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Original article

A Pilot Study on Effects of Concomitant Usage of Acetaminophen and N-Acetylcysteine to Prevent Possible Acetaminophen Toxicity

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This study was designed to evaluate the effects of acetaminophen (APAP) and N-acetylcysteine (NAC) in combination on the protective effect of NAC for possible APAP toxicity determining oxidative stress and inflammation parameters in a rat model. DMSO as vehicle, 50 mg/kg APAP and 50 mg/kg APAP plus 100 mg/kg NAC were administrated to Wistar Albino rats by intra-peritoneal for five days. Plasma TNF-α, IL-2 and IL-6 levels and level of malondialdehyde (MDA), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) activities in liver and kidney were analyzed. APAP administration produced an increase in hepatic and renal MDA levels and co-administration of APAP plus NAC resulted in decreases in the kidney MDA levels and an increase in hepatic and renal superoxide scavenging enzyme, SOD, activity. Taken together, the results of this study suggest that combination of APAP and NAC may be a valuable combination to protect hepatotoxicity and nephrotoxicity induced by APAP. Furthermore, pharmaceutical preparation with combination of APAP and NAC may be useful alternative to prevent from possible APAP related toxicity. However, further investigations are needed to understand the underlying protection mechanism of NAC combination with APAP.

Key words: Acetaminophen, N-acetylcysteine, Oxidative stress, Inflammation, Liver, Kidney

Asetaminofen ve N-Asetilsistein'in Birlikte Kullanılmasının Olası Asetaminofen Toksisitesini Önlemedeki Etkisinin Belirlemesi İçin Pilot Bir Çalışma

Bu çalışma asetaminofen (APAP) ve N-asetilsistein (NAC) kombinasyonunun sıçan modelinde oksidatif stress ve inflammasyon parametreleri kullanılarak olası parasetamol toksisitesine karşı koruyucu etkisini değerlendirmek için tasarlanmıştır.Wistar sıçanlara intra-peritonel olarak 5 gün boyunca taşıyıcı olarak DMSO, 50 mg/kg APAP ve 50 mg/kg APAP ve 100 mg/kg NAC kombinasyonu uygulanmıştır. TNF-α, IL-2 ve IL-6 düzeyleri plazmada ve malondialdehit (MDA) düzeyleri, glutatyonperoksidaz (GSH-Px), katalaz (CAT) ve süperoksitdismutaz (SOD) aktiviteleri karaciğer ve böbrekte analiz edilmiştir. APAP uygulaması karaciğer ve böbrekte MDA düzeylerini arttırmış ve NAC ile kombinasyonu böbrek MDA düzeylerinde azalmaya ve karaciğer ve böbrek süperoksit süpürücü enzim, SOD aktivitesinde artışa neden olmuştur.Birlikte ele alındığında, bu çalışmanın sonuçları APAP ve NAC kombinasyonunun APAP ile indüklenen hepatotoksisite ve nefrotoksisiteyi önlemek için iyi bir kombinasyon olabileceğini göstermiştir. Ayrıca, APAP ve NAC kombinasyonu olan farmasötik preparatların muhtemel APAP toksisitesini engellemede iyi bir alternative olabilceği düşünülmüştür. Ancak NAC'ın APAP ile kombinasyonunun koruyucu mekanizmasının anlaşılması için ileri/ek çalışmalara ihtiyaç olduğu değerlendirilmiştir.

Anahtar kelimeler: Asetaminofen, N-asetilsistein, Oksidatifstres, İnflamasyon, Karaciğer, Böbrek

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INTRODUCTION

Acetaminophen (APAP) is a worldwide used analgesic and antipyretic drugand it is safe enough when administrated at therapeutic doses. However, ingestion of overdoses result in increased reactive toxic metabolite N-acetyl-p-benzoquinoneimine (NAPQI) and thereby, formation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and subsequently lipid peroxidation (LPO) and protein oxidationincrease and finally loss of cellular functions and cellular death (1). Furthermore, recent studies showed that hepatotoxicity induced by APAP cause inflammatory response and release of cytokines. Activated kupffer cells play a key role in the APAP dependent liver injury and stimulate production of proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (2).While TNF-α and IL-6 induce acute-phase responses by activating hepatocytes, IL-2 induce chronic inflammation (3).

Since increased NAPQI formation first depletes glutathione stores, N-acetylcysteine (NAC) therapy as a precursor of reduced glutathione is still the best therapeutic option for the APAP overdosesince1970s (4). In addition, NAC also decreases the synthesis and release of hepatic pro-inflammatory cytokines such as TNF- α and IL-6 (5). NAC is most effective when given as early as possible after APAP intoxication (4). But there is no enough report regarding the effects of NAC on APAP toxicity when administrated in combination with APAP. Although APAP is normally metabolized in the liver and kidney, liver is the primary while kidney the second target organ of APAP toxicity (1).The aim of this study was to investigate the protective effect of NAC on APAP related induced oxidative stress on rat liver and kidney when concomitant usage. In addition the effects of NAC plus APAP combination on cytokine levels were evaluated in this study.

EXPERIMENTAL

Materials

Chemicals

1,1,3,3- tetromethoxypropane, iodonitro tetrazolium chloride (INT), bovine serum albumin (BSA), ethylene diamine tetra acetic acid (EDTA) disodium dihydride, N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), xanthine oxidase (XO), xanthine sodium, glutathione reductase, trizma base, 2,4,6-tribromoanisol (TBA), reduced GSH, sodium azide, tert-butyl hydroperoxide were obtained from Sigma (St. Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) and sodium dodecyl sulphate (SDS) were obtained from Merck (Darmstadt, Germany). Disodium phosphate (Na_2HPO_4) was obtained from Fluka (Switzerland). Acetic acid (CH_3COOH) was obtained from J.T. Baker (Netherlands). Sodium hydroxide (NaOH), sodium potassium tartrate ($C_4H_4KNaO_4.4H_2O$), copper sulfate ($CuSO_4$), sodium carbonate (Na_2CO_3), hydrogen peroxide (H_2O_2), trichloroaceticasit(TCA) were obtained from Riedel-De-Haen (Germany); APAP was obtained from Mallinckrodat (Ireland); N-acetylcytseine was obtained from Moehs (Spain) and dimethyl sulfoxide (DMSO) was obtained from Alfa Aesar (Germany).

Animals and experimental design

All procedures involving the use of laboratory animals were reviewed and approved by the Animal Ethics Committee of Yeditepe University. Twenty Female Wistar Albino ratsaged 6 weeks (275 g to 350 g body weight) obtained from Yeditepe University Medical School Experimental Research Center (YUDETAM). The rats were housed in cages under standard hygienic conditions, with light and dark cycles exchanging every 12 h. They received standard rat feed and had free access to water.

Rats were randomly divided into 4 groups (5 rats/group) and administered intra-peritoneal %50 DMSO as vehicle (control group), 50 mg/kg acetaminophen (APAP group), 50 mg/kg acetaminophen and 100 mg/kg NAC (APAP plus NAC group) for 5 days. The APAP doses were identified according to

previous study conducted by Manimaran et al (1).

The rats were observed daily for gross clinical signs and symptoms of toxicity and mortality. Body weights of rats were recorded daily. The animals were sacrificed 24 h after the last administration. At the end of the experiment, blood samples were collected into the tubes containing lithium heparin and liver and kidney were dissected and frozen immediately on dry ice. All samples werestored at -80° C until analysis.

Analysis of oxidative stress parameters
TissueSamples

Tissue samples (1 g) were homogenized in 9 mL of cold KCl solution (1.15 %) and centrifuged at 4,400 rpm for 10 minutes +4 °C and the supernatant was stored at -80 °C until assay. The supernatants were then used to analyze MDA, SOD, GSH-Px and CAT as described below:

Determination of total protein content

Protein concentration of samples was measured by the method described by Lowry et al (6). Briefly, there are three main reagents as follows: Reagent A (2% Na_2CO_3 in 0.10 N NaOH), Reagent B (0.5 % $CuS0_4$ in distillated water) and Reagent C (1% NaKtartrate in distillated water). 50 mL of Reagent A, 0.5 mL Reagent B and 1 mL Reagent C were mixed to obtain alkaline copper reagent. Folin-Ciocalteu phenol reagent (1:2 v/v) was diluted with distillated water to prepare Folin's reagent. Working standards were prepared with BSA in concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 mg/mL. For protein content measurement, 10 µL of BSA or sample, 1000 µL distillated water and 4500 µL alkaline copper reagent mixed and kept at room temperature for 10 minutes. At the end of this process, 500 µL Folin's Reagent was added and incubatedat room temperature for 20 minutes. Optical density of standards and samples were measured at 640 nm by spectrophotometer (Thermo Scientific, Evolution 300, USA).

Determination of SOD activity

SOD activities in supernatant of tissue homogenates were measured using the method described by Celepet al (7). 50 µL of supernatant was mixed with 1700 µL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L INT in a buffer solution containing 50 mmol/L CAPS and 0.094 mmol/LEDTA (pH 10.2). 250 µLof xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 minutes. SOD activity was expressed in U/mg protein.

GSH-Px activity measurement

GSH-Px activities in supernatant were measured by using the mixture of 1 mmol/L Na_2EDTA, 2 mmol/L reduced glutathione, 0.2 mmol/L NADPH, 4 mmol/L sodium azide and 1000 U glutathione reductase in 50 mmol/L tris buffer (pH 7.6) (8). Ten µL of the supernatant and 990 µL of the reaction mixture were mixed and incubated at 37 °C for 5 min. The reaction was initiated by adding 10 µL tert-butyl hydroperoxide and a decrease in absorbance was recorded at 340 nm for 3 min. Absorbance of each sample was measured at every 30 seconds. GSH-Px activity was expressed in U/g protein.

Measurement of MDA levels

Lipid peroxidation was determined by using tetrametoxy propane solution as standard (8). Briefly, the reaction mixture containing 200 µL of each sample, 0.2 mL SDS (8.1%), 1.5 mL acetic acid (20%), 1.5 mL TBA (0.8%) and 0.6 mL doubly distilled deionized water was prepared and incubated at 95°C on water bath for 1 hour. Then, they were cooled under tap water and 2 mL of the mixture was added to 2 mL of TCA. This assay is based on the formation of red adduct in acidic medium between TBA and MDA. The product of lipid peroxidation was measured at 532 nm.MDA level was expressed in nmol/g protein.

CAT activity measurement

50 mM phosphate buffer pH 7.0, 30 mM H_2O_2 and tissue homogenate were mixed and the reduction rate of H_2O_2 was followed at 240 nm for 4 times at every 15 s at room temperature. Catalase activity was expressed in nmol/g protein (9).

Determination of IL-2, IL-6 and TNF-α levels

The plasma IL-2, IL6 or TNF-α levels were measured by using a commercially available

quantitative enzyme-linked immunosorbent assay (ELISA) system according to manufacturer's instructions (eBioscience high performance immune assay, Vienna, Austria).

Statistical analysis

All results were expressed as the mean ± standard deviation (SD). The differences between the groups were evaluated with Kruskal-Wallis analysis of variance and comparisons between two independent groups were made with the Mann-Whitney *U*-test. $p<0.05$ was considered statistically significant.

RESULTS

Effects on MDA

As seen in Figure 1, liver MDA levels of APAP (56.12 ± 7.60 nmol/g protein) and APAP plus NAC (72.72 ± 18.00 nmol/g protein) groups were found to be higher than control group (38.95± 10.18 nmol/g protein) ($p< 0.05$). MDA levels of APAP were slightly lower than APAP plus NAC.

Kidney MDA levels were increased in both of groups treated with APAP (111.83± 21.79 nmol/g protein) and APAP plus NAC (96.16 ±11.35 nmol/g protein) (Figure 2).

Effects on SOD activity

Liver SOD levels of rats treated with APAP (177.99±149.31U/mg protein) and APAP plus NAC group (269.55 ±132.99U/mg protein) showed significant increase compared to control group (29.49 ± 13.77 U/mg protein) ($p<0.05$) (Figure 1).

Significant increase of renal SOD activity were also observed on APAP (182.74±64.48 U/mg protein) and APAP plus NAC (298.41±92.65 U/mg protein) as compared with control (64.39±35.21 U/mg protein) ($p<0.05$) (Figure 2).

Effects on GSH-Px activity

As shown in the Figure 1, the liver GSH-Px activities of control (2982.33 ± 607.56 U/g protein) were higher than APAP (2825.84 ± 631.24 U/g protein) and APAP plus NAC (2843.09±523.07 U/g protein).

Kidney GSH-Px activity of APAP plus NAC group was 2689.16 ± 436.77 U/g proteins and it was higher compared to control (2587.37 ± 1182.61) and APAP (2226.85 ± 684.58) groups, but not significant differences.

Effects on CAT activity

The administration of APAP alone resulted in an elevation of liver CAT activity (28.51 ±13.95 U/g protein) as compared to control (15.89± 7.33 U/g protein). CAT activity in liver was reduced in APAP plus NAC group (23.00 ± 5.49 U/g protein) as compared with APAP. CAT activity in kidney decreased in APAP group (9.79 ± 4.81 U/g protein) when compared with control (11.07 ± 3.10 U/g protein) and APAP plus NAC groups (14.71 ± 6.96 U/g protein). However, there were no significant differences among all groups in terms of liver as well as kidney.

IL-2, IL-6 and TNF-α levels in rat plasma

We found that plasma TNF-α levels of APAP and APAP plus NAC groups were markedly higher compared to control ($p<0.05$) (Table 1). In the APAP plus NAC group, plasma TNF-α level was slightly higher than APAP but there were no significant differences among groups.

DISCUSSION

We investigated the protective effect of NAC on APAP related induced oxidative stress on rat liver and kidney when concomitant usage. The maximum APAP dose for adult was reported to be 4000 mg/day which is equivalent to 420 mg/kg for rats (1). The APAP doses used for this study were below the maximum dose for rats and thereby were in therapeutic range approximately.

Previous studies showed that lipid peroxidation (LPO) is closely related to APAP-induced toxicity and MDA, the end product of LPO, was reported to be increased in the liver tissue following APAP toxicities (10). In accordance with this study, MDA levels in the APAP were found significantly higher than control group, indicating oxidative damage in liver tissue even at therapeutic dose. On the other hand, NAC did not show protective effect against oxidative damage in liver in combination with APAP.

The enzymatic antioxidant defense system is composed of direct acting proteins such as SOD, CAT and GSH-Px (10, 11). As superoxide scavenging enzyme, SOD is consider as a first line protection against free radicals. CAT is extensively involved in the degradation of H_2O_2 resulted from peroxidation of superoxide radicals by SOD. Also GPx catalyzes the reduction of H_2O_2 at the expense of GSH (11). According to our result, liver SOD activity was increased significantly in APAP and APAP plus NAC treated groups compared to control. This may be explained by the adaptive response of liver to the increased generation of free radicals during APAP exposure (12). Although no statistically significant differences were observed between APAP and APAP plus NAC groups, the higher SOD levels in APAP plus NAC may indicate the protective role of NAC against to oxidative stress.

According to our results, no significant differences were found in CAT activity between groups in liver tissue. However, slightly increase in CAT activity was observed in APAP treated group comparing to all other groups. This up-regulation in CAT activity may be due to the high levels of H_2O_2 formation following APAP administration without NAC.

protective agent against tissue injury by conjugation of the sulfhydryl part of glutathione with electrophilic and highly reactive NAPQI but detoxification is limited because of insufficient glutathione stores (13). It has been reported that glutathione production is a cycle and GSH-Px enzyme is a critical constituent of this cycle (14). Kuvandik et al. reported that depletion of liver GSH-Px activity showed APAP-toxicity in treated rats (13). Although no significant differences were found in GSH-Px activity of liver among the groups in this study, slight decrease in GSH-Px activity was observed after APAP administration and NAC seems to prevent the depletion of GSH-Px.

In the kidney, MDA levels of APAP treated group were higher than other groups. However, this increase was not found statistically significant. This observation indicates the oxidative damage resulted from APAP administration. Although slightly increase in MDA levels of APAP plus NAC group were observed versus to control, decreased levels of MDA comparing to APAP group suggesting the protective effect of concomitant administration of NAC with APAP against oxidative damage. This result is in accordance with the study conducted by Ucar et al. Authors reported that APAP

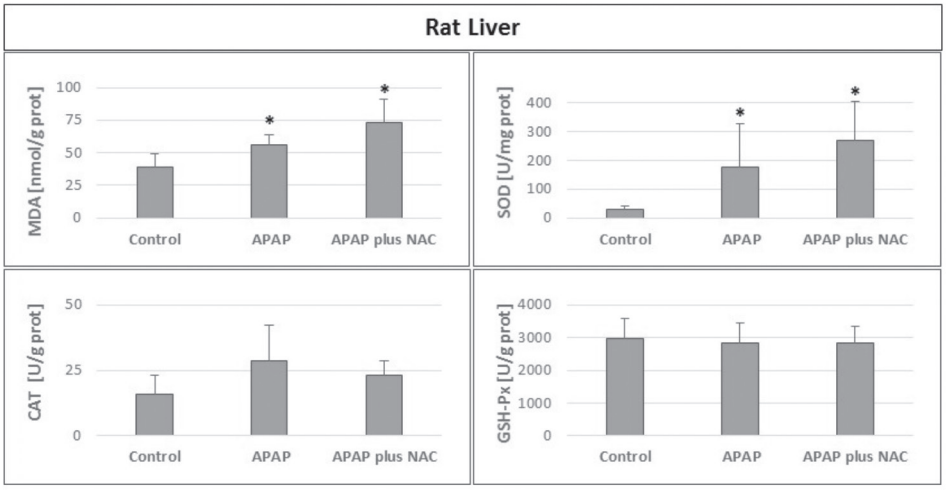

Figure 1. Effects of control, APAP and APAP plus NAC on MDA, GSH-Px, SOD and CAT activities in rat liver tissue. Data are means ±SD, n=5. $^{*}p<0.05$ compared with control.

Previous studies demonstrated that high doses of APAP cause formation of increased level of NAPQI. Glutathione is an important

induced renal injury in rat model and treatment with NAC results in decreased renal MDA levels (15).

In the term of renal SOD activity, statistically significant increase was observed in APAP group. This increase suggests that reactive metabolite was formed due to APAP toxicity and cells became activated to up regulate the SOD levels as a protective measure. In APAP plus NAC group, SOD activity decreased versus APAP alone group but it was also significantly higher than control. It can be concluded that NAC added an antioxidative potential to cells.

In a previous study, the decreased activities of renal GSH-Px suggested that APAP alone was mediated renal damage through oxidative stress in rats (1). In contrast to that study, we could not observe significant changes at kidney GSH-Px activities after APAP administration. However APAP plus NAC group has higher GSH-Px activity, but this increase was not significant.With our results, NAC seems to induce GSH formation and prevent the depletion of GSH-Px in APAP toxicity.

The pro-inflammatory cytokines such as TNF-α, IL-6 andinflammatory cytokine likeIL-2 are produced by macrophages /monocytes. According to the severity of the inflammation, levels of cytokines- TNF-α, IL-2 and IL-6- can be increased in the liver and kidney. In some studies have shown that APAP treatment led to increased levels of TNF-α and IL-6 and thereby inflammation in the liver and kidney.In addition, decreased levels were seen in the NAC treatment (5, 15, 16). As expected that in our study, formation of TNF-α was significantly higher in APAP group than control. However, combination of APAP plus NAC was not decreased the level of TNF-α in plasma. Effectiveness of NAC seems to be not enough to decrease in APAP related inflammation. Ucar et al reported that serum IL-6 levels were not significantly altered in the APAP-treated rats compared to the control group (15). Similarly, in the current study, any differences were not observed in IL-6 level among the groups.

Table1. The levels of IL2, IL6 and TNF-in plasma of control, APAP, APAP plus NAC and NAC groups.

Groups	**IL2 (pg/mL)**	**IL6 (pg/mL)**	**TNF-a (pg/mL)**
	Mean ± SD		
Control	255.61 ±74.27	198.40 ± 2.31	149.70 ± 11.82
APAP	220.83 ± 24.45	203.60 ± 8.32	170.80 ± 13.89*
APAP plus NAC	276.83 ± 67.41	210.80 ±9.10	173.60 ± 9.32*
NAC	239.50 ± 43.92	207.60 ± 8.67	164.80 ± 9.63

p<0.05 versus control.

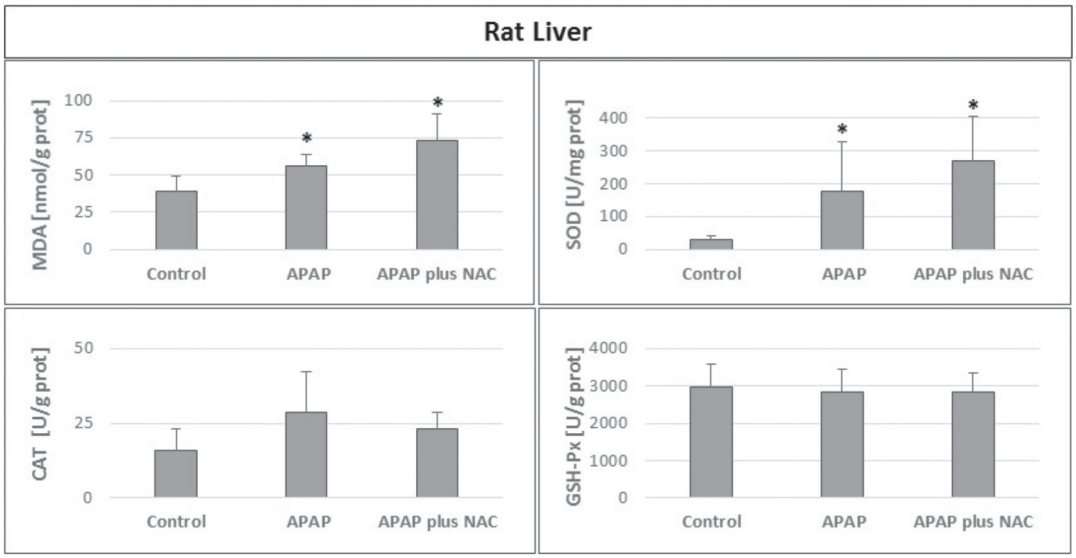

Figure 2. Effects of control, APAP and APAP plus NAC on MDA, GSH-Px, SOD and CAT activities in rat kidney tissue. Data are means ±SD (two replicates in each assay), n=5. *p<0.05 compared with control.

CONCLUSION

The physiopathology mechanisms of nephrotoxicity and hepatotoxicity caused by APAP are complex and may include oxidative stress and inflammation.

The findings of our study have shown that the combination of APAP and NAC may protect the liver and kidney from hepatotoxicity and nephrotoxicity caused by APAP in a rat model. Consequently, pharmaceutical preparation with combination of APAP and NAC may be useful alternative to prevent from possible APAP related toxicity. However, further investigations are needed to demonstrate the exact protection mechanism of APAP plus NAC combination in the liver and kidney.

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