The Effect of N-acetylcysteine on Pyrrolized Protein, Lipid Hydroperoxide and Thiol Levels in the Carbon Tetrachloride Hepatotoxicity

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This study was performed, on the rat model, to form oxidative stress by carbon tetrachloride and to reveal the relationship between toxicity and oxidative/thiol stresses through lipid/protein oxidation and to investigate the effects of N-acetylcysteine on the hepatotoxicity induced oxidative stress. Wistar albino male rats were divided into four groups as carbon tetrachloride, N-acetylcysteine, carbon tetrachloride-N-acetylcysteine and control, each of ten rats. Carbon tetrachloride (1.0 ml, 800 mg/kg; single dose) and N-acetylcysteine (200 mg; three doses) were intraperitoneally applied to corresponding groups as per kg of rat weight. Hepatotoxicity was identified with histopathological methods. Besides the thiol levels in plasma/tissue samples, pyrrolized protein and total lipid hydroperoxide levels in serum samples were measured. Carbon tetrachloride-induced hepatotoxic lesions remarkably improved in the presence of N-acetylcysteine. There was no significant difference between control and N-acetylcysteine groups, in terms of parameters measured in serum/plasma and tissue samples. When compared to these groups, pyrrolized protein and total lipid hydroperoxide levels were found to be higher; whereas plasma/tissue thiol levels lower in the carbon tetrachloride group. N-acetylcysteine in combination with carbon tetrachloride significantly lowered pyrrolized protein and total lipid hydroperoxide levels, and increased thiol levels, so that the values were reached to those of control and N-acetylcysteine groups. As reflected by higher pyrrolized protein and total lipid hydroperoxide, and lower thiol levels, enhanced free radical production in the carbon tetrachloride hepatotoxicity may lead to oxidative and thiol stresses mediated lipid/protein oxidation. In addition, N-acetylcysteine, a powerful antioxidant, may be added as a thiol source to the treatment protocols of several diseases whose pathogenesis is of oxidative and thiol stress.

Key words: Carbon tetrachloride, N-acetylcysteine, protein oxidation, pyrrolized protein, rat

Oxidative stress (OS), defined as an imbalance of oxidant/antioxidant systems depending on increased production of reactive oxygen species (ROS) or insufficient antioxidant mechanisms, is reflected as the accumulation of toxic compounds in cells and tissues, interacton of ROS with nucleic acids, polysaccharides, lipid and proteins; and ROS dependent changes are observed in cellular functions. OS also plays an important role in inflammation; and arises in many pathological incidents such as cancer, diabetes, liver diseases, cardiovascular and pulmonary diseases[1].

Because of their relative early formation, greater stability and reliability, and their longer life span, the usage of protein oxidation products have become widespread as OS markers, in the place of lipid peroxidation products, such as malondialdehyde (MDA), which have shorter half-life, low specificity, sensitivity and stability[2,3]. For instance, pyrrolized protein (PP), formed widely in the reaction of protein amino groups with lipid peroxidation products such as MDA, lipid hydroperoxide (LHP), epoxyl- and hydroxyl-alkenals, has been proposed as can be used as a marker in demonstrating the exposure to OS[4,5].

Otherwise, the nature of ROS is also important. For instance, hypochlorous acid (HOCl), induces mainly oxidation of proteins but causes little modification of DNA/lipids[2]. HOCl has also been suggested in particularly oxidizing the thiol groups, and therefore, selectively chooses plasma albumin as a target, and thiols are also known to selectively capture HOCl/chloramines[6]. Therefore, the oxidation of thiol groups may be considered as the indicator of protein oxidation. Since thiol loss due to OS is called thiol stress[7], it will reflect both the loss of antioxidant power and the degree of protein oxidation.

In recent years, the usage of antioxidant agents became prevalent to strengthen the endogenous defense system for diseases that are thought to have OS in their pathogenesis[2,3]. N-acetylcysteine (NAC), known as the precursor of reduced glutathione (GSH), and is used for years as a mucolytic agent against pulmonary diseases[8], has been subject to clinical[9] and experimental[10-12] studies. NAC has been reported to be effective in the treatment of various cancer types, hemodialysis and cardiac patients, that OS is involved in their pathophysiology[8].

In the studies on toxicology, OS is discussed as a possible mechanism of toxicity. While OS can be generated by many agents in animal experiments,

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carbon tetrachloride (CCl_4) (Chemical Abstracts Service No. 56-23-5) is regarded as a model which best characterizes xenobiotic-mediated-hepatotoxicity, as its toxicity is similar to the development of cirrhosis[13]. The hepatotoxicity that CCl_4 generates in many species including rats, mice, rabbits and humans allows monitoring of oxidative damage processes[13] as well as may reveal the potential benefits of antioxidants such as NAC[10-12] and melatonin[12], suggested as hepatoprotective agents[14].

This study on the rat model was conducted to investigate the presence of lipid/protein oxidation in rats which generated ROS-mediated hepatotoxicity induced by CCl_4 and, by this way, to reveal the relationship of toxicity with oxidative and thiol stress, and further to research the effects of NAC, which may be a thiol source of organism, on the OS due to hepatotoxicity.

MATERIALS AND METHODS

A total of 40 male Wistar albino rats, weighing 250-300 g, grown at Erciyes University Hakan Çetinsaya Experimental and Clinical Research Center, were included in the study. The rats, acclimatized for one week before the experiments, were kept on the 12-hour light/dark cycle and at normal room temperature (22±1°) and moisture during the study. The rats, kept in standard wire cages and fed *ad libitum* with standard pellet chow and tap water, were randomly divided into four groups of 10 rats each to form the control, the CCl_4, the NAC and the CCl_4–NAC groups.

Study design:

Experimental design of the study, of which the protocol was approved by Erciyes University Experimental Animal Ethics Committee (No: 08/68;12.11.2008), was given in Table 1. The rats were weighed prior to the study, and the doses of CCl_4 (319961-Sigma-Aldrich; 99.9%, d: 1.594 g/ml) and/or NAC (112422; Merck) were adjusted to be applied as per kilogram (kg) of rat weight intraperitoneally (ip). Olive oil and/or normal saline (NS; 0.9% NaCl) were used as solvents. In the ratio of 1/1, the mixtures of NS/olive oil and CCl_4/olive oil were administered to the rats in the groups of control and CCl_4, respectively. In NAC group, 200 mg of NAC in 0.5 ml of NS, and also 0.5 ml of solvent mixture (NS/olive oil) were injected to the rats for three consecutive days. Following NAC injection, CCl_4-olive oil mixture was administered after exactly 30 min in CCl_4–NAC group, and NAC injection was repeated for two consecutive days. Care was taken to make daily applications at the same time (9:00-10:00 am).

At the end of the treatments, rats were fasted overnight with free access to water. Blood was taken from the abdominal aorta under anesthesia, by i.p. 80 mg of ketamine HCl (Ketalar®, 50 mg/ml, Pfizer) and 10 mg of xylazine HCl (Rompun®, 20 mg/ml, Bayer), per kg of rat weight. The livers were removed immediately and washed out three times with ice-cold NS. For histological analysis, a portion of liver tissues was fixed in 10% formalin for 24 h; the rest was kept frozen at -70° for the measurement of thiol levels until study day. Heparinized/flat blood tubes were kept at 4° for 30 min and then separated by centrifugation at 2000×g at 4° for 10 min. Obtained plasma/serum samples were kept, in aliquots, at -70° until the determinations of PP, total LHP and thiol levels.

Histopathological analysis:

Monitoring of liver samples was performed with a tissue tracking device (Leica ASP300; D-69226 Nussloch/Germany), in the Pathology Department. Tissue sections of 5 µm thick were taken from paraffin blocks by microtome. The sections were stained with Hematoxylin-Eosin and evaluated under light microscope.

In histopathological examination; the hepatocytes, which have centrally located round nuclei containing prominent 1-3 nucleoli, and that extends from central vein to portal areas as columns, were accepted as "normal". Liver damage was determined by observation of the microscopic findings such as steatosis, inflammation and necrosis[15]. Tissue

TABLE 1: THE EXPERIMENTAL DESIGN OF THE RATS IN THE STUDY GROUPS

GROUPS	Applied Agent	Applied Dose	Application	
			Route	Duration (days)
Control	Solvent mixture (1/1 v/v)	1.0 ml*	i.p.	1 (single dose)
CCl_4	CCl_4-olive oil mixture (1/1 v/v)	1.0 ml*	i.p.	1 (single dose)
NAC	NAC/solvent mixture	200 mg /1.0 ml•	i.p.	3
CCl_4-NAC	CCl_4 /olive oil mixture	1.0 ml*	i.p.	1 (single dose)
	NAC/solvent mixture	200 mg /1.0 ml		3

NS: Normal Saline (0.9% NaCl); i.p.: intraperitoneally, *: ml of mixture/kg rat weight/day. •: mg of the agent/ml of solvent mixture/kg rat weight/day samples were evaluated according to the definitions and scoring, as shown in Table 2.

Biochemical analysis:

Serum PP levels were determined using Ehrlich's reagent [p-(dimethylamino) benzaldehyde; DMAB] by the method of Hidalgo *et al.*[4], modified by Martinez-Cruz *et al*[5]. The principle was based on the measurement of color intensity of Ehrlich adducts at 570 nm, resulted from the interaction of pyrroles on proteins and DMAB under high temperature and acidic conditions. The evaluation was made by extinction coefficient of 35.000 $M^{-1}cm^{-1}$ of ε-N-pyrrolylnorleucine as standard, and PP values were expressed in nano moles per mg protein determined at 280 nm (n mol/mg protein)[5].

Total plasma LHP levels were assayed by the method of Nourooz-Zadeh[16], ferrous ion oxidation in xylenol orange (FOX) assay. The method is based on the oxidation of ferrous ions to ferric ions, by peroxides under acidic conditions, complexed by the xylenol orange (ferric ion indicator), generating a blue-purple complex with an absorbance at 550 nm. Total LHP levels were calculated using extinction coefficient of 4.31×10^4 $M^{-1}cm^{-1}$ of "Fe^{3+}.XO" complex and given as micromoles per liter (µmol/l).

Thiols were determined by the methods of Hu *et al*[6] in plasma and Forster *et al*[17] in tissue samples. The principle was based on the measurement of dark yellow color intensity of 5-thio-2-nitrobenzoic acid (TNB) formed by depending on the reduction of Ellman's reagent [5,5′-dithiobis (2-nitrobenzoic acid); (DTNB)] by free thiol groups, at 412 nm. During measurement, plasma samples were used directly[6]. Thawed liver samples, washed with colder NS, were weighed 0.1 grams and homogenized by 2.0 ml of 0.02 M EDTA, and used directly in thiol assay on the same day[17]. The evaluation was performed using a standard GSH curve. Thiols were indicated as µmol/l and nano moles per mg protein determined at 280 nm (nmol/mg protein), in plasma and tissue samples, respectively. Variation coefficient of the methods of PP, total LHP and plasma/tissue thiols was found as 3.36, 3.11 and 1.36/5.15%, respectively.

Statistical analysis:

The results were evaluated using a statistical computer package (SPSS, version 15 for Windows, Chicago, IL, USA). Data normality was assessed with Shapiro–Wilk test. As the tissue thiol, PP and histopathological scoring values did not conform to a normal distribution; Kruskal-Wallis test was used for between-group comparisons. Binary comparisons were performed using Mann-Whitney U test and evaluated by Bonferroni correction. The significance level was considered as $P<0.0125$. Total plasma LHP and thiols showing a normal distribution, were compared by analysis of variance (ANOVA). Homogeneity of variances was assessed by Levene test. Binary comparisons were made with Tukey post-hoc test. Differences were considered significant at $P<0.05$. Data were expressed as mean±SD, or median and minimum (min) – maximum (max) ranges (by grey zone), at the tables.

RESULTS

Following the NAC and/or the CCl_4 applications, when microscopic changes such as inflammation, steatosis and necrosis in liver sections were graded according to the Table 2 as "none (0), mild (1), medium (2) and severe (3)"; although no pathological findings in the control and NAC groups, the data shown in Table 3 were obtained in the rats of CCl_4 and CCl_4-NAC groups. When compared to the control and NAC groups, the signs of the steatosis, inflammation and necrosis observed in the CCl_4 group were found to be statistically significant ($P<0.05$). On the other hand, according to the CCl_4 group, CCl_4-induced steatosis in the CCl_4-NAC group was determined to improve and statistically at the level of the control and NAC groups ($P>0.05$). The inflammation and the necrosis caused by CCl_4 also significantly improved with concomitant administration of NAC and CCl_4 ($P<0.05$); but could not reach the levels of Control and NAC groups ($P<0.05$). When these findings taken together; hepatotoxicity occurred in CCl_4 group ($P<0.05$), hepatotoxic lesions were improved in the presence of NAC ($P<0.05$); but the degree of improvement was not identified in the levels of the control and NAC groups ($P<0.05$, Table 3).

PP and total LHP levels of study groups were given in Table 4. PP and total LHP were found to be statistically the same levels in the control and NAC groups ($P>0.05$). When compared to these groups,

TABLE 2: SCORING FOR HISTOPATHOLOGICAL EVALUATION OF STUDY GROUPS

	None	Mild	Moderate	Severe
Inflammation	0	1	2	3
Steatosis (parenchymal involvement) (%)	0	1 (0–33)	2 (33–66)	3 (66–100)
Necrosis	0	1 (zone 3, focal)	2 (zone 3, confluent)	3 (massive)

TABLE 3: THE EFFECTS OF CCL_4 AND/OR NAC COMBINATIONS ON THE LIVER TISSUE

	Histopathological Findings							
	Steatosis		Inflammation		Necrosis		Total score	
GROUPS	MeanSD	Median (min-max)	Mean±SD	Median (min-max)	Mean±SD	Median (min-max)	Mean±SD	Median (min-max)
Control	0	0	0	0	0	0	0	0
NAC	0	0	0	0	0	0	0	0
CCl_4	1.5±0.71	1* (1-3)	2.60.52	3* (2-3)	2.7±0.48	3* (2-3)	6.81.1	7* (5-9)
CCl_4-NAC	0▪	0	1.0±0.82•,▪	1 (0-2)	1.2±0.92	1.5•,▪ (0-2)	2.2±1.7	2.5•,▪ (0-4)

10 rats were in each group. Data presented as mean±SD and median (min-max; grey zone). Significant findings were obtained, when compared with Control and NAC groups *(p=0.001); •(p=0.007), and also CCl_4 group ▪(p=0.001).

both higher PP and total LHP were determined in the CCl_4 group (P<0.05). Increased PP and total LHP levels induced by CCl_4 were statistically decreased in the presence of NAC (P<0.05), and reached to those of the control and NAC groups (P>0.05).

Plasma and tissue thiol levels of study groups were presented in Table 5. When compared to the control, plasma/tissue levels of thiol showed significantly increased in the NAC group (P<0.05). The decreases in plasma/tissue thiol levels were found to be statistically significant in CCl_4 group, according to the control and NAC groups (P<0.05).

On the other hand, concomitant administration of NAC and CCl_4 significantly increased thiol levels according to the CCl_4 group (P<0.05), and the increases in liver tissues statistically reached to the values of the control and NAC groups (P>0.05). However, the increase in plasma thiol levels in CCl_4-NAC group was found to be lower than that of only the NAC group (p<0.05, Table 5).

DISCUSSION

The importance of clinical and experimental studies on OS, and on strengthening endogenous defense system, is gradually increasing. Experimentally generated liver damage by CCl_4, easily provided as pure substance, is similar to cirrhosis development in humans; and further, the modification of hepatotoxicity is also possible by changing CCl_4 dose and/or administration period[13]. CCl_4-induced hepatotoxicity in rats has been shown to be a well standardized experimental model which has high reproducibility.

According to Lu *et al*[18], steatosis, necrosis, inflammation and ballooning, and massive necrosis in zone 3 where cytochrome P_{450} system is dense, are expected consequences of CCl_4 hepatotoxicity. Parallel to hepatic necrosis studies[13,18]; in the present study, steatosis, inflammation and necrosis were observed in liver of CCl_4-given rats; therefore, a good OS model was assumed to be generated with CCl_4-hepatotoxicity.

Several mechanisms supported by experimental studies are suggested to explain CCl_4-hepatotoxicity. The evidence for CCl_4-hepatotoxicity is the generation of ROS by cytochrome P_{450} system, where CCl_4 is metabolized[1]. CCl_4-derived radicals lead to cellular damage, either by binding to the membrane protein or lipid peroxidation; that is, CCl_4 causes fibrosis and cirrhosis[13]. Considering the facts that cytochrome P_{450} system produces superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2)[1], and that CCl_4 is metabolized via CYP2E1; CCl_4 may be thought to lead to ROS generation by this system. In addition, one of the findings of hepatotoxicity is inflammation[18,19], as also shown in the present study, neutrophil-mediated ROS formation, primarily HOCl[20], may contribute to CCl_4-hepatotoxicity.

When the literature, suggesting CCl_4 leads to OS, is evaluated; possibility that CCl_4 may lead to OS by stimulating ROS production is strengthened. The presence of CCl_4-mediated OS is proven by increased MDA[11,19]. In the present study, instead of MDA which has shorter half-life, low specificity, sensitivity/stability[2,3], LHP, the stable product[16], found higher in CCl_4-given rats, as shown previously[21]. Considering the histopathological findings on liver sections and high total LHP levels in the study, it may be concluded that peroxidation chain reactions may spread rapidly to an extent that leads to LHP formation in hepatocytes with CCl_4; that LHP, which can easily pass the membrane, may be in high levels in circulation, and that oxidative damage may occur in the presence of CCl_4.

Now a days, protein oxidation products as markers of OS are becoming increasingly common[2,3]. PP generation is an indicator of protein oxidation[4]. Lipid peroxidation products cause some changes on proteins defined as oxidized lipid/amino acid reaction products (OLAARP), including pyrroles. OLAARP formation is accepted as an ultimate step in the lipid

TABLE 4: SERUM PYRROLIZED PROTEIN AND TOTAL LHP LEVELS OF STUDY GROUPS

	Pyrrolized Protein (nmol/mg protein)		Total LHP (µmol/l)	
GROUPS	Mean±SD	Median (min-max)	Mean ± SD	Median (min-max)
Control	1.27±0.28	1.24 (0.91-1.8)	9.51±1.79	9.92 (6.7-11.86)
NAC	1.07±0.20	1.09 (0.67-1.35)	9.32±1.93	9.86 (6.45-11.86)
CCl_4	2.05±0.54	1.77*,n1 (1.57-3.13)	18.02±3.77*, n1	17. 01 (13.15-23.98)
CCl_4-NAC	1.23±0.31	1.20▪ (0.80-1.96)	12.61±2.65 n2,▪	12. 89 (8.51-16.76)

10 rats were in each group. Data presented as mean±SD and median (min-max; grey zone). Significant findings were obtained, when compared with the groups of Control *(p=0.001); NAC (n1: p=0.001; n2: p=0.042), and also CCl_4 group ▪(p=0.001)

TABLE 5: PLASMA AND LIVER TISSUE THIOL LEVELS OF STUDY GROUPS

	Plasma (µmol/L)		Tissue (nmol/mg protein)	
GROUPS	Mean±SD	Median (min-max)	Mean±SD	Median (min-max)
Control	218.8±49.7	205.1 (154.3-326.0)	96.09±27.57	96.4 (59.9-155.6)
NAC	314.0±54.1*	318.5 (251.0-434.3)	156.13±29.32	146.2* (130.1-231.3)
CCl_4	98.9±27.3*,•	97.6 (56.0-152.6)	38.01±9.25	40.4*,• (24.2-49.5)
CCl_4-NAC	189.6±54.4 •,▪	164.3 (132.6-281.0)	119.77±33.34	103.5▪ (91.3-176.8)

10 rats were in each group. Data presented as mean±SD and median (min-max; grey zone). Significant findings were obtained, when compared with Control *(p=0.001) and NAC •(p=0.001) groups and also CCl_4 group ▪(p=0.001)

peroxidation process, in the presence of proteins. Therefore, the detection of PP has been suggested to be used as a marker of OS[4,5].

Increased plasma PP was reported in atherosclerosis and renal failure[22]. In the present study, serum PP was found to be higher in CCl_4-treated rats and a similar study was not identified in the literature. Considering the findings of LHP and PP levels; high LHP may be an important parameter for determination of OS, since it indicates the presence of CCl_4-mediated lipid peroxidation and it directly participates in the formation of PP, and it is the precursor of carbonyl compounds that are to form modifications like OLAARP and PP.

Free thiols prevent oxidative damage of biomolecules by reacting with ROS and are consumed by oxidation. Therefore, thiol oxidation may also be considered as the marker of protein oxidation. Considering albumin, the primary thiol source in plasma is the main protein exposed to OS; the decrease in total thiol levels may be associated with consumption of thiol against oxidants produced under OS conditions. Furthermore, determination of thiols will reflect the loss of antioxidant power as well as the degree of protein oxidation. In accordance with studies showing low thiols with CCl_4-treated rats[19,23], plasma/tissue thiols were also low in the present study. These findings in CCl_4-treated rats may be presumed as important in demonstrating the presence of protein oxidation.

In the literature, NAC has been shown to be effective and safe in ‘‘non-acetaminophen’’ acute liver failure in a meta-analysis of prospective clinical trials[14]. Many experimental studies are also available using NAC on ROS-mediated hepatotoxicity induced by CCl_4[10,11,24], as it is the case in the present study**.** Experimental evidence indicates NAC is able to enhance the intracellular biosynthesis of GSH[9-12,24], and therefore NAC increases GSH levels and replenish GSH store[8].

Moreover, NAC is a powerful scavenger of HOCl, and also capable of directly scavenging ROS[25]. Maksimchik *et al.*[11] defined that NAC prevents the generation of mixed disulphides and lipid peroxidation while increasing low molecular weight thiol levels in liver cells of CCl_4-hepatoxicated rats. Although oxidative damage of proteins reflected by high levels of protein carbonyl compounds in CCl_4 hepatotoxicity has been shown to prevent in the presence of NAC[11,12]; the present study is the first and only study in the literature showing the generation of PP as an indicator of protein oxidation and also protective effect of NAC, in the same conditions.

In the present study, the therapeutic effect on the hepatotoxic findings such as steatosis, inflammation and necrosis; the decreases in PP and total LHP levels and increases in plasma/tissue thiol levels were observed by NAC administration in combination with CCl_4 to rats. Since the antioxidant effect of NAC depends on free thiol groups in its structure, NAC used in various pathological conditions will primarily support the tissue and plasma thiol content, and will provide to keep thiol at a certain level and even higher, rapidly consumed in OS circumstances.

Based on these findings, it may be said that the usage of NAC may be protective on CCl_4-hepatoxicity.

Consequently, it may be said that CCl_4 generates ROS-mediated hepatotoxicity in the rat model, depending on the findings of histopathology and also the levels of PP, total LHP and thiols, first determined together in this study. Moreover, NAC may protect cells against lipid peroxidation and protein oxidation, and helps maintain the integrity of cellular organelles, by acting as a GSH precursor and by enhancing intracellular concentrations of GSH. Therefore, NAC, has no serious side effects, may be added as a thiol source to the treatment protocols of several diseases whose pathogenesis is of oxidative and thiol stress.

FINANCIAL ASSISTANCE

This paper is supported by Research Fund of the Erciyes University (project number: TSD-09-660).

CONFLICT OF INTERESTS

None declared.

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Accepted 23 Apr 2016
Revised 12 Mar 2016
Received 30 Jun 2015
Indian J Pharm Sci 2016;78(2):267-272