14.4 ± 0.5	909,670 ± 145,532
L-carnitine (10)	9146 ± 1971 ^g	14.5 ± 0.5	836,780 ± 182,828
N-acetylcysteine (10)	7229 ± 1825 ^b	14.5 ± 0.5	921,430 ± 117,372
Genistein (10)	9594 ± 1432 ^g	14.3 ± 0.7	908,140 ± 139,791
CCl ₄ + L-carnitine (7)	10,342 ± 3246 ^g	14.1 ± 1.3	993,830 ± 308,237
CCl ₄ + N-acetylcysteine (9)	5178 ± 2986 ^{a,b,c,e,f,h}	14.7 ± 1.1	667,800 ± 380,485
CCl ₄ + Genistein (7)	10,277 ± 4320 ^g	14.3 ± 0.9	920,140 ± 275,800

SD: standard deviation; CCl₄: carbon tetrachloride.

Comparisons with ^acontrol group, ^bCCl₄-treated group, ^cL-carnitine-treated group, ^dN-acetylcysteine-treated group, ^egenistein-treated group, ^fCCl₄ + L-carnitine-treated group, ^gCCl₄ + N-acetylcysteine-treated group, ^hCCl₄ + genistein-treated group: P < 0.05.

in the CCl₄-treated group compared to the control group ($P > 0.05$). There was no difference in hemoglobin and thrombocyte levels ($P > 0.05$) among the groups (Table 2).

The levels of blood TNF- α , PDGF-BB and IL-6 were higher in group II than in the control group ($P < 0.05$). Groups VI, VII, and VIII had lower levels than group II ($P < 0.05$). The level of liver GSH was lower in group II than in the control group ($P < 0.05$). There were slight increases in the levels of liver GSH in groups VI, VII, and VIII, but these differences did not reach statistical significance compared with group II ($P > 0.05$) (Table 3).

There was no significant difference in the TAS values between the control group and group II ($P > 0.05$). There was no difference between group II and groups VI, VII, and VIII ($P > 0.05$). The TAS values significantly increased in groups VII and VIII, but not in group VI, when compared with the control group. The TOS values increased in group II compared with the control group ($P < 0.05$). The TOS value in group VI was higher than that in the control group ($P < 0.05$). When compared to group II, the TOS value in group VIII was lower and that of group VII was similar, but these differences were not statistically significant ($P > 0.05$). The difference in OSI levels between the CCl₄-treated group and the control group did not reach statistical significance ($P > 0.05$). No significant differences were present between group II and groups VI, VII, and VIII ($P > 0.05$). Group VI had a higher level than the control group ($P < 0.05$), but the levels in groups VII and VIII were not significantly different from the control group ($P > 0.05$) (Table 4).

Liver histopathological and immunohistochemical examinations results

Steatosis, necrosis, inflammation, fibrosis and α -SMA expression scores increased in group II compared to the control group ($P < 0.05$). There was no significant difference in the steatosis scores between group II and groups VI, VII, and VIII ($P > 0.05$). When compared to group II, groups VII and VIII had significantly lower necrosis and fibrosis scores, group VII had a significantly lower inflammation score, and groups VI, VII and VIII had significantly lower α -SMA expression scores ($P < 0.05$).

The histopathological and immunohistochemical characteristics of livers of the groups are shown in Table 5, and representative images are shown in Fig. 2.

Discussion

The hepatoprotective and antioxidant effects of L-carnitine, N-acetylcysteine and genistein in liver damage induced by CCl₄ have been reported in different experimental studies [18,19,21,27–29,42]. However, these studies were mostly short in duration (1–8 days). In contrast, in our study, CCl₄ was administered for a longer period of time (6 weeks) to generate a model of more severe fibrosis. Additionally, L-carnitine, N-acetylcysteine and genistein were administered for a longer time, and their effects were observed more reliably and accurately. In addition, the effects of these agents were compared.

In the present study, CCl₄, L-carnitine, N-acetylcysteine, and genistein were administered at the doses of 1.5 mL/kg,

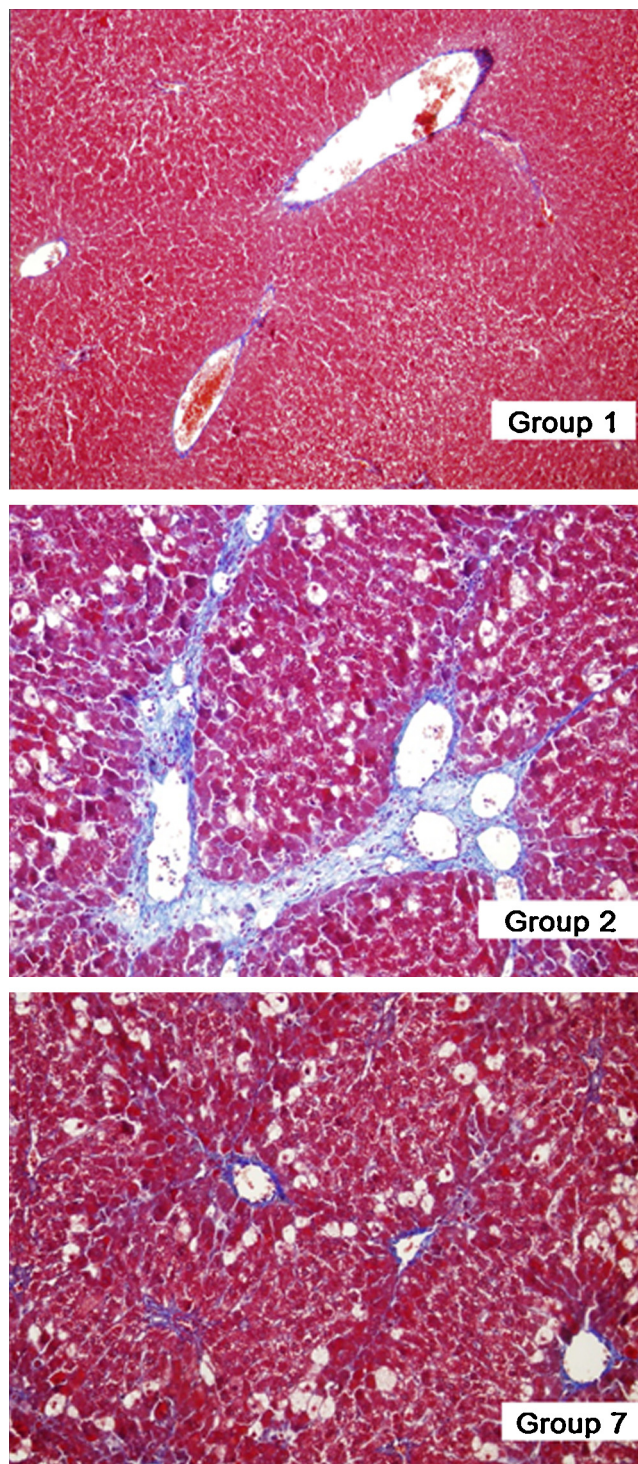


Figure 2 The liver histological examination samples (hematoxylin-eosin, $\times 400$) in three groups (Group I: normal liver, Group II: evident fibrotic liver, Group VII: less fibrotic liver than in group II).

200 mg/kg, 50 mg/kg, and 1 mg/kg, respectively. CCl₄, L-carnitine, N-acetylcysteine have been administered in different doses in literature reviewing. CCl₄ had been administered at the doses of 2 mL/kg [19,43,44], 1.5 mL/kg [29,41], 1.25 mL/kg [42,45], and 1 mL/kg [18,20,32]. L-carnitine had been used at the doses of 500 mg/kg [23],

Table 3 Comparisons of blood TNF- α , PDGF-BB, IL-6 and liver GSH levels (mean \pm SD) of the groups.

Groups (n)	TNF- α (pg/mL)	PDGF-BB (pg/mL)	IL-6 (pg/mL)	Liver GSH (μ mol/mL)
Control (10)	103.4 \pm 21.9 ^{b,d}	25.9 \pm 16.7 ^{b,e,g}	31 \pm 18 ^{b,f,h}	23.7 \pm 4.5 ^{b,c,e,f,g,h}
CCL ₄ (8)	210.1 \pm 6.6 ^{a,c,d,e,f,g,h}	751 \pm 123.5 ^{a,c,d,e,f,g,h}	128.7 \pm 31.6 ^{a,c,d,e,f,g,h}	10.2 \pm 2.9 ^{a,c,d,e}
L-carnitine (10)	107.5 \pm 26.5 ^{b,d}	28.3 \pm 26.6 ^{b,e,f,g}	26.4 \pm 10 ^{b,f,g,h}	16.8 \pm 6.2 ^{a,b}
N-acetylcysteine (10)	80.6 \pm 12 ^{a,b,c,f,g,h}	24.9 \pm 9.2 ^{b,e,f,g}	32.7 \pm 11.4 ^b	21.9 \pm 6.3 ^{b,f,g,h}
Genistein (10)	86.6 \pm 33.2 ^b	46.4 \pm 20.9 ^{a,b,c,d,f,g}	51.4 \pm 29.6 ^b	17.7 \pm 1.6 ^{a,b,h}
CCL ₄ + L-carnitine (7)	104.6 \pm 4.9 ^{b,d}	199.5 \pm 111 ^{b,c,d,e,g}	56.2 \pm 42 ^{a,b,c}	13.7 \pm 5.8 ^{a,d}
CCL ₄ + N-acetylcysteine (9)	108.1 \pm 17.1 ^{b,d}	338.3 \pm 167.9 ^{a,b,c,d,e,f}	59.6 \pm 39.5 ^{b,c}	13 \pm 4.9 ^{a,d}
CCL ₄ + Genistein (7)	111.3 \pm 17.9 ^{b,d}	197.7 \pm 203.7 ^b	59.7 \pm 36.4 ^{a,b,c}	13.8 \pm 5.4 ^{a,d,e}

TNF- α : tumor necrosis factor- α ; PDGF-BB: platelet-derived growth factor-BB; IL-6: interleukin-6; GSH: reduced glutathione; SD: standard deviation; CCL₄: carbon tetrachloride.

Comparisons with ^acontrol group, ^bCCL₄-treated group, ^cL-carnitine-treated group, ^dN-acetylcysteine-treated group, ^egenistein-treated group, ^fCCL₄ + L-carnitine-treated group, ^gCCL₄ + N-acetylcysteine-treated group, ^hCCL₄ + genistein-treated group: *P* < 0.05.

Table 4 Comparisons of blood TAS, TOS and OSI test results (mean \pm SD) of the groups.

Groups (n)	TAS (mmol Trolox equivalent/L)	TOS (μ mol H ₂ O ₂ equivalent/L)	OSI
Control (10)	1.9 \pm 0.4 ^{c,d,g,h}	45.9 \pm 23.9 ^{b,f}	2.7 \pm 1.9 ^f
CCL ₄ (8)	2.3 \pm 0.7	96.1 \pm 25.8 ^{a,c,d,e,f}	4.5 \pm 1.6 ^{c,d,e}
L-carnitine (10)	2.6 \pm 0.5 ^a	44.4 \pm 33.9 ^{b,f}	1.9 \pm 1.8 ^{b,f}
N-acetylcysteine (10)	2.5 \pm 0.6 ^a	25.9 \pm 23.3 ^{b,f,g}	1.2 \pm 1.2 ^{b,f,g}
Genistein (10)	2.4 \pm 0.5	52.9 \pm 34.4 ^{b,f}	2.2 \pm 1.4 ^{b,f}
CCL ₄ + L-carnitine (7)	2.4 \pm 0.9	163.9 \pm 85.3 ^{a,b,c,d,e}	8.2 \pm 6.4 ^{a,c,d,e}
CCL ₄ + N-acetylcysteine (9)	2.9 \pm 0.7 ^a	102.2 \pm 81.8 ^d	3.6 \pm 2.9 ^d
CCL ₄ + Genistein (7)	2.7 \pm 0.5 ^a	64 \pm 66.8	2.7 \pm 3.2

TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative stress index; SD: standard deviation; CCL₄: carbon tetrachloride.

Comparisons with ^acontrol group, ^bCCL₄-treated group, ^cL-carnitine-treated group, ^dN-acetylcysteine-treated group, ^egenistein-treated group, ^fCCL₄ + L-carnitine-treated group, ^gCCL₄ + N-acetylcysteine-treated group, ^hCCL₄ + genistein-treated group: *P* < 0.05.

300 mg/kg [22,24], 100, and 200 mg/kg [26], 125, and 250 mg/kg [25], 200 mg/kg [19] and 50 mg/kg [18,21]. The doses of N-acetylcysteine were quite different in similar studies. Wong et al. [45] tested three dosages of N-acetylcysteine (150, 300 and 600 mg/kg), and they reported that the most effective hepatoprotection against CCL₄-induced liver injury in rats were provided with the high dose of N-acetylcysteine. Galicia-Moreno et al. [27] administered N-acetylcysteine at the dose of 300 mg/kg for 8 weeks but their dose routes were per oral. Maksimchik et al. [42] administered N-acetylcysteine at the dose of 150 mg/kg only three times in their study. N-acetylcysteine had been administered at the doses of 20 mg/kg in two studies [43,44], and 10 mg/kg in a study [28]. It was reported that the hepatoprotective effects were provided in these studies. Finally, we endeavored to prefer an accurate and administrable mean dose of N-acetylcysteine. Genistein had been administered at the dose of 1 mg/kg [29,33] and approximately 1.5 mg/kg [32,35] in previous studies.

Demirdag et al. [21] reported in their one-week study that L-carnitine reduced lipid peroxidation products, steatosis, inflammation and necrosis but had no effect on fibrosis. Kuzu et al. [29] reported that genistein reduced liver malondialdehyde, increased GSH levels, and had positive effects on inflammation, necrosis and α -SMA expression but did

not change the fibrosis score. In both studies, administering L-carnitine and genistein together with CCL₄ significantly reduced the ALT and AST levels compared to control groups that were only given CCL₄. Annudurai et al. [19] showed that L-carnitine increased the levels of antioxidant enzymes and liver GSH. Additionally, Galicia-Moreno et al. [27] reported that N-acetylcysteine increased liver GSH levels, reduced lipid peroxidation products and transforming growth factor (TGF)- β levels, and prevented collagen accumulation. Pereira-Filho et al. [28] reported that N-acetylcysteine decreased the fibrosis scores, increased glutathione peroxidase levels and reduced inducible nitric oxide synthase levels.

In our study, the rats in groups administered CCL₄ did not gain weight and had significantly higher ALT and AST levels. The control group was not significantly different from any other group, except for group II. This result indicated that L-carnitine, N-acetylcysteine and genistein improved ALT and AST levels.

The most striking observation from the blood count data was the decreased leukocyte count in the CCL₄ + N-acetylcysteine group. N-acetylcysteine was also found to be the most successful agent for suppressing inflammation, as determined histologically. A decrease in leukocyte count may contribute to the anti-inflammatory effect of

Table 5 Comparisons of histopathological and immunohistochemical examination results (mean \pm SD) of the groups.

Groups (n)	Steatosis	Necrosis	Inflammation	Fibrosis	α -SMA expression
Control (10)	0 \pm 0 ^{b,f,g,h}	0.14 \pm 0.38 ^{b,f,g,h}	2.14 \pm 0.9 ^{b,f,g,h}	0 \pm 0 ^{b,f,g,h}	6.14 \pm 1.68 ^{b,c,e,f,g,h}
CCl ₄ (8)	1.38 \pm 0.52 ^{a,c,d,e}	6.38 \pm 3.2 ^{a,c,d,e,g,h}	48 \pm 22.19 ^{a,c,d,e,g}	2.63 \pm 0.52 ^{a,c,d,e,g,h}	35.63 \pm 11.19 ^{a,c,d,e,f,g,h}
L-carnitine (10)	0.2 \pm 0.42 ^{b,f,g,h}	0.1 \pm 0.32 ^{b,f,g,h}	3.5 \pm 1.43 ^{b,d,f,g,h}	0 \pm 0 ^{b,f,g,h}	10.6 \pm 2.63 ^{a,b,f,g,h}
N-acetylcysteine (10)	0 \pm 0 ^{b,f,g,h}	0.1 \pm 0.32 ^{b,f,g,h}	1.8 \pm 0.92 ^{b,c,f,g,h}	0 \pm 0 ^{b,f,g,h}	9.1 \pm 3.9 ^{b,f,g,h}
Genistein (10)	0.2 \pm 0.42 ^{b,f,g,h}	0.1 \pm 0.32 ^{b,f,g,h}	2.4 \pm 1.07 ^{b,f,g,h}	0 \pm 0 ^{b,f,g,h}	9.4 \pm 2.76 ^{a,b,f,g,h}
CCl ₄ + L-carnitine (7)	1.14 \pm 0.38 ^{a,c,d,e}	4.29 \pm 1.11 ^{a,c,d,e,g}	49.14 \pm 12.8 ^{a,c,d,e,g}	2.71 \pm 0.49 ^{a,c,d,e,g,h}	23.86 \pm 5.01 ^{a,b,c,d,e,g}
CCl ₄ + N-acetylcysteine (9)	0.78 \pm 0.67 ^{a,c,d,e}	2.44 \pm 1.51 ^{a,b,c,d,e,f}	22.67 \pm 13.35 ^{a,b,c,d,e,f,h}	1.44 \pm 0.73 ^{a,b,c,d,e,f}	16.22 \pm 4.68 ^{a,b,c,d,e,f}
CCl ₄ + Genistein (7)	1.14 \pm 0.69 ^{a,c,d,e}	3.43 \pm 1.62 ^{a,b,c,d,e}	35.57 \pm 13.5 ^{a,c,d,e,g}	1.57 \pm 0.53 ^{a,b,c,d,e,f}	20.43 \pm 7.63 ^{a,b,c,d,e}

SD: standard deviation; α -SMA: α -smooth muscle actin; CCl₄: carbon tetrachloride.

Comparisons with ^acontrol group, ^bCCl₄-treated group, ^cL-carnitine-treated group, ^dN-acetylcysteine-treated group, ^egenistein-treated group, ^fCCl₄ + L-carnitine-treated group, ^gCCl₄ + N-acetylcysteine-treated group, ^hCCl₄ + genistein-treated group: $P < 0.05$.

N-acetylcysteine during the cirrhotic process. The CCl₄ group did not show an increase in leukocyte count compared to the control group. This result may be because the blood specimens were collected at the end of the study, which corresponds to the chronic phase of toxicity.

In our study, *L*-carnitine, *N*-acetylcysteine and genistein decreased the levels of TNF- α , PDGF-BB and IL-6, which were elevated by CCl₄. No difference in the efficacies of the agents was found. HSC activation represents a critical event in fibrosis because these cells are the primary source of extracellular matrix during liver injury. The most important growth factors implicated in HSC activation and collagen synthesis are PDGF-BB and TGF- β [46,47]. The main role of TNF- α in hepatic fibrosis is the initiation of HSC activation [48]. IL-6, a main fibrogenic cytokine that is produced by HSC from normal or cirrhotic livers, up-regulates TGF- β expression in HSC from cirrhotic livers [49]. IL-6 exerts a direct anti-apoptotic effect; therefore, it has been suggested that IL-6 may counteract the hepatotoxic effect of CCl₄ [16]. It has been shown that *L*-carnitine accelerates hepatic regeneration by increasing TNF- α levels and decreasing malondialdehyde [26], *N*-acetylcysteine suppresses TGF- β in HSCs [27], and genistein prevents oxidative damage by decreasing TNF- α and IL-6 [33]. In our study, the effects of *L*-carnitine, *N*-acetylcysteine, and genistein on the levels of TNF- α , PDGF-BB and IL-6 were similar to that which has been reported in the literature.

Reduced glutathione (GSH) plays a key role in detoxifying the reactive toxic metabolites of CCl₄, and fibrotic process begins when the GSH stores are markedly depleted [19]. In our study, the preventive agents partially increased liver GSH levels compared to the control CCl₄ group, but these increases were not statistically significant. In two different studies [19,29] aiming to prevent the damage of acute toxicity, *L*-carnitine and genistein increased GSH levels. In another study [27], *N*-acetylcysteine, which increased GSH, was administered at a dose of 300 mg/kg orally for 8 weeks. In our study, we administered 50 mg/kg intraperitoneally, and therefore, our results may be associated with the lower dosage of these compounds. In another study [28], CCl₄ was administered for 10 weeks, after which the development of cirrhosis was determined via histological inspection and liver enzymes. The administration of *N*-acetylcysteine was then started at 10 mg/kg/day, and the study was terminated at the 16th week. At the end of the study, liver GSH levels were not directly measured and the glutathione peroxidase levels were measured instead.

Reactive oxygen species are molecules containing oxygen that are produced during normal metabolism. Organisms have a system for neutralizing the negative effects of reactive oxygen species, and when an interruption in antioxidant mechanism and/or an increase in oxidative status occurs, the oxidative/antioxidative balance shifts towards being more oxidative [50]. The serum or plasma levels of individual oxidant and antioxidant products can be measured in the laboratory, but this is very difficult, time consuming, expensive and requires complicated methods [51]. Therefore, methods measuring the total amount of reactive oxygen species have been developed. In our study, TAS and TOS

values were studied by pre-established methods [37,38]. The high value of TAS indicates antioxidant properties, while TOS and OSI indicate oxidant properties. The OSI considers both the TOS and TAS.

In the present study, CCl₄ increased the TOS value. The CCl₄+*N*-acetylcysteine and CCl₄+genistein groups showed increased TAS levels compared with the control group. The CCl₄+*L*-carnitine group showed increased TOS levels compared with the CCl₄ group. The CCl₄+genistein group showed decreased TOS compared with the CCl₄ group, though this decrease was not statistically significant. Therefore, *N*-acetylcysteine and genistein may have positive effects on the impaired oxidant/antioxidant balance. The methods that were used in our study were previously applied in similar studies. While all three agents have been reported to have antioxidant effects [18,19,21,27–29], we did not observe this for *L*-carnitine. This situation may be due to our methods for measuring antioxidant species, which were more comprehensive.

In our study, the histopathological and immunohistochemical analyses of liver sections showed higher steatosis, necrosis, inflammation, fibrosis, and α -SMA positive cell scores in the groups administered CCl₄ compared with the control group. The increase in α -SMA release results from HSC activation due to liver damage. Following liver injury, HSCs show a reduction in vitamin A content and an increase in expression of α -SMA [52]. Therefore, immunohistochemical staining by monoclonal α -SMA antibody is indicative of activated HSCs.

In the present study, *L*-carnitine, *N*-acetylcysteine and genistein were not shown to have significant effects on hepatic steatosis. *N*-acetylcysteine and genistein decreased hepatic fibrosis and necrosis, *N*-acetylcysteine decreased inflammation, and all three agents decreased α -SMA expression. In other studies, *N*-acetylcysteine, but not *L*-carnitine [21] and genistein [29], improved the fibrosis score [28].

In conclusion, *L*-carnitine, *N*-acetylcysteine and genistein improved ALT, AST, TNF- α , PDGF-BB and IL-6, which were increased by CCl₄. They increased liver GSH, which was decreased by CCl₄, but this difference did not reach statistical significance. *N*-acetylcysteine and genistein had positive effects on the oxidant/antioxidant balance that was impaired by CCl₄. In liver histological examinations, *L*-carnitine reduced α -SMA expression; *N*-acetylcysteine reduced necrosis, inflammation, fibrosis, and α -SMA expression; and genistein reduced necrosis, fibrosis, α -SMA expression. Thus, it may be considered that *N*-acetylcysteine and genistein have a curative effect on fibrosis, whereas *L*-carnitine has a preventive effect in mild pathological situation. We assume that *N*-acetylcysteine has an important effect on acute injury because of its anti-inflammatory effect. Furthermore, it may be speculated that *N*-acetylcysteine and genistein may be effective at all stages of cirrhotic process by their antioxidant effects. The present study supports that *L*-carnitine, *N*-acetylcysteine and genistein have positive effects on liver damage and fibrosis. These agents may potentially be used for the prevention and treatment of liver fibrosis. Pre-clinical and clinical studies are needed to validate these findings.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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