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Effects of Concomitant Use of N-acetylcysteine and Cyclosporine A on Acetaminophen-induced Acute Kidney Injury in Mice

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ABSTRACT

Background: Acetaminophen (APAP), a commonly used analgesic, causes acute kidney injury (AKI) in overdose although it is rare. Mitochondrial dysfunction plays a major role in the pathophysiology of renal damage, although the exact molecular mechanism is unknown. This study aimed to evaluate the potential therapeutic effect of cyclosporin A (CsA), a mitochondrial membrane permeability transition pore (MPTP) inhibitor, with N-acetylcysteine (NAC) in APAP-induced AKI.

Methods: Male BALB/c mice were divided into Control, APAP, APAP+NAC, APAP+CsA and APAP+NAC+CsA groups (n=6). A single dose of APAP (400 mg/kg) was administered intraperitoneally. All other treatments (1200 mg/kg NAC, 50 mg/kg CsA) were performed intraperitoneally 3h after APAP administration. All animals were decapitated and blood samples and kidney tissue samples were collected for evaluation. Serum creatinine (Cr) and blood urea nitrogen (BUN) levels were measured. The kidney tissue 8-hydroxy-deoxyguanosine (8-OHdG), cytochrome c (Cytc) and 3-nitrotyrosine (3-NT) levels and cytochrome c (Cytc) expressions were determined.

Result: Increased Cr and BUN levels, histopathological examinations and expressions of 8-OHdG, 3-NT and Cytc were detected in the APAP group. Combined NAC+CsA treatment sufficiently reversed oxidative stress, serum Cr and BUN levels and histopathological alterations induced by APAP. Moreover, cytc levels and renal tubular injury were remarkably reduced by combined drug treatment compared to the APAP+NAC group. These data suggest that the therapeutic effect of combined NAC+CsA treatment in mice with APAP-induced nephrotoxicity can be related to the combination of the antioxidant effect of NAC and the mitochondrial MPTP inhibitor effect of CsA.

Key words: 3-nitrotyrosine, 8-Hydroxy-deoxyguanosine, Combined drug treatment, Cytochrome c, Mitochondrial dysfunction, Nephrotoxicity.

INTRODUCTION

Acetaminophen (APAP), one of the most commonly used analgesics, is safe at therapeutic dosages. However, its overdose can lead to acute liver failure (ALF) and/or acute kidney injury (AKI). Renal tubular damage and acute renal failure may occur in nearly 2% of patients (Ko *et al.,* 2017) and AKI can occur in patients with APAP overdose without liver failure. AKI is a syndrome associated with many etiologies and pathophysiologic processes leading to impaired kidney function (Akakpo *et al.,* 2020). Regarding this clinical condition, there are many case reports in the literature. Such that the hemodialysis requirement of the patients has indicated the seriousness of these cases (Jeffery and Lafferty, 1981; Blakely and McDonald, 1995; Mazer and Perrone, 2008; Waring, 2009; Saleem and Iftikhar, 2019). Although the precise mechanism of APAP-induced AKI is obscure, a wide variety of processes from oxidative stress to mitochondrial dysfunction may have a crucial role in the primary mechanism of AKI (Ruan *et al.,* 2019).

It is well known that oxidation of APAP in the liver led to a toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI) production, which is detoxified by hepatic glutathione (GSH). APAP overdose-mediated high NAPQI levels cause depletion of GSH, so reactive oxygen species (ROS) levels increase and oxidative stress occurs (Abdeen *et al.,* 2019). Besides, it has been reported that APAP overdose-related excessive NAPQI product is excreted by the kidney, so it

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can conduce to renal injury (Chen *et al.,* 2015). N-acetylcysteine (NAC), which is an amino acid that contains the thiol group, is a safe and cheap drug. NAC is a precursor of GSH, which is one of the most important antioxidants. Therefore, it is used for treating diseases induced by free oxygen radicals. Moreover, it has been used for the therapy of APAP-induced toxicity for a long time. It is universally effective in preventing hepatotoxicity if administered within 10 h of APAP overdose (Mokhtari *et al.,* 2017). Although the efficacy of NAC against APAP-induced hepatoxicity has been reported many times in experimental studies, some studies have shown that it cannot effectively attenuate kidney damage caused by APAP overdose (Mazer and Perrone, 2008; Akakpo *et al.,* 2020). Therefore, there is a need for additional drugs that can significantly improve kidney damage resulting from APAP overdose.

The kidney is a vital organ involved in the filtration of various substances and is particularly sensitive to drugs taken in toxic doses (Ansari *et al.,* 2020). In kidneys, which require high ATP in terms of function, the density of mitochondria is high, especially in the renal tubules. Moreover, it is well known that mitochondria play important roles in many cellular processes, such as signal transduction, cell proliferation, cell growth and cell death. Therefore, mitochondrial dysfunction or damage could lead to impaired ATP generation and ROS balance. On the other hand, most therapeutic drugs may cause AKI because of their negative effects on the mitochondrion of the renal tubule (Hua *et al.,* 2018). Moreover, mitochondrial damage through the opening of the mitochondrial membrane permeability transition pore (MPTP) has an important role in the mechanism of APAP-induced toxicity. Thus, mitochondria are a potential target for treating AKI. Cyclosporin A (CsA) is an immunosuppressive drug (Taylor *et al.,* 2005) that prevents the opening of MPTP by binding to cyclophilin D, which penetrates the inner and outer membranes of mitochondria (Kim *et al.,* 2003; Niimi *et al.,* 2012). The opening of MPTP plays a crucial role in the mitochondrial dysfunction-induced cell death mechanism. Thus, the inactivation of cyclophilin D using CsA can block MPTP and suppress APAP-induced mitochondrial damage (Niimi *et al.*, 2012).

In our previous study, it was revealed that hepatotoxicity occurred because of APAP overdose in mice and combined NAC+CsA treatment could be beneficial against hepatotoxicity (Kaya Tektemur *et al.,* 2021). However, considering that hepatotoxicity caused by high-dose APAP is rarely seen together with renal damage and because NAC treatment alone is not sufficient to improve, especially, APAP overdose-related renal injury, we aimed to evaluate the potential therapeutic effects of NAC+CsA combined treatment on APAP-induced nephrotoxicity in mice.

MATERIALS AND METHODS

Ethical statement

A total of 30 adult male BALB/c mice were used in this study. Mice were housed under optimal conditions (food, temperature, water, light) at the Firat University Animal Research Center (Elazig, Turkey). All experimental procedures were conducted according to the European Union guidelines (2010/63/EU) and ethical approval was obtained from the Firat University Animal Experiments Local Ethics Committee (Ethical number: 30.08.2021/3348).

Experimental procedure

Mice were randomly divided into five groups (n=6): Control, APAP, APAP+NAC, APAP+CsA and APAP+NAC+CsA. To induce the AKI model, APAP was administered in a single dose of 400 mg/kg (Salama *et al.,* 2015). All other treatments were performed 3 h after APAP administration. NAC was administered in a single dose of 1200 mg/kg and/or CsA was administered in a single dose of 50 mg/kg. All drug administrations were performed intraperitoneally. However, no administration was made to the mice in the control group during the experimental period. Twelve hours after the administration of APAP, all animals were decapitated and blood samples were collected and stored -20°C for the evaluation of biochemical parameters. In addition, kidney tissue samples were rapidly removed and to evaluate the histological changes, one of the kidney tissues for each mouse was taken into 10% formalin and fixed. In addition, other kidney tissue samples were stored at -80°C after being frozen in liquid nitrogen to analyze the alterations in the gene expression levels.

Histological evaluations

To perform routine microscopic assessment, kidney samples fixed in 10% formalin solution were embedded in paraffin blocks. Subsequently, 5 µm of sections were taken from paraffin-embedded tissue samples and stained with hematoxylin and eosin (H&E). Histopathological alterations of kidney sections for each group were evaluated using a light microscope. On the other hand, the acute tubular injury score in kidney samples, stained with H&E, was conducted according to the scoring system used by Chen *et al.* (2013). In brief, 20 fields per kidney samples (×200 magnification) were evaluated in terms of tubular damage and scored from 0 to 5 (Score 0: no damage; Score 1: <10% damage; Score 2: 10-25% damage; Score 3: 25-50% damage; Score 4: 50-74% damage; Score 5: >75% damage).

Evaluation of renal function

The collected mouse blood samples were centrifuged at 4000 rpm for 10 min and their serumwas separated. Serum creatinine (Cr) and blood urea nitrogen (BUN) levels were measured as markers of renal function using an autoanalyzer (Gesan chem 200 Gesan Production srl, Campobello, Italy).

Immunohistochemistry (IHC)

The avidin-biotin-peroxidase complex method was used to evaluate the immunoreactivity levels of 3-nitrotyrosine (3-NT) (bs-8551R, Bioss, USA), cytosolic cytochrome c (Cytc) (bs-0013R, Bioss, USA) and 8-hydroxy-deoxyguanosine

(8-OHdG) (ab48508, abcam) primary antibodies in kidney tissue samples. All sections were evaluated using a Leica DM500 microscope (DFC295; Leica, Wetzlar, Germany). Staining was evaluated by both its prevalence (0.1:<25%, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and severity (0: no, +0.5: very little, +1: little, +2: medium, +3: severe) (Histoscore=prevalence×severity).

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was performed to determine 3-NT and 8-OHdG levels in kidney tissue samples. Commercially available mice-specific 3-NT (E0019Ra, BT LAB, China) and 8-OHdG (YLA1580RA, YL Biont, China) ELISA kits were purchased. Briefly, kidney tissue samples were homogenized in phosphate-buffered saline (PBS) solution with a Bullet Blender Homogenizer (Next Advance, USA), centrifuged at 3000 rpm for 20 min and the obtained homogenates were evaluated in terms of protein concentration using a micro-spectrophotometer device (Allsheng, China). ELISA analyses were performed according to recommended protocols and a spectrophotometer (Multiskan FC, Thermo Scientific) was used to measure the optical density (OD) of kidney homogenate samples.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from mouse kidney samples using TRIzol Reagent (RiboEx, GeneAll, Korea) according to the recommended protocol. The quantity and purity of the obtained RNA samples were evaluated using a microspectrophotometer (Nano-400A, Allsheng, China). RNA samples were converted into complementary DNAs (cDNAs) using a reverse transcription kit (VitaScript, Procomcure, Austria). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using an ABI 7500 Real-Time PCR device (Applied Biosystems, Singapore) and BrightGreen 2X qPCR MasterMix (abm, Canada). To analyze the Cytc mRNA expression level, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene (Table 1). At the end of the analysis, the $2^{-\Delta\Delta Ct}$ method was used to calculate the variations in mRNA expression levels.

Statistical analysis

Statistical analysis was performed using the IBM SPSS 22.0 package program. The Shapiro-Wilk test was used for normalizing all data. To compare median values among the groups, Kruskal-Wallis and Mann-Whitney U tests were used. On the other hand, for statistical analysis of qPCR data, the online Qiagen Gene Globe PCR data analysis method was used. All data are expressed as mean ± standard error (SE). $p<0.05$ was considered statistically significant.

Table 1: Primer list of genes used for qPCR in the study.

Symbol		Primer sequence (5′-3′)
Gapdh	F	ACCACAGTCCATGCCATCAC
	R	TCCACCACCCTGTTGCTGTA
Cytochrome c	F	ACCAGCCCGGAACGAATTAAA
	R	CCGAACAGACCGTGGAGATT

F: Forward; R: Reverse.

Results and Discussion

APAP-induced histopathological alterations and effects of NAC and/or CsA on kidney tissue samples of mice

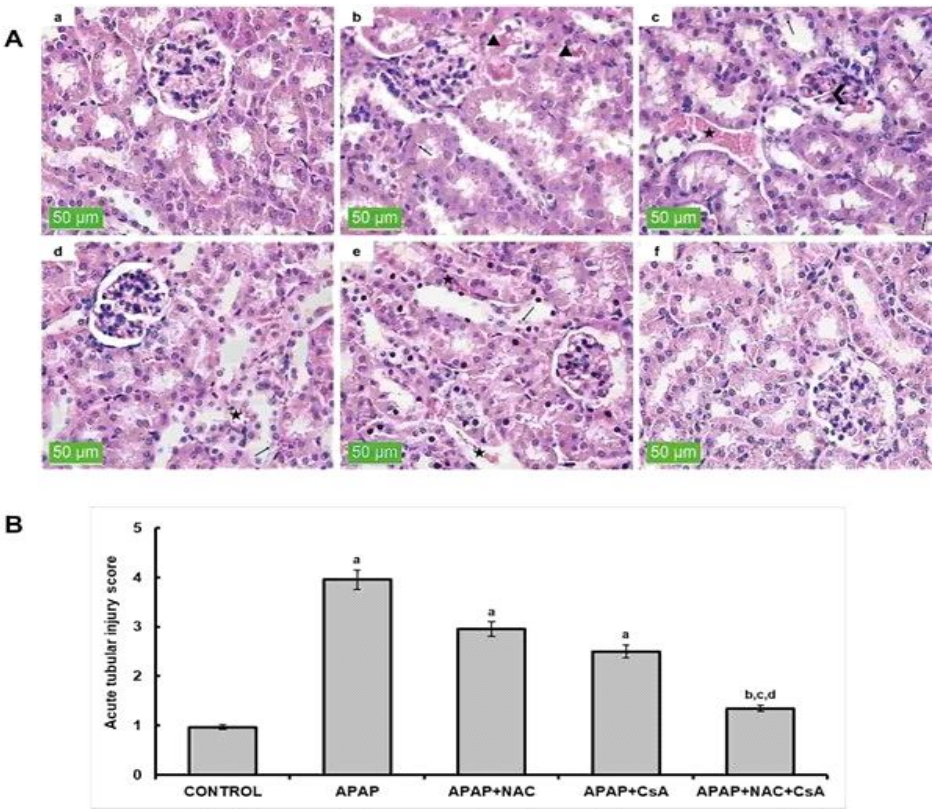
We first histopathologically examined the effect of APAP overdose on the kidneys of mice (Fig 1A). Although normal kidney morphology was observed in the control group [Fig 1A (a)], mice subjected to 400 mg/kg APAP showed noticeable congestion and hemorrhage, intratubular cast formation, glomerular atrophy and excessive renal tubular injury [Fig 1A (b,c)]. NAC or CsA treatments reduced APAP-induced kidney injury indications, but marked congestion and hemorrhage and moderate renal tubular injury were observed [Fig 1A(d,e; respectively)]. On the other hand, combined NAC+CsA treatment improved almost all of the histological alterations caused by APAP in mouse kidney tissue samples [Fig 1A(f)]. In addition, when histological data were evaluated particularly in terms of renal tubular injury, statistically significant increases were seen in the APAP, APAP+NAC and APAP+CsA groups compared with the control group ($p<0.001$). Also, APAP+NAC+CsA group observed a significant decrease compared with the APAP group ($p<0.001$). Compared with the APAP+NAC and APAP+CsA groups, a significant decrease was detected in the APAP+NAC+CsA group ($p<0.001$) (Fig 1B).

Effects of APAP, NAC and/or CsA treatment on serum creatinine (Cr) and blood urea nitrogen (BUN) levels

Cr and BUN levels, which are considered markers of renal function, were analyzed in serum samples of mice. Serum Cr and BUN levels were markedly increased in the APAP, APAP+NAC and APAP+CsA groups compared with the control group ($p<0.001$). On the other hand, serum Cr and BUN levels were remarkably decreased in the APAP+NAC, APAP+CsA and APAP+NAC+CsA groups compared with the APAP group ($p<0.001$). Moreover, significantly decreased serum Cr and BUN levels were detected in the APAP+NAC+CsA group compared with the APAP+NAC and APAP+CsA groups ($p<0.001$), however, no marked changes were observed against the control group ($p>0.05$) (Fig 2).

Effects of NAC and/or CsA treatment on 8-OHdG levels in APAP-induced acute nephrotoxicity

8-OHdG, a marker of oxidative DNA damage, was evaluated in terms of both immunoreactivity and protein concentration in kidney tissue samples of mice. In the immunoreactivity levels, a significant increase was observed in the APAP, APAP+NAC and APAP+CsA groups compared with the control group ($p<0.001$). Also, a remarkable decrease was detected in the APAP+NAC and APAP+NAC+CsA groups compared with the APAP group ($p<0.001$). Besides, a



(A) Histopathology of the kidney with H&E stain (x400). **a.** Control group. **b-c.** APAP group. **d.** APAP+NAC group. **e.** APAP+CsA group. **f.** APAP +NAC+CsA group. Histological changes such as congestion and hemorrhage (black star), intratubular cast formation (black triangle), glomerular atrophy (black arrowhead) and excessive renal tubular injury (black arrow) are marked. **(B)** Acute tubular injury score. [a] p<0.001 versus CONTROL group. [b] p<0.001 versus APAP group. [c] p<0.001 versus APAP+NAC group. [d] p<0.001 versus APAP+CsA group.

Fig 1: Histopathological examination findings of the kidney tissues.

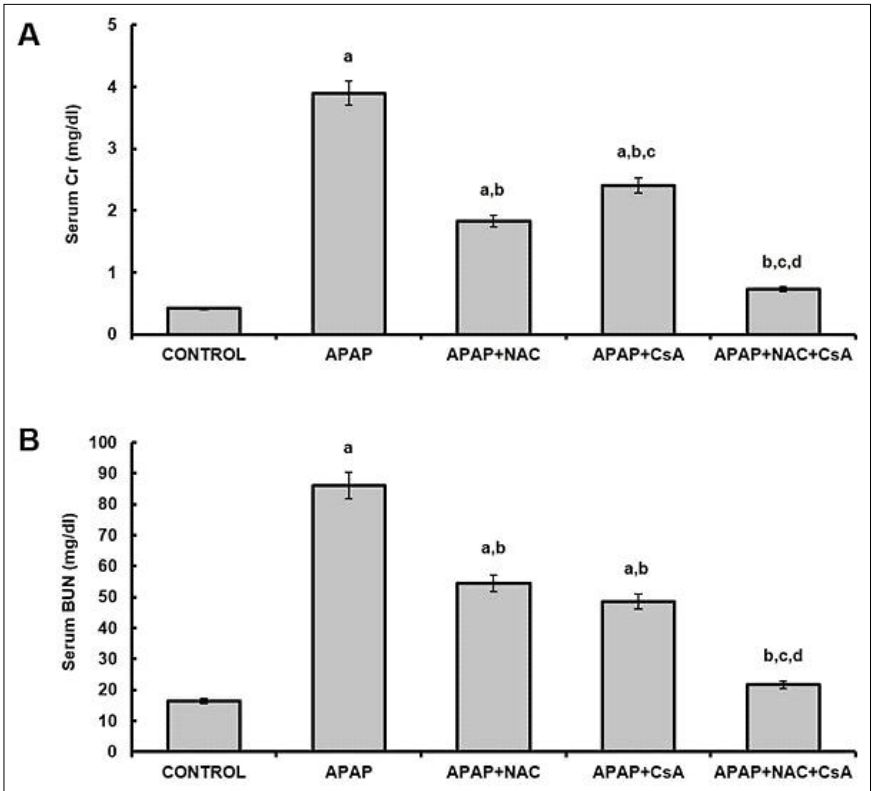
marked decrease was noticed in the APAP+NAC+CsA group compared with both APAP+NAC and APAP+CsA groups (p<0.001) (Fig 3A-B). On the other hand, in the protein concentration, a remarkable increase was observed in the APAP and APAP+CsA groups compared with the control group (p<0.001). Moreover, a significant decrease was detected in the APAP+NAC+CsA group compared with the APAP and APAP+CsA groups (p<0.001) (Fig 3C).

Effects of NAC and/or CsA treatment on 3-nitrotyrosine levels in APAP-induced acute nephrotoxicity

3-NT, a molecule that is among the potential markers of oxidative stress, was evaluated in terms of both immunoreactivity and protein concentration in mouse kidney tissue samples. In the immunoreactivity levels, a remarkable increase was detected in the APAP, APAP+NAC and APAP+CsA groups compared with the control group (p<0.001). In addition, a marked decrease was detected in the APAP+NAC+CsA group compared with the APAP, APAP+NAC and APAP+CsA groups (p<0.001) (Fig 4A-B). On the other hand, in the protein concentration levels, a significant increase was observed in the APAP+NAC and APAP+CsA groups compared with the control group (p<0.001). Also, a significant decrease was detected in the APAP+CsA and APAP+NAC+CsA groups compared with the APAP group (p<0.001). Moreover, a remarkable decrease was detected in the APAP+NAC+CsA group compared with the APAP+CsA group (p<0.001) (Fig 4C).

Effects of NAC and/or CsA treatment on cytochrome c levels in APAP-induced acute nephrotoxicity

In our study, Cytc, a potential biomarker for AKI, was assessed in terms of both immunoreactivity and mRNA levels in the kidney tissue samples of mice. When evaluated in terms of cytosolic immunoreactivity levels, a statistically significant increase was detected in the APAP, APAP+NAC and APAP+CsA groups compared with the control group (p<0.001). In addition, a remarkable decrease was detected in the APAP+NAC+CsA group compared with the APAP, APAP+NAC and APAP+CsA groups (p<0.001) (Fig 5A-B). Moreover, when evaluated in terms of mRNA levels, a statistically significant increase was detected only in the



(A) The serum level of Cr **(B)** The serum level of BUN. [a] $p<0.001$ versus CONTROL group. [b] $p<0.001$ versus APAP group. [a] $p<0.05$ versus CONTROL group. [b] $p<0.05$ versus APAP group. [c] $p<0.05$ versus APAP+NAC group. [d] $p<0.05$ versus APAP+CsA group.

Fig 2: Biochemical analysis findings of the serum samples.

APAP group compared with the control group ($p<0.05$). In addition, a remarkable decrease was detected only in the APAP+NAC+CsA group compared with the APAP group ($p<0.05$) (Fig 5C).

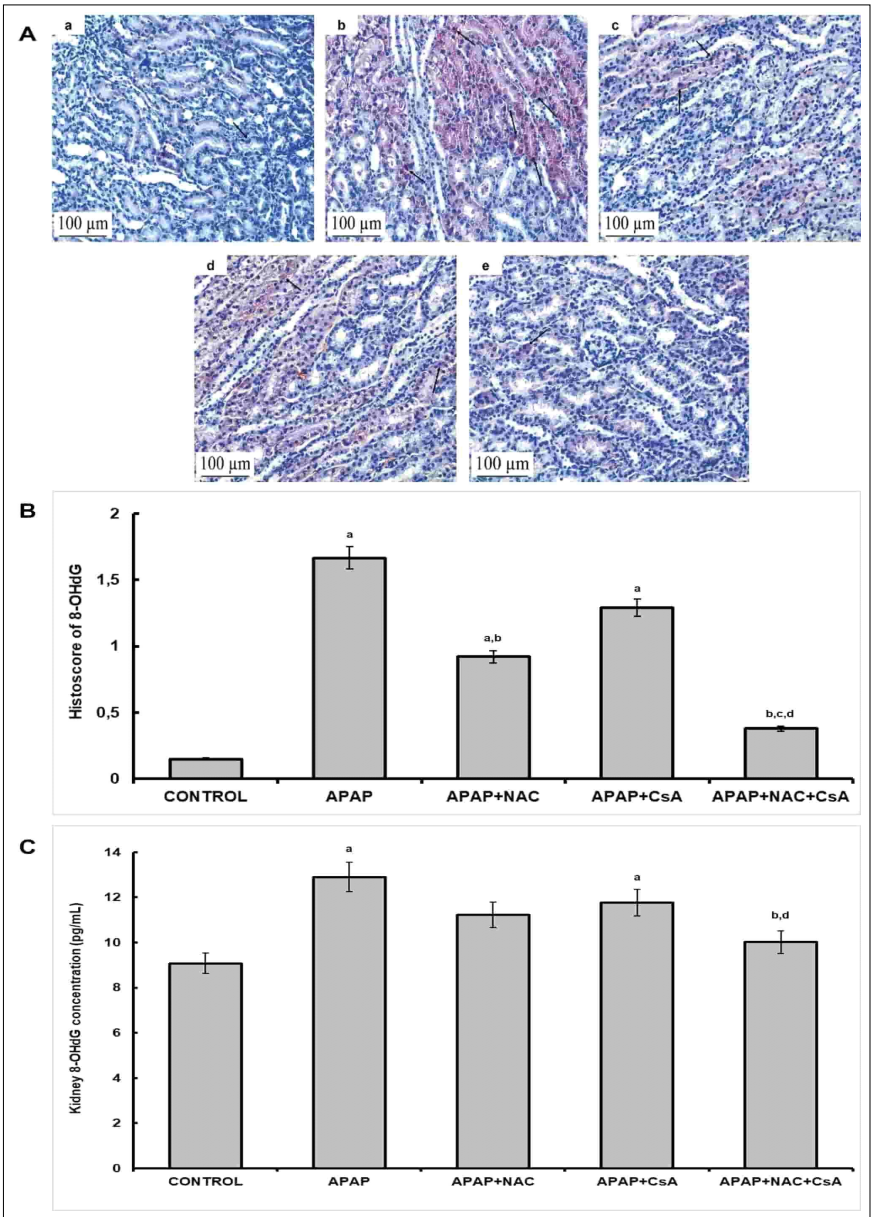
There are previous experimental studies showing the safety effects of some plant extracts on kidney histology and blood parameters (Ammari *et al*., 2023; Ansari *et al*., 2020). On the other hand its known that kidney damage induced by drugs or chemicals has recently become a major clinical problem (Zhao et al., 2011). Several studies have indicated that an overdose of APAP induces nephrotoxicity, associated with AKI and death, both in humans and experimental animals (Karaali *et al*., 2019). Treatment of APAP-induced nephrotoxicity has life-saving significance because of the life-threatening outcomes of APAP overdose on kidneys (Demirbağ *et al*., 2010). However, a lack of effective treatment options for such diseases has drawn attention (Zhao *et al*., 2011). Thus, it is crucial to develop novel therapeutic approaches for APAP-induced nephrotoxicity (Ucar *et al.,* 2013). To the best of our knowledge, for the first time, the potential therapeutic effect of NAC+CsA drug combination on APAP-induced nephrotoxicity in mice was evaluated in the current study. Our results demonstrated that combined NAC+CsA treatment provides remarkable ameliorative effects on APAP-induced nephrotoxicity. These were determined by histopathological, immunohistochemical, biochemical and molecular analysis. This approach may provide reinforcement to conventional NAC therapy, which indicates some restrictions for APAP poisoning.

To induce APAP-mediated toxicity, a single dose of APAP (400 mg/kg to 2000 mg/kg) was used in the majority of the experimental research (Chinnappan *et al.,* 2019). The APAP-induced damage model used in our study was constituted according to the model in mice previously developed by Salama *et al*. (2015). It was shown histopathologically that overdose of APAP led to pathological changes in mouse kidney samples such as focal tubular cell detachment, vacuolar degeneration, necrosis and apoptosis (Zhao *et al.,* 2011; Salama *et al.,* 2015; Karaali *et al*., 2019). Similarly, it was determined histopathologically that APAP overdose caused critical negative results in mouse kidney tissues and in our study. Moreover, the increase in serum Cr and BUN levels, which are renal function markers, after APAP overdose also supports this finding.

In both the liver and kidney, APAP is oxidized by cytochrome P450 enzymes and the toxic electrophile N-acetyl-p-benzoquinone imine (NAPQI) is generated. At therapeutic doses of APAP, the produced NAPQI level is quite low and is conjugated with reduced GSH to produce

nontoxic metabolites. However, toxic doses of APAP lead to the production of high NAPQI and only a part of it can be detoxified by GSH. Consequently, cellular GSH depletion occurs, which produces an imbalance between prooxidants and antioxidants (Zhao *et al*., 2011). In addition, the remaining free NAPQI binds to cellular proteins and other macromolecules. As a result, the production of superoxides (ROS and/or RNS)induces oxidative stress (Das *et al*., 2010; Ansari *et al*., 2020; Haidara *et al*., 2020; Alshahrani *et al*., 2021) and nitrosative stress (Xie *et al*., 2016; Yan-Zi *et al*., 2018). In addition, it can lead to DNA damage (McGill *et al*., 2012), mitochondrial MPT induction (Brown *et al*., 2012; McGill *et al*., 2012) and mitochondrial dysfunction (Xie *et al*., 2016; Haidara *et al*., 2020).

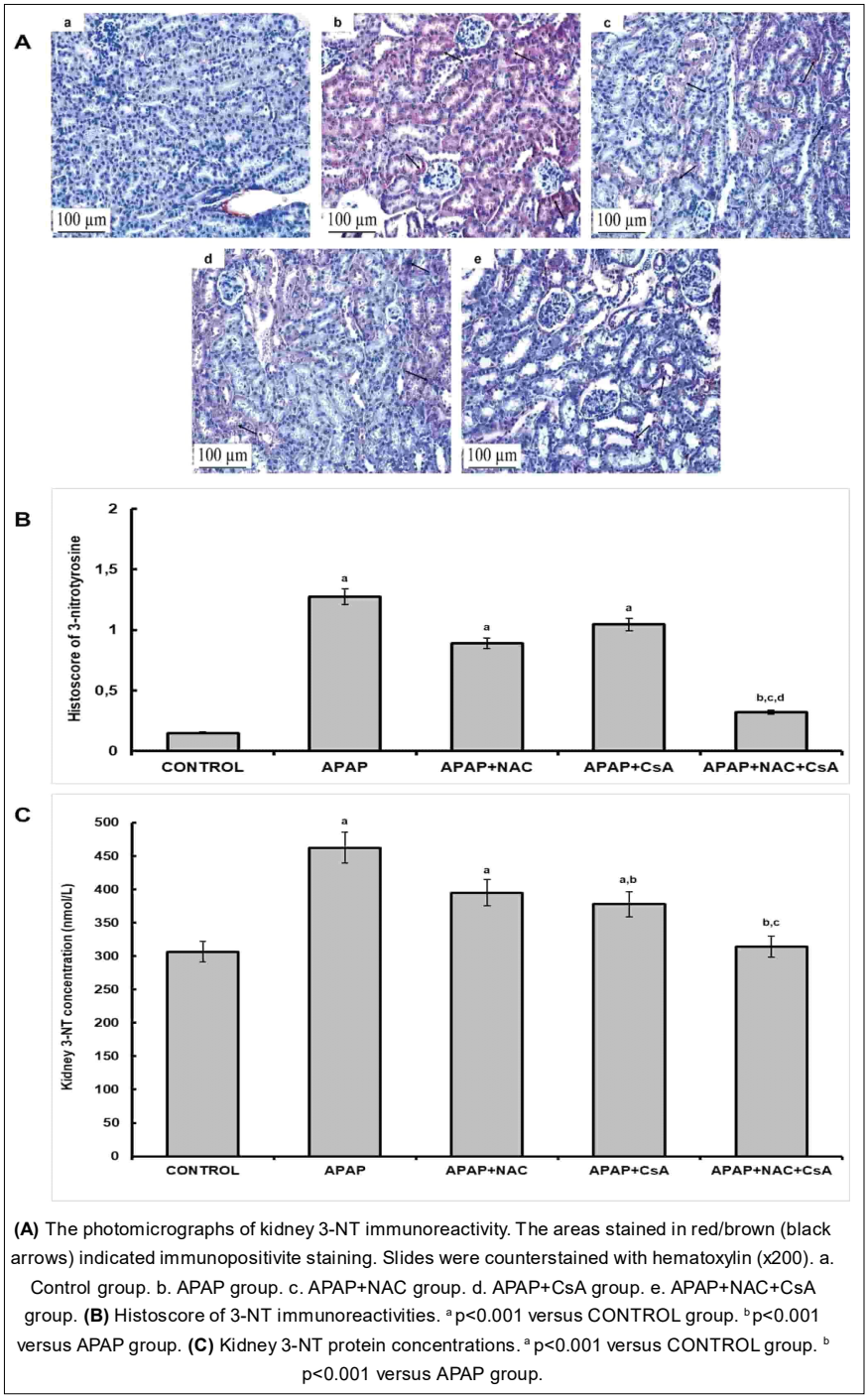
The precise molecular mechanism of kidney injury induced by APAP overdose remains obscure. However, the increase in ROS produced by NAPQI and consequent oxidative stress plays a crucial role in APAP-caused



(A) The photomicrographs of kidney 8-OHdG immunoreactivity. The areas stained in red/brown (black arrows) indicated immunopositivite staining. Slides were counterstained with hematoxylin (x200). **a.** Control group. **b.** APAP group. **c.** APAP+NAC group. **d.** APAP+CsA group. **e.** APAP +NAC+CsA group. **(B)** Histoscore of 8-OHdG immunoreactivities. [a] $p<0.001$ versus CONTROL group. [b] $p<0.001$ versus APAP group. **(C)** Kidney 8-OHdG protein concentrations. [a] $p<0.001$ versus CONTROL group. [b] $p<0.001$ versus APAP group.

Fig 3: Alterations in 8-OHdG expression levels in kidney tissue samples.

nephrotoxicity. Research in experimental animals demonstrated that APAP overdose increases lipid peroxidation and inhibits antioxidant defense in kidney tissue (Ansari *et al*., 2020). Moreover, previous studies have shown that APAP overdose leads to DNA damage and impaired double-strand DNA break repair (Napirei *et al.,* 2006; Borude *et al*., 2018). It is well known that this DNA damage occurs through oxidative stress-induced hydroxyl radicals that cause base modifications and breaks in DNA. 8-OHdG is located in both nuclear and mitochondrial DNA and is considered one of the most important markers of DNA damage (Benzer *et al.,* 2018; Turk *et al.,* 2019; Gelen *et al.,* 2021). In addition, several studies have shown that ROS-mediated DNA damage can be prevented by inhibiting 8-OHdG (Cao *et al.,* 2006; Kowluru and Kanwar, 2007; Turk *et al.,* 2019; Gelen *et al.,* 2021). Although DNA damage

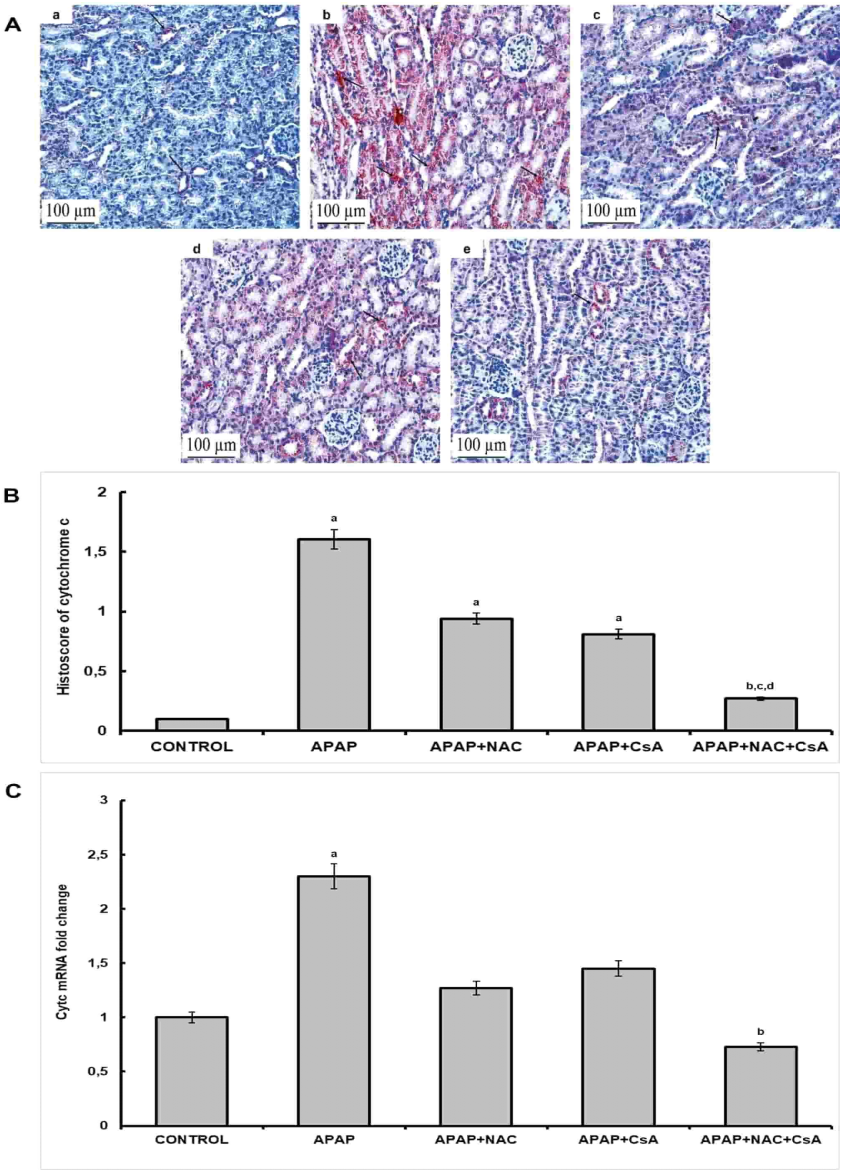


(A) The photomicrographs of kidney 3-NT immunoreactivity. The areas stained in red/brown (black arrows) indicated immunopositivite staining. Slides were counterstained with hematoxylin (x200). a. Control group. b. APAP group. c. APAP+NAC group. d. APAP+CsA group. e. APAP+NAC+CsA group. **(B)** Histoscore of 3-NT immunoreactivities. [a] $p<0.001$ versus CONTROL group. [b] $p<0.001$ versus APAP group. **(C)** Kidney 3-NT protein concentrations. [a] $p<0.001$ versus CONTROL group. [b] $p<0.001$ versus APAP group.

Fig 4: Alterations in 3-NT expression levels in kidney tissue samples.

analysis was not performed in our study, in parallel with the literature, the remarkable increase in the 8-OHdG level due to APAP overdose suggested that DNA damage occurred in the kidney tissue.

The production of reactive nitrogen species (RNS) due to APAP-induced toxicity causes nitrotyrosine adduction to proteins after GSH depletion. Protein nitration is believed to occur by peroxynitrite (ONOO-), a highly reactive molecule produced by superoxide and nitric oxide (James *et al.*, 2003; Kowluru *et al.,* 2007; Brown *et al.,* 2012; Xie *et al.,* 2016; Yan-Zi *et al.*, 2018). 3-NT, a diagnostic marker of peroxynitrite, is a stable end product frequently used to detect nitrosative stress (Ishii *et al.*, 2006). Previous studies have reported the presence of 3-NT in APAP toxicity,



(A) The photomicrographs of kidney Cytc immunoreactivity. The areas stained in red/brown (black arrows) indicated immunopositivite staining. Slides were counterstained with hematoxylin (x200). a. Control group. b. APAP group. c. APAP+NAC group. d. APAP+CsA group. e. APAP+NAC+CsA group. **(B)** Histoscore of Cytc immunore activities. [a] $p<0.05$ versus CONTROL group. [b] $p<0.05$ versus APAP group. [c] $p<0.05$ versus APAP+NAC group. [d] $p<0.05$ versus APAP+CsA group. **(C)** Kidney Cytc protein concentrations. [a] $p<0.05$ versus CONTROL group. [b] $p<0.05$ versus APAP group. [c] $p<0.05$ versus APAP+NAC group. [d] $p<0.05$ versus APAP+CsA group.

Fig 5: Alterations in Cytc expression levels in kidney tissue samples.

particularly in the pathogenesis of APAP-induced hepatotoxicity (Banerjee *et al.*, 2017; Yan-Zi *et al.*, 2018). Similarly, the current research demonstrated that APAP overdose leads to increased 3-NT immunoreactivity and protein concentration in kidney tissue.

Mitochondrial dysfunction is a characteristic of APAP-induced toxicity and is related to increased oxidative stress in APAP overdose. The role of reactive metabolite NAPQI generation in mitochondrial dysfunction related to APAP overdose toxicity is well known. In addition, peroxynitrite formation due to APAP toxicity can lead to mitochondrial dysfunction (Brown *et al.,* 2012). Moreover, it has been confirmed that numerous proteins related to antioxidant defense, energy supply, or fatty acid metabolism are nitrated by peroxynitrite in APAP hepatotoxicity. Mitochondrial dysfunction can also be caused by this condition (Xie *et al.,* 2016). In addition, mitochondrial damage has been demonstrated in APAP overdose-induced hepatotoxicity in our previous study (Kaya Tektemur *et al.,* 2021). Besides, Cytc release is one of the critical endpoints of cell death signaling and mitochondrial damage (Sepand *et al.,* 2016). Similarly, the current research demonstrated that APAP overdose leads to increased Cyc immunoreactivity in the cytosol and mRNA levels in kidney tissue.

NAC, a well-known free radical scavenger, has universally confirmed beneficial effects on the prevention of APAP-induced hepatotoxicity (Mokhtari *et al.,* 2017). This preventive effect of NAC in the liver occurs by replenishing depleted hepatic GSH (Mokhtari *et al.*, 2017; Akakpo *et al.*, 2020). Moreover, treatment with NAC decreases nitration of proteins in APAP-induced hepatotoxicity (Banerjee *et al.*, 2017). Additionally, in our previous study, we showed that NAC treatment in APAP-mediated hepatotoxicity led to a certain level of improvement by reducing increased cytochrome c, AST and ALT levels and increasing decreased GSH levels, alleviating extremely massive and severe histopathological changes (Kaya Tektemur *et al.*, 2021). On the other hand, it has been demonstrated that NAC cannot provide sufficient protection against APAP-induced nephrotoxicity (Akakpo *et al.*, 2020). In addition, the current study clearly demonstrated that NAC therapy alone is not effective enough against APAP-induced nephrotoxicity, especially in terms of acute tubular damage. The reason for this may be that because the kidney is not a real producer of GSH like the liver, getting enough GSH from the circulation is critical for kidney tissue (Ucar *et al.*, 2013). This reveals that searching for another protective molecule that would offer effective prevention against APAP-induced nephrotoxicity is required (Das *et al.*, 2010). In kidneys, which require high ATP in terms of function, the density of mitochondria is high, especially in the renal tubules. Moreover, it is well known that mitochondria play important roles in many cellular processes such as signal transduction, cell proliferation, cell growth and cell death. Therefore, mitochondrial dysfunction or damage could lead to impaired ATP generation and ROS balance. On the other hand, most therapeutic drugs may cause AKI because of their negative effects on the mitochondrion of the renal tubule (Hua *et al.*, 2018). It has been revealed that APAP-induced toxicity triggers the opening of mitochondrial MPTP. Thus, mitochondria are a potential target for treating AKI. Given the central role of mitochondria in the pathophysiology of AKI, in our study, it is not surprising that the therapeutic effects of CsA, an immunosuppressive drug known to inhibit the opening of MPTP (Taylor *et al.*, 2005), together with NAC on APAP-induced renal damagewere evaluated. The opening of MPTP is characterized by the loss of membrane potential and cellular ATP depletion and has a crucial role in the mitochondrial dysfunction-induced cell death mechanism (Brown *et al.*, 2012; Niimi *et al.*, 2012). Additionally, the opening of MPTP is a prerequisite stage of Cytc release into the cytosol and consequently results in the swelling of mitochondria (Borutaite *et al.*, 2003; Sepand *et al.*, 2016). The observation of increased Cytc levels in mouse kidney tissue with APAP administration in our study may be related to this. On the other hand, Cytc levels and acute tubular injury were significantly reduced by combined NAC and CsA treatment compared to the APAP+NAC group indicating that mitochondrial MPTP-mediated mitochondrial damage may play a role in APAP-induced nephrotoxicity.

CONCLUSION

NAC or CsA treatments alone have not been sufficient to reduce APAP-mediated acute nephrotoxicity. However, the current study indicated that combined NAC and CsA treatment can effectively reduce APAP toxicity in mice kidney tissue. Based on the obtained data, we propose that CsA treatment contributes to NAC in providing protection against APAP-induced nephrotoxicity by inhibiting the opening of mitochondrial MPTPs in renal tubular cells.

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Conflict of interest

All authors declare no conflict of interest.

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