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N-Acetyl cysteine alleviates carbon-tetrachloride induced acute liver injury in rats

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Abstract

The liver is a vital organ that performs most of the body's metabolic and detoxifying functions. There are various exogenous and endogenous factors that might cause liver issues. Carbon tetrachloride (CCL_4) is non-inflammable colorless organic compound and employed in a variety of industrial fields. N-acetylcysteine (NAC) is one of the prospective pharmaceutical candidates possesses multiple clinical applications. The purpose of this study is to determine whether NAC has a protective effect in cases of liver injury or not. Thirty male albino rats were involved in the study, which lasted one month. They were categorized into three groups: control, liver injury group (0.5 ml/kg rat body weight (Bwt) CCL_4 administered orally twice a week), and protective group (150 mg/kg Bwt NAC supplied orally). Serum lipid and protein profiles and liver enzymes activities were evaluated. Antioxidant, oxidative stress, and anti-inflammatory parameters were also assessed. Additionally, hepatic tissue was subjected to a histopathological investigation. The biochemical and histopathological results revealed dramatic improvement of studied parameters in NAC protective group comparing to liver injury one. Hence, we can conclude that, NAC shown a great potential in attenuating liver injury induced by CCL_4 via refinement tumor necrosis factor-alpha, interleukin-6 and reduced glutathione pathway.

Keywords: Liver injury, Carbon tetrachloride, N-acetylcysteine, Oxidative stress, Inflammatory markers.

Introduction

Liver is one of the dynamic organs that has a crucial role in macromolecules metabolism, bile acid biosynthesis and detoxification process of circulating agents (Zheng et al., 2022). Liver injury is attributed to multiple issues as subjection to repeated doses of drugs, medicine-based treatment, and toxic substances in the surrounding environment (Attallah et al., 2022). It also occurs as consequence of some diseases like non-insulin dependent diabetes mellitus, chronic kidney, and heart failure (Byrne and Targher, 2022). In many cases of untreated liver injury is progressed to liver fibrosis, cirrhosis, and cancer (Kermanizadeh et al., 2022).

Carbon tetrachloride (CCL_4), tetrachloromethane, is a colorless chlorinated hydrocarbon that has various industrial benefits in degreasing process and enters in refrigerant and fire extinguisher's structure (Aramjoo et al., 2022). However, continuous daily exposure to CCL_4 leads to cell destruction and necrosis as it is considered one of non-inflammable environmental poison (Popoola et al., 2022). N-acetylcysteine (NAC) is sulfhydryl compound which is the acetylated amino acid L-cysteine (Mohamed et al., 2022). It possesses multiple biological influences including respiratory tract (Guerini et al., 2022), neurodegenerative (Sharma et al., 2022), renal (Nejati et al., 2022) and cardiac disorders (Li et al., 2022). This study emphasizes the theory of protective effect of NAC on liver injury

induced by CCL_4 via oxidants-inflammatory biomarkers inhibition.

Materials and Methods

Chemicals, reagents, and kits

5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), CCL_4, reduced glutathione (GSH) and thiobarbituric acid (TBA) were got from Sigma-Aldrich (USA). N-naphthyl ethylenediamine dihydrochloride was purchased from Coinbrook Bucks (England). Disodium hydrogen phosphate, ethyl alcohol, formalin, phosphoric acid, sodium dihydrogen phosphate, sodium nitroprusside and sodium nitrite were obtained from Merck. Sulfanilamide and sulfosalicylic acid were got from Win Lab (UK). Trichloroacetic acid (TCA) and xylene were obtained from LOBA-Chemie and Taiwan, respectively. Hematoxylin and eosin (H and E) stains purchased from Ricca Chemical Company (Arlington).

Further, kits of alanine aminotransferase (ALT), aspartate aminotransferase (AST), high density lipoprotein-cholesterol (HDL-c), total cholesterol (TC) and triacyl glycerol (TG) were got from Biomed Co. (Egypt). However, albumin and total protein (TP) kits were purchased from Spectrum Co. (Egypt). ELISA kit of rat interleukin-6 (Il-6) and tumor necrosis factor-alpha (TNF-α) were obtained from MyBiosource (INC). Finally, NAC, olive oil and isoflurane were obtained from the pharmacy.

Animals and treatment

Thirty male albino *Wistar* rats of Bwt of 120-180 g and 8-10 weeks old age were got from the animal house in Faculty of Medicine, Assiut University, Assiut, Egypt. Rats were kept two weeks for acclimatization in polycarbonate cages in controlled environmental conditions of temperature (22 ± 2°C), relative humidity (45-46%) and 12 h/12 h light/dark cycle. Food and water were provided ad-libitum. The protocol was approved according to the Ethics Committee on Animal Experimentation of Assiut University, Faculty of Veterinary Medicine and the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication No. 8023, revised 1978). The experimental period was conducted for 30 days. Rats were randomly divided into 3 groups of 10 animals in each group that were categorized as follows:

Group 1: Control group (CN), rats in this group were healthy and free from any diseases and received only distilled H_2O daily by intra gastric tube all over the experimental period.

Group 2: Liver injury-induced group (CCL_4 group), where the liver injury was induced in rats according to Rahmouni, Hamdaoui, Badraoui, and Rebai (2017) with minor modification, where rats were received 0.5 ml/kg Bwt CCL_4 dissolved in olive oil in ratio 1:3 twice a week for one month by oral gavage.

Group 3: Liver injury NAC protected group (CCL_4 - NAC), rats were orally daily administrated NAC at dose 150 mg/kg Bwt (Khalifa, Bakr, & Osman, 2017) and CCL_4 twice a week at dose 0.5 ml/kg Bwt dissolved in olive oil for 30 days at the same time of NAC administration.

Sample preparation

Blood samples

At the end of experiment, one month, rats were fasted overnight. Before scarification, rats were anesthetized using 1% isoflurane, then blood samples were collected from eye-canthus in wisterman tubes, tubes were kept in inclined position till time of centrifugation at 3000 rpm for 15 min. After centrifugation, clear non hemolyzed serum was separated and stored at -20°C till time of biochemical analysis.

Liver samples

Liver was dissected out during scarification, divided into 3 parts, one part used for biochemical assays, second part used for TNF-α and Il-6 determination at protein level and the third part of liver was put in falcon tube containing 10% formalin for histopathological examination. The first and second part of liver were homogenized in phosphate buffer saline, 0.1 M, pH 7.4 immediately before parameters estimation.

Serum lipid profile assays

Serum TC, TG and HDL-c was determined using commercial kits. However, low density lipoprotein-cholesterol (LDL-c) and very density lipoprotein-cholesterol (vLDL-c) levels were calculated according to previous formula of Friedewald, Levy, and Fredrickson (1972) and Srilatha, Bobby, Subrahmanyam, and NithinKumar (2017) respectively.

Serum protein profile assays

Determination of serum TP was determined according to previous method of Yatzidis (1987). Further, serum albumin was analyzed by commercial kits according to (Doumas, Watson, & Biggs, 1971). Globulin concentration in samples was calculated by subtraction of TP content from albumin concentration

and expressed as g/dl (Samanta, Sharma, Das, Mallick, & Kumar, 2016). Finally, A/G ratio was done by dividing concentrations of albumin on globulin of each individual sample.

Liver enzymes activity assays

Determination of serum ALT and AST was performed via kinetic commercial kits using UV spectrophotometer (JENWAY 61431, 6705, UK) according to (Bergmeyer, Bowers Jr, Hørder, & Moss, 1977).

Pro-oxidant and antioxidant parameters evaluation in hepatic tissue

Lipid peroxidation level was measured as an index of malondialdehyde (MDA) production. The level of lipid peroxidation was determined in liver homogenate according to the previous method of Lukaszewicz, Moniuszko, and Rogalska (2007) where one molecule of MDA was reacted with two molecules of TBA and produce pink pigment with a maximum absorbance of 540 nm.

Further, nitric oxide (NO) level was determined according to the pervious method (Montogomery & Dymock, 1961)**.** The assay was based on, NO production from sodium nitroprusside at physiological PH. The produced NO reacted with oxygen and gave nitrite ions (NO_2^-). Then NO_2^- reacted with *Griess* reagent and formed reddish-purple azo-dye product that detected at 540 nm via spectrophotometer. Hepatic GSH content was also determined according to Ellman (1959) procedure.

Inflammatory markers estimation in hepatic tissue

Both TNF-α and Il-6 assays were performed according to manufacture of instruction and it was employed the quantitative sandwich enzyme immunoassay technique (Aydin, 2015). Antibody specific for TNF-α (Catalog No: MBS2507393) and Il-6 (Catalog No: MBS726707) has been pre-coated onto separate microplates. The assay was depended on combination of either samples or standard with the TNF-α or Il-6 antibody. Developing of the color has been appeared due to the enzymatic reactions which yielded blue product that turned to yellow when stop solution was added. The absorbance was read at 450 nm using ELISA reader (HEEPF D-080-HO, Biotec, USA). The concentration of liver homogenate TNF-α and Il-6 in pg/mg tissue protein was determined using standard curve.

Histopathological study of hepatic tissue

All histopathological procedures were performed in Histology Unit, City of Scientific Research and Technological Application City, Alexandria, Egypt. Following scarification, specimens were collected from the livers of rats in all groups, then fixed in 10% neutral buffered formalin solution. After 24 hrs., following steps of dehydration in ascending grades of ethyl alcohol, cleared in xylene, and then embedded in paraffin wax. Tissue sections (3-5 microns thick) were cut and stained with H and E according to Banchroft, Stevens, and Turner (1996) and subjected to the light microscope (Germany) for the histopathologic evaluation.

Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations. Statistical analysis was applied to determine differences between studied groups ($P < 0.05$). Statistical data analysis was undertaken using SPSS Version-25 software and the comparison of means between each experimental groups was done by one way ANOVA test.

Results

Effect of NAC on serum lipid profile

Serum TC was declined by 35.31% and 30.66% in CN and CCL_4 -NAC groups, respectively compared to CCL_4 group. Also, TG was decreased by 25.79% in CN and 28.43% in CCL_4-NAC compared to CCL_4. However, in comparison to CCL_4 group, there was significant increase in HDL-c by 73.38% in CN and 8.47% in CCL_4-NAC. Moreover, LDL-c was diminished nearly by 1.9- and 0.8-folds in CN and CCL_4 groups respectively comparing to CCL_4 group. Also, there was notable lessening in vLDL-c in CN by 25.81% and in CCL_4-NAC by 28.45 in comparison to CCL_4 group **(Table 1 and Fig.1)**.

Table.1. Serum levels of TC, TG, HDL-c, LDL-c and vLDL-c (mg/dl) of rats after 30 days administration of NAC with CCL_4. Values are expressed as means ± SE (n=10).

Groups	TC (mg/dl)	TG (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)	vLDL-c (mg/dl)
CN	85.44 ± 1.38[a]	115.17 ± 0.86[a]	102.92 ± 2.72[a]	-40.52 ± 2.99[a]	23.03 ± 0.17[a]
CCL_4	132.07 ± 4.17	155.19 ± 2.96	59.36 ± 3.57	41.67 ± 5.72	31.04 ± 0.59
CCL_4-NAC	91.58 ± 1.74[a]	111.07 ± 3.24[a]	64.39 ± 1.29	4.98 ± 1.44[a]	22.21 ± 0.65[a]

ANOVA (one way). Means with letters (a), (b) and (c) were statistically represented compared to CCL_4 group as follow: aP < 0.001, bP < 0.01, cP < 0.05

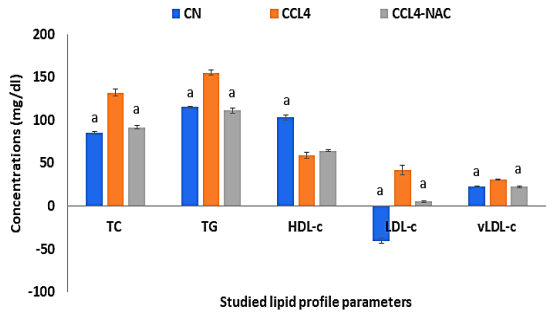

Fig.1. Serum TC, TG, HDL-c, LDL-c and vLDL-c concentrations of CN, CCL_4 and CCL_4-NAC groups. Values are expressed as mean ± SE (n=10).
Significance: aP < 0.001, bP < 0.01, cP < 0.05 comparing to CCL_4.

Effect of NAC on serum protein profile

Data presented in **Table 2 and Fig.2** exhibited reduction of TP in both CN and CCL_4-NAC groups nearly by 0.77- and 0.92- folds when compared to CCL_4 group. However, both globulin and A/G ratio were folded raised by 2.31 and 1.31 in CN group and by 2.24 and 1.41 in CCL_4-NAC, respectively.

Table.2. Serum levels of TP, albumin, and globulin (g/dl) and A/G ratio of rats after 30 days administration of NAC with CCL_4. Values are expressed as means ± SE (n=10).

Groups	TP (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio
CN	7.74 ± 0.20[a]	3.21 ± 0.13[a]	4.53 ± 0.26[a]	0.72 ± 0.07[a]
CCL_4	4.36 ± 0.07	7.81 ± 0.34	-3.45 ± 0.29	-2.30 ± 0.12
CCL_4-NAC	8.36 ± 0.38[a]	4.08 ± 0.32[a]	4.28 ± 0.17[a]	0.96 ± 0.08[a]

ANOVA (one way). Means with letters (a), (b) and (c) were statistically represented compared to CCL_4 group as follow: aP < 0.001, bP < 0.01, cP < 0.05

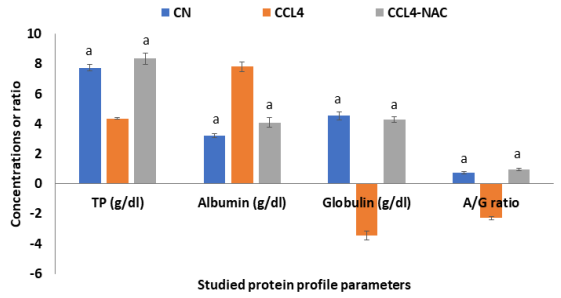

Fig.2. Serum TP, albumin and globulin concentrations and A/G ratio of CN, CCL_4 and CCL_4-NAC groups. Values are expressed as mean ± SE (n=10).
Significance: aP < 0.001, bP < 0.01, cP < 0.05 comparing to CCL_4.

Effect of NAC on serum liver enzymes activity

Activity of liver enzymes revealed significant reduction in CN and CCL_4-NAC groups in comparison to CCL_4, where ALT was diminished by 62.27% in CN and 50.59% in CCL_4-NAC and AST was declined by 31.68% and 21.63% in CN and CCL_4 -NAC, respectively **(Table 3 and Fig.3)**.

Table.3. Serum activity of ALT and AST (U/ml) of rats after 30 days administration of NAC with CCL_4. Values are expressed as means ± SE (n=10).

Groups	ALT (U/ml)	AST (U/ml)
CN	71.47 ± 2.26[a]	66.70 ± 1.99[a]
CCL_4	189.41 ± 3.00	97.63 ± 1.62
CCL_4-NAC	93.59 ± 2.07[a]	76.51 ± 2.66[a]

ANOVA (one way). Means with letters (a), (b) and (c) were statistically represented compared to CCL_4 group as follow: aP < 0.001, bP < 0.01, cP < 0.05

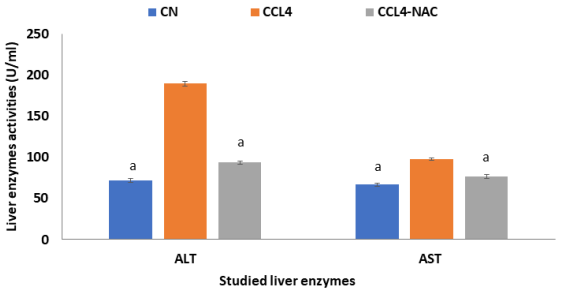

Fig.3. Serum ALT and AST activities of CN, CCL_4 and CCL_4-NAC groups. Values are expressed as mean ± SE (n=10).
Significance: aP < 0.001, bP < 0.01, cP < 0.05 comparing to CCL_4.

Effect of NAC on pro-oxidant and anti-oxidant parameters

Results in **Table 4 and Fig.4** revealed lessening of TBARS and NO concentration by 65.71% and 72.72% in CN group and 48.05% and 72.72% in CCL_4-NAC when compared to CCL_4 group. On the other hand, GSH content in CN group was improved nearly 2.25-fold and in CCL_4-NAC elevated nearly 1.42-fold comparing to CCL_4 group.

Table.4. Hepatic TBARS (nM/mg protein), NO (nM/mg protein) and GSH (µmol/mg protein) concentrations in rats after 30 days administration of NAC with CCL_4. Values are expressed as means ± SE (n=10).

Groups	TBARS (nM/mg protein)	NO (nM/mg protein)	GSH (µmol/mg protein)
CN	1.32 ± 0.19[a]	0.03 ± 0.00[a]	0.39 ± 0.17
CCL_4	3.85 ± 0.42	0.11 ± 0.01	0.12 ± 0.13
CCL_4-NAC	2.00 ± 0.30[a]	0.03 ± 0.00[a]	0.29 ± 0.09

ANOVA (one way). Means with letters (a), (b) and (c) were statistically represented compared to CCL_4 group as follow: aP < 0.001, bP < 0.01, cP < 0.05

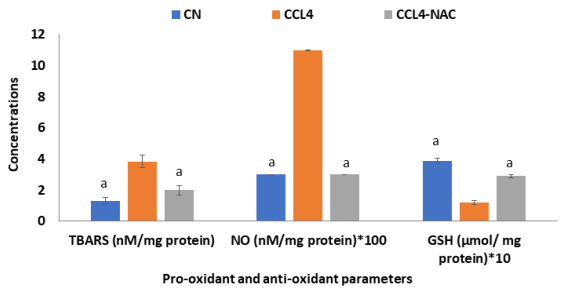

Fig.4. TBARS, NO and GSH concentrations in hepatic tissue of CN, CCL_4 and CCL_4-NAC groups. Values are expressed as mean ± SE (n=10).
Significance: aP < 0.001, bP < 0.01, cP < 0.05 comparing to CCL_4.

Effect of NAC on inflammatory markers

Protective effect of NAC against liver injury exposed anti-inflammatory influence where data in **Table 5 and Fig.5** displayed reduction of TNF-α and Il-6 by 43.08% and 59.84% in CCL_4-NAC group respectively when compared to CCL_4 group.

Table.5. Hepatic TNF-α and Il-6 (pg/mg protein) concentrations in rats after 30 days administration of NAC with CCL_4. Values are expressed as means ± SE (n=10).

Groups	TNF-α (pg/mg protein)	Il-6 (pg/mg protein)
CN	7.52 ± 0.31[a]	4.90 ± 0.39[a]
CCL_4	16.48 ± 0.43	11.58 ± 0.36
CCL_4-NAC	9.38 ± 0.65[a]	4.65 ± 0.49[a]

ANOVA (one way). Means with letters (a), (b) and (c) were statistically represented compared to CCL_4 group as follow: aP < 0.001, bP < 0.01, cP < 0.05

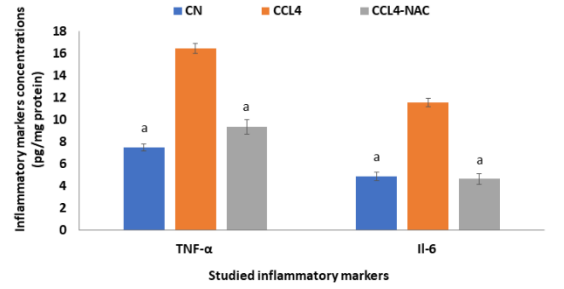

Fig.5. TNF-α and Il-6 concentrations in hepatic tissue of CN, CCL_4 and CCL_4-NAC groups. Values are expressed as mean ± SE (n=10).

Significance: aP < 0.001, bP < 0.01, cP < 0.05 comparing to CCL_4.

3.1. Histopathological investigation results of hepatic tissue

The results in **Fig.6. A.** disclosed normal hepatocytic cords (Black arrows) crowded around a clear central vein (CV). On the other side CCL_4 group showed excess mononuclear cell infiltration (Black arrows), large areas of centrilobular congestion (Red arrows) and dilated portal tract (Green arrows), also the hepatocytes are crowded and have small dark nuclei (Blue arrows) and some pyknotic nuclei were seen (Arrows head) **(Fig.6. B)**. The histopathological examination of hepatic tissue after using of NAC together with CCL_4 showed vacuolar and hydropic degeneration in some of hepatocytes (Black arrows) with normal hepatocytic cords **(Fig.6. C)**.

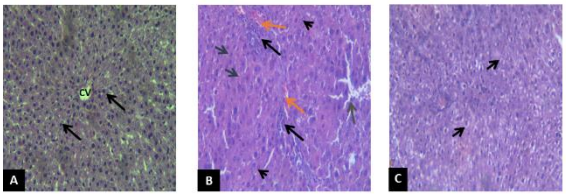

Fig.6. Hepatic tissue sections microscopical images in different rat's groups stained with H & E. (A) CN, (B) CCL_4 and (C) CCL_4-NAC (X= 400).

Discussion

In recent years and due to a lot of environmental stressors either exogenous or endogenous, liver becomes easily supposed to the damage that may lead to acute or chronic liver disorders. CCL_4 is one of hazardous poisonous chemicals that causes tissue destruction as it initiates secretion of reactive oxygen species (ROS) in all body tissues once human or animal subjected to single overdose or multiple low doses of it (Shahat et al., 2022). The destructive action of CCL_4 is accredited to bio-alteration of cytochrome P4502E1 (CYP2E1) that produces vastly reactive trichloromethyl (CCL_3•) free radicals which reacts with oxygen and gives trichloromethyl peroxyl (CCL_3OO•) radicals that can attack the lipids and protein of cell membrane bounded to various organs (Zhao et al., 2022). The current study revealed that CCL_4 disrupted the lipid metabolism by increasing TC, TG, LDL-c and vLDL-c and lowering HDL-c and these findings matched with previous work of (Shaban et al., 2022). Hypercholesteremia after CCL_4 administration may be attributed to hindering the β-oxidation of fatty acids (Mahmoodzadeh et al., 2017) and activation the esterification process of lipid (Mesalam et al., 2021). On the same hand, hypertriglyceridemia ascribed to the capability of CCL_4 to transfer the acetate into hepatocytes (Weber et al., 2003) and hinder the action of lysosomal lipase enzyme (Marimuthu et al., 2013).The current study

exposed elevation in the activity of transaminases after CCL_4 administration and that was allied to hyperlipidemia which interferes with liver function and also out flow of liver cytosolic content into the circulation due to oxidative stress of CCL_4 that lead to imbalance between antioxidant and prooxidant system (Xu et al., 2021). These results were in accordance with the previous findings reported by (Haddar et al., 2021). Further lipid peroxidation was raised because of covalent binding of CCL_4 metabolites, CCL_3• and CCL_3OO•, to the protein in the liver cell causing destructive effects (Abbasi et al., 2021). In addition, hepatic GSH content was depleted after CCL_4 administration which might be due to the extreme need of GSH to eradicate the CCL_3OO• radicals and hypochlorous acid, where GSH is the key mediator in detoxification process inside the body (Habashy et al., 2021). This result corroborates with the reports of (Emmanuel et al., 2021). It was further noted that upregulation of inflammatory cytokines, TNF-α and Il-1ß, which credited to macrophage activation (Ebaid et al., 2021). Our results are consistent with the previous mentioned data of Tsai et al. (2017). It is known that liver is the main organ involved in protein biosynthesis, chiefly albumin. Although CCL_4 induces hypoproteinemia, but hyperalbuminemia was reported in our study and that may be due to the liver is not the only site of albumin creation, it can be synthesized in extrahepatic tissues as intestine, kidney pancreas, brain, and reproductive organs (Shevtsova et al., 2021). Hence from the biochemical background, because of destructive action of ROS on the cell membrane, the tissues released albumin content into the blood circulation leading to albumin over level. In harmony of our findings Ojeaburu and Oriakhi (2021) were demonstrated significant elevation of serum albumin in CCL_4 rat model. Nowadays much attention has been focused on using antioxidants to ameliorate the incidence of liver injury. NAC is one of thiol molecules that augments the detoxification process through various theories. Firstly, NAC administration could decrease the hyperlipidemia noticed in our study via its repressing influence on hydroxy-3-methyl glutaryl-CoA reductase enzyme, thus decreasing mevalonic acid that is required for cholesterol biogenesis (Hammerschmidt et al., 2022). NAC influences the synthesis of long chain fatty acids and hinders the aggregation of lipid in adipose tissue (Wolosowicz et al., 2022). NAC hampers lipid accumulation via hindering the pathway of lipid metabolism regulator genes like peroxisome proliferator-activated receptor gamma and sterol regulatory element binding protein (Dludla et al., 2019). The current findings validate the previous findings of Ma et al. (2016), where receiving the obese mice NAC for 8-weeks disclosed hypolipidemic effect. Further, NAC improved the oxidative stress-antioxidant status produced from liver injury through its capability to inspire sulfhydryl groups that act as a scavenger of free radicals by interaction with ROS specially hydrogen peroxide (Esalatmanesh et al., 2022). NAC is also able to penetrate the hepatic cell membrane and catabolized into cysteine that is the precursor of GSH (Liu et al., 2022). Many in vivo and in vitro studies exhibited the antioxidant activity of NAC (Elsayed et al., 2021; Moens et al., 2022). Also, NAC ameliorated the alteration of TBARS as a measure of lipid peroxidation and NO due to nitric oxide synthase enzyme inhibition (Tsikas andMikuteit, 2022). Additionally, liver enzymes activity and protein and albumin levels were improved during NAC administration and that was in accordance with (El-Maddawy andEl-Sayed, 2018). Results revealed NAC alleviated hepatic injury in rats by abolishing the inflammatory markers, TNF-α and Il-6. Anti-inflammatory activity of NAC is attributed to various theories. NAC inhibits the adhesion molecule expression that responsible for recruitment of neutrophils to the site of inflammation (Habas and Shang, 2018) and it also interferes the phosphorylation process of nuclear factor kappa-B (Zheng et al., 2019). These sentinel findings confirmed with previously published studies (Satvati et al., 2022). Finally, the histopathological evaluation of hepatic tissue section agrees and confirmed our biochemical results.

Conclusion

Overall, our study found that NAC might recover liver injury induced by CCL_4 by obstructing hepatic oxidative stress and inflammation. Based on antioxidant and anti-inflammatory influence of NAC, its oral administration has attained a good clinical outcome.

Conflict of interest

The authors haven't conflict of interest to declare.

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