Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2022;47(2):519-525
DOI: 10.17826/cumj.783088

ARAŞTIRMA / RESEARCH

Effect of N-acetylcysteine on cisplatin induced apoptosis in rat kidney

N-asetilsisteinin rat böbreklerinde sisplatin ile indüklenen apoptoz üzerine etkisi

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Cukurova Medical Journal 2022;47(2):519-525

Abstract

Purpose: Cisplatin is one of the most potent and widely used chemotherapeutic agents for the treatment of a wide variety of solid organ cancers. However, due to various side-effects such as nephrotoxicity, its therapeutic applications are limited. In the current study, it was aimed to investigate the effects of N-acetylcysteine (NAC), which is an effective antioxidant and anti-inflammatory agent, on cisplatin-induced apoptosis in rat kidneys.

Materials and Methods: Twentyfour male Wistar rats were separated into 4 equal groups: Control, NAC-250, cisplatin (CP), and CP+NAC groups. Rats in the experimental groups were treated with intraperitoneally (i.p.) single-dose cisplatin (10 mg/kg) and NAC (i.p., 250 mg/kg) for 3 days.

Results: At the end of the experiment, nephrotoxicity was confirmed by blood urea nitrogen and creatinine levels, and the apoptotic changes were demonstrated by TdT-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and caspase-3 levels in rat kidneys. The number of TUNEL-positive cells and caspase-3 levels were significantly increased by cisplatin. Treating the rats with NAC significantly decreased TUNEL-positive cells and caspase-3 levels.

Conclusion: These data suggest that apoptotic cell death is involved in the pathogenesis of cisplatin-induced nephrotoxicity, and that the inhibition of apoptosis plays a central role in the beneficial effects of NAC.

Keywords:. Cisplatin, apoptosis, N-acetylcysteine, Rat, TUNEL

Öz

Amaç: Sisplatin, çeşitli solid organ kanserlerinin tedavisi için en güçlü ve yaygın olarak kullanılan kemoterapötik ajanlardan biridirAncak nefrotoksisite gibi çeşitli yan etkileri nedeniyle terapötik uygulamaları sınırlıdır. Bu çalışmada, etkili bir antioksidan ve antienflamatuar ajan olan N-asetilsistein (NAC)'in rat böbreklerinde sisplatin kaynaklı apoptoz üzerindeki etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Wistar cinsi erkek ratlar eşit sayıda olacak şekilde 4 gruba ayrıldı: Kontrol, NAC-250, sisplatin (CP) ve CP+NAC. Gruplardaki ratlara tek doz sisplatin (10 mg/kg) ve 3 gün NAC (250 mg/kg) intraperitoneal (i.p.) olarak uygulandı.

Bulgular: Deneyin sonunda, nefrotoksisite, kan üre nitrojen ve kreatinin seviyeleri ile doğrulandı ve apoptotik değişiklikler, sıçan böbreklerinde TdT aracılı deoksiüridin trifosfat nick-end etiketleme (TUNEL) ve kaspaz-3 seviyeleri ile gösterildi. TUNEL pozitif hücre sayısı ve kaspaz-3 seviyeleri sisplatin ile önemli ölçüde arttı. Sıçanları NAC ile tedavi etmek, TUNEL pozitif hücreleri ve kaspaz-3 seviyelerini önemli ölçüde azalttı.

Sonuç: Bu veriler, apoptotik hücre ölümünün sisplatin ile indüklenen nefrotoksisitenin patogenezinde etkili olduğunu ve apoptoz inhibisyonunun NAC'ın faydalı etkilerinde merkezi bir rol oynadığını göstermektedir.

Anahtar kelimeler: Sisplatin, apoptoz, N-asetilsistein, Rat, TUNEL

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Geliş tarihi/Received: 09.12.2021 Kabul tarihi/Accepted: 16.03.2022

INTRODUCTION

Cisplatin is a commonly used chemotherapeutic drug against a wide spectrum of solid neoplasms, including bladder, lung, ovarian, testicular, and head and neck cancers[1-3]. Despite its therapeutic effectiveness, the drug leads to serious side-effects such as nephrotoxicity, ototoxicity, and neurotoxicity as well as nausea and vomiting[3]. Nephrotoxicity, as dose-limiting toxicity, occurs in nearly one-third of treated patients and is the most significant adverse effect of cisplatin chemotherapy[1,2,4].

The pathogenesis of cisplatin-induced nephrotoxicity is complex and causes tubular cell injury and renal dysfunction through several mechanisms, including oxidative stress, inflammation, apoptosis, and necrosis[2,4,5]. Of these, apoptosis has been identified as a major mechanism, and several apoptotic pathways including the extrinsic pathway activated by death receptors, the intrinsic mitochondrial pathway, and the endoplasmic reticulum (ER)-stress pathway play crucial roles in cisplatin-induced nephrotoxicity[6-10]. While, the mitochondrial pathway involves the release of cytochrome c and apoptosis-inducing factor from the mitochondria. the death receptor pathway leads to activation of the apoptotic cascade via caspase-8. Cytocrom c is responsible for caspase-9 activation. As a result, both pathways initiate apoptotic cell death by causing degradation of cellular and nuclear proteins, especially with the activation of caspase-3 and 7[11].

As cisplatin is a potent chemotherapeutic agent, various studies have been conducted to reduce the side-effects with the use of antioxidants and anti-apoptotic agents together with cisplatin without altering its properties and beneficial effects[12]. However, there is no consensus on an agent and dose range that can be used yet.

N-acetylcysteine (NAC), has been used in clinical practice as a thiol-containing antioxidant, anti-inflammatory, and mucolytic agent[13]. NAC is also a free radical scavenger, which acts as a precursor of reduced glutathione[14]. The previous study by the current authors showed that through antioxidative mechanisms NAC also has renoprotective against the effects of cisplatin-induced nephrotoxicity[15]. Moreover, in a study with different cell lines, the protective effect of NAC on cisplatin-induced apoptosis was demonstrated. According to Wu et al. NAC administration reversed the cytotoxic and apoptotic effects if added concurrent with cisplatin or up to 2 hours after cisplatin by blocking both the death receptor and the mitochondrial apoptotic pathways[16]. As such, NAC may provide possible beneficial effects against cisplatin adverse effects through apoptosis. However there are very limited studies on this subject. Luo et al. reported that NAC, administered 2 days before cisplatin injection, has reduced cisplatin induced apoptosis, renal dysfunction and morphological damage in rats[17].

Nonetheless, chemoprotectants have had relatively limited clinical use due to inability to present a common view on the appropriate dose and duration of use, possibility of drug interactions, and decreased antitumor effects. For this reason, before clinical trials the use of an agent such as NAC, which has been shown to be beneficial against cisplatin-induced toxicity, needs to be investigated in more detail at different doses and/or durations. Therefore, the aim of this study was to investigate the effects of NAC on cisplatin-induced apoptosis in rat kidneys.

MATERIALS AND METHODS

Animals

In a one-way ANOVA study, sample sizes of 6, 6, 6, and 6 are obtained from the 4 groups whose means are to be compared. The total sample of 24 subjects achieves 94% power to detect differences among the means versus the alternative of equal means using an F test with a 0,05000 significance level. A total of 24 male Wistar Albino rats, each weighing between 280–445 g, 6-8 months old were used in the study. All the rats were housed in cages under conditions of a 12 h light/dark cycle at a controlled temperature (22 ± 3 °C), and 50–55% humidity during the experimental period, with unrestricted access to fresh water and food. All the rats were acclimatised to the housing conditions for at least one week before the start of the study.

Experimental design

The rats were randomly separated into four equal groups of six, as follows: control (C), cisplatin (CP), NAC (NAC-250) and cisplatin plus NAC (CP+NAC) groups. The control group was administered 1 mL/kg rat body weight (RBW) normal Saline (0.9% NaCl) for 3 days; the CP group received a single dose 10 mg/kg RBW CP; the NAC-250 group received 250 mg/kg RBW NAC (i.p.) for 3 days; and the CP+NAC group received 10 mg/kg RBW single

dose cisplatin + 250 mg/kg RBW NAC, administered four hours later cisplatin, for 3 consecutive days.

At the end of the experiment, the rats were anesthetized with an i.p. injection of 80 mg/kg ketamine (Ketalar®- Pfizer) and 10 mg/kg xylazine (Rompun®- Bayer). Blood samples were taken and the right kidney was rapidly excised from each animal for TUNEL assay and fixed in 10% neutral formaldehyde.

Measurement of plasma BUN and creatinine levels

Nephrotoxicity was evaluated from the blood urea nitrogen (BUN) and creatinine levels. Blood samples were centrifuged at 1500 g for 15 min at +4 °C. The plasma BUN and creatinine levels were determined with the Roche/Hitachi Cobas c501 system (Roche Diagnostics, Indianapolis, IN, USA) in Erciyes University Gulser-Dr. Mustafa Gundogdu Central Laboratory. In Central Laboratory Units registered to External Quality Control Programs, all tests are performed in accordance with these control programs and daily control values are recorded for each test.

Examination of apoptosis

The apoptotic changes were demonstrated by the Terminal deoxynucleotidyl transferase (TdT)--mediated dUTP Nick-End Labelling (TUNEL) assay and cysteinyl aspartate specific proteinases 3 (caspase-3) levels. All procedures were performed by a blinded histologist at Erciyes University Histology Embryology Department.

Apoptosis detection using the TUNEL assay

To determine apoptotic cells, the TUNEL assay was performed in the rat kidney sections using the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Cat#S7110, EMD Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain to stain the DNA. TUNEL-positive cells were evaluated using an immunofluorescence microscope. Semi-quantitative analysis results were shown by counting the TUNEL-positive cells per single field at ×400 magnification.

Determinations of caspase-3 levels

Tissue caspase-3 levels were measured using the Rat Caspase-3 ELISA kit (Sunred Biological Technology Company; Shanghai, China) according to the manufacturer's instructions. The intra - and inter-assay coefficients of variation (CV) were <8% and <10 % respectively. The caspase-3 results were obtained as a proportion of the protein values calculated by the Lowry method in the same tissues[18]. All experimental studies were carried out in Erciyes University Clinical Biochemistry Research Laboratory. Ethical approval for this study was obtained from Erciyes University Animal Experiments Local Ethics Committee (Ethics Committee Decision No:13/150 Date: Dec 2013). All the procedures were performed in Erciyes Universıty, Experimental Research and Application Center, in compliance with the Helsinki Declaration and International Guiding Principles for Biomedical Research Involving Animals.

Statistical analysis

IBM-SPSS version 23.0 software was used for all analyses. Conformity of the data to normal distribution was analyzed with the Shapiro Wilk test. The results were presented as mean ± standard deviation (SD) values. One-way ANOVA and post-ANOVA tests were used to compare BUN, creatinine and apoptosis parameters between study groups. A value of $p< 0.05$ was accepted as statistically significant.

RESULTS

The CP group showed significantly increased BUN and creatinine levels compared to the control and NAC-250 groups ($p <0.05$). No statistically significant difference was observed between the control group and the NAC-250 group ($p>0.05$). Treatment with NAC significantly decreased the elevated levels of BUN and creatinine compared to the CP-treated group ($p< 0.05$) (Table 1). A significant increase was observed in the caspase-3 levels in the NAC-250 and CP groups compared to the control group. The most prominent increase was demonstrated in the CP group. Treatment with NAC after cisplatin significantly decreased the elevation of caspase-3 levels compared to the CP group ($p< 0.05$) (Table 2).

Table 1. Post-study BUN and plasma creatinine levels of the rats

	Groups (N=6)			
	Control	**NAC-250**	**CP**	**CP+NAC**
BUN (mg/dL)	18.77 ±1.90	16.09 ± 2.10	137.09 ± 33.82*,a	102.4 ±4.67*,a,b
Creatinine (mg/dL)	0.44 ± 0.08	0.38 ± 0.04	3.59 ± 1.01*,a	1.87 ± 0.20*,a,b

Data were expressed as mean ± SD. Significant findings compared to: *CONTROL group a:NAC-250 group b: comparisons of other groups with CP group; p<0.05. BUN: Blood Urea Nitrogen; NAC: N-acetylcysteine; CP: Cisplatin.

Table 2. Changes in the apoptosis parameters of the rats in the study groups

Groups (N=6)				
	Control	**NAC-250**	**CP**	**CP+NAC**
Caspase-3 (ng/ mg protein)	0.41 ± 0.16	1.20 ± 0.28*	1.72 ± 0.35*,a	1.10 ± 0.13*,b
TUNEL (+) Cells	0.21 ± 0.12	0.60 ±0.28*	1.75±0.60*,a	0.49 ± 0.26b

Data were expressed as mean ± SD. Significant findings: *:with CONTROL group a:NAC-250 group b: comparisons of other groups with CP group; p<0.05. TUNEL: TdT-mediated deoxyuridine triphosphate nick-end labeling; NAC: N-acetylcysteine; CP: Cisplatin.

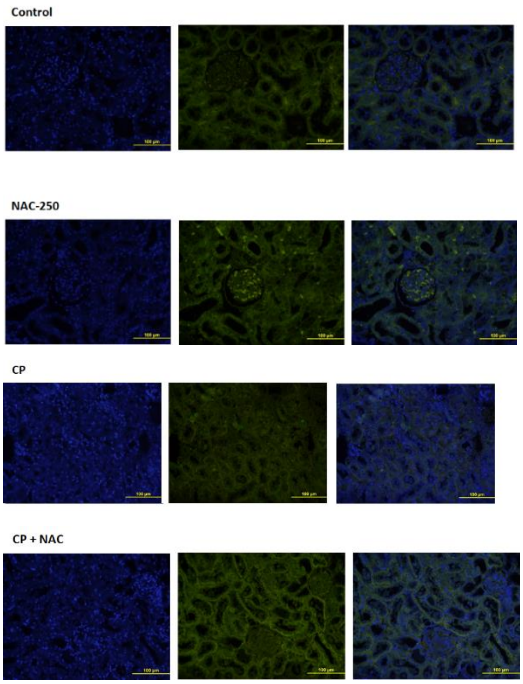

Figure 1. The effects of NAC on cisplatin-induced apoptotic cell death. Histological examination demonstrated cisplatin-induced TUNEL staining (green) in the kidney and TUNEL staining was significantly attenuated with NAC administration. Nuclei were stained with Hoechst 33342 (blue).

A statistically significant increase was observed in the number of TUNEL (+) cells in the NAC-250 and CP groups compared with the control group (p<0.05). Similar to caspase-3 levels, the most prominent increase was shown in the CP group. A decrease was seen in the CP+NAC group, which was not different from that of the control group (Table 2, Figure 1).

DISCUSSION

Although cisplatin is a frequently preferred chemotherapeutic agent, clinical use is highly limited by its nephrotoxic effects. The primary consequence of cisplatin-induced nephrotoxicity is the loss of renal functions, which include decreased glomerular filtration rate, creatinine clearance, and increases in serum creatinine and BUN[19]. In the results of the present study, the increased BUN and creatinine levels demonstrated the development of nephrotoxicity due to cisplatin use. The molecular and cellular mechanisms involved in drug-induced nephrotoxicity are still not fully understood, although previous studies have demonstrated the cellular mechanisms by which cisplatin causes cell death in renal tubular epithelial cells. At the crossroads of DNA damage, oxidative stress, and inflammation, all of which have critical roles in cisplatin-induced cell death, there are two different metabolic pathways, apoptosis, and necrosis. Apoptosis is mediated mainly by caspases produced as inactive proteins. To summarize, first an extracellular/intracellular stimulus causes an initiator caspase cleavage, then an executioner caspase resulting in apoptotic cell death. Although there are several initiator caspases, such as caspase-3 and caspase-7, the main executioner caspase implicated in cisplatin-induced nephrotoxicity is caspase-3[20].

In vitro studies have indicated that necrotic cell death occurs with high concentrations and apoptosis with lower concentrations of cisplatin[21]. However, in the current study, although high single dose cisplatin (10 mg/kg RBW) was used, increased TUNEL (+) cells and kidney tissue caspase-3 levels were observed in the CP group.

Yano et al. reported that short-term, high doses of cisplatin increased the number of PI-stained cells, and the long-term low doses increased, especially the number of annexin V-stained cells in the renal tubular cell line[22]. However, similar to the current study results, in the in vivo part of the same study, 7.5 mg/ kg RBWcisplatin demonstrated the presence of TUNEL positive cells indicating apoptosis in renal tubules. The authors noted that the drug administered at high doses remained in the tissue at low doses and long term after the initial excretion. However, there are also studies in literature showing the presence of apoptosis in high-dose cisplatin applications[23,24]. Several strategies including hydration and supplementation are used to prevent cisplatin-induced kidney injury but an effective solution has not yet been revealed[25]. There are many studies in the literature on this subject and many agents with different characteristics to help prevent toxicity have been tried[3].

Although cisplatin-induced renal tubular cell death is a secondary result of oxidative stress, it is considered to be a decisive point of therapeutic intervention[20]. Therefore, in the current study, it was preferred to use NAC, a clinically proven agent with antioxidant properties that has been used in many pathologies associated with oxidative stress[26]. Moreover, Wu et al. demonstrated that NAC prevents caspase-mediated apoptosis in different cell lines[16]. Sancho Martinez et al. showed that in HK-2 cells, the increased necrosis and apoptosis caused by cisplatin treatment decreased with NAC administration, and this decrease was a result of the reduction of platinum DNA adducts in the cell[27]. Similar to these results, Luo et al. reported that NAC decreased caspase-3 cleavage and apoptosis in rat kidneys[17].

In the present study, it can be said that the nephrotoxic state which developed as a result of cisplatin administration significantly improved with NAC application, as assessed by BUN and creatinine levels. Moreover, considering the decreases in caspase-3 and TUNEL (+) cell numbers, NAC was seen to be effective in the decrease in apoptotic cells, which is one of the mechanisms known to contribute to nephrotoxicity. However, it is noteworthy that this improvement is still not sufficient when compared to the control group values. Although the decrease in the number of TUNEL (+) cells became more pronounced, the fact that the BUN and creatinine levels remained significantly higher than those of the control group can be considered to be a result of the large number of mechanisms involved in nephrotoxicity[9].

The most important issue in studies related to chemoprotectants is the interaction with a chemotherapeutic. Therefore, in the current study, NAC application was performed at the 4th hour after cisplatin administration when drug interaction was at the lowest level, as stated by Muldoon et al[28].

The most surprising finding in this study was that the apoptotic cell levels seen in the NAC group alone were statistically increased compared to the control group. Although no similar findings have been found in experimental rat studies in literature, Liu et al. showed that NAC paradoxically caused caspase-3 and 9 mediated apoptosis in the H9c2 cell line, causing glutathione levels to be further reduced, thereby resulting in an imbalance in redox potential[29]. However, this is not the direct subject of this study and further studies are needed.

Nonetheless, there are some limitations to the present study. First the change in the response of NAC at different dose ranges have not been evaluated. Second, perhaps most importantly, the effect of NAC applied on the antitumoral efficacy of cisplatin could not be examined, since the animals did not have tumor tissue.

On the other hand, the results of this study showed that NAC reduces apoptotic cell death, which is known to be particularly effective in cisplatin-mediated nephrotoxicity, and consequently improves the renal injury. Therefore, the current study will guide new studies to determine the appropriate dose and duration for the use of NAC, which has clinical use and more importantly, a wide therapeutic range as a chemoprotectant.

Yazar Katkıları: Çalışma konsepti/Tasarımı: İG, CY, KK, FD; Veri toplama: İG, CY, AY, FD; Veri analizi ve yorumlama: İG, FD, GŞS; Yazı taslağı: İG, GŞS, CY, AY; İçeriğin eleştirel incelenmesi: İG, CY, KK; Son onay ve sorumluluk: İG, GŞS, FD, AY, CY, KK; Teknik ve malzeme desteği: IG, CY, AY; Süpervizyon: İG, CY, KK; Fon sağlama (mevcut ise): yok.
Etik Onay: Bu çalışma için Erciyes Üniversitesi Hayvan Deneyleri Yerel Kurul Başkanlığından 11.12.2013 tarih ve 13/150 sayılı kararı ile etik onay alınmıştır.
Hakem Değerlendirmesi: Dış bağımsız.

Çıkar Çatışması: Yazarların açıklayacak rakip çıkarları yoktur. Bu yazının yapımında hiçbir yazı yardımı kullanılmamıştır.
Finansal Destek: Bu çalışma Erciyes Üniversitesi Araştırma Fonu tarafından desteklenmiştir (Hibe No: TDK-2014-5056).
Yazarın Notu: Bu araştırma 27-31 Ekim 2019 tarihleri arasında Antalya/Türkiye, 27. Balkan Klinik Laboratuvar Federasyonu (BCLF) Kongresi ve 30. Ulusal Biyokimya Kongresi'nde (NBC) sözlü sunum olarak kabul edilmiştir.
Author Contributions: Concept/Design : İG, CY, KK, FD; Data acquisition: İG, CY, AY, FD; Data analysis and interpretation: İG, FD, GŞS; Drafting manuscript: İG, GŞS, CY, AY; Critical revision of manuscript: İG, CY, KK; Final approval and accountability: : İG, GŞS, FD, AY, CY, KK; Technical or material support: IG, CY, AY; Supervision: İG, CY, KK; Securing funding (if available): n/a.
Ethical Approval: Ethical approval was obtained from Çukurova University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee for this study. (With the date 6.3.2020 and the number 97/1).
Peer-review: Externally peer-reviewed.
Conflict of Interest: The authors have no competing interests to disclose. No writing assistance was utilized in the production of this manuscript.
Financial Disclosure: This study was supported by the Research Fund of Erciyes University (Grant No: TDK-2014-5056).
Acknowledgement: This research was accepted as oral presentation at the 27th Balkan Clinical Laboratory Federation (BCLF) Congress and 30th National Biochemistry Congress (NBC), 27-31 October 2019 Antalya/Turkey.

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