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Research Article
Protective Effect of Alpha-Linolenic Acid on Cisplatin Induced Ototoxicity in Mice

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Abstract

Background and Objective: Alpha-linolenic acid (ALA), has anti-inflammatory and antioxidant activity and has been shown to prevent cell damage by reducing oxidative stress. Cisplatin (CIS) has been shown to increase reactive oxygen radicals and reduce endogenous antioxidant enzyme activity in cochlear cells. Therefore, this study measured the protective effect of alpha-linolenic acid on cisplatin-induced ototoxicity. **Materials and Methods:** The mice were given 100 mg kg^{-1} cisplatin and 200 mg kg^{-1} alpha-linolenic acid together with cisplatin for 9 days. On days 9 and 10, a rotarod test was performed to evaluate the effect of cisplatin and alpha-linolenic acid on the motor coordination of mice. Then, the rotarod-tested mice were killed by cervical dislocation. The organ of Corti explants was dissected to use for ELISA experiments to analyze inflammatory and apoptotic mediators. **Results:** The CIS administration reduced the fall time of mice. Administration of alpha-linolenic acid together with cisplatin prolonged this period. The CIS treatment increased expression of inflammation mediators (Phospholipase A_2 (PLA_2)), Cyclooxygenase-2 (COX-2) and Inducible Nitric Oxide Syntheses (iNOS) and apoptotic mediators (bax and caspase-3) CIS also decreased bcl-2 which is an antiapoptotic protein. Administration of alpha-linolenic acid reversed these effects. **Conclusion:** This study showed that ALA would be useful in preventing CIS-induced ototoxicity by reducing inflammatory and apoptotic mediators.

Key words: Cisplatin, ototoxicity, alpha-linolenic acid, motor coordination, apoptosis and inflammation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cisplatin is an antineoplastic agent used in adults and children in the treatment of many malignant diseases, especially head and neck tumors, urogenital system, central nervous system, respiratory system and esophageal cancers. Cisplatin has significant side effects such as nephrotoxicity and irreversible ototoxicity[1].

Balance disorder is caused by damage to the vestibular apparatus. Loss of hair cells in this apparatus as a result of ototoxicity causes balance disorders[2].

Alpha-linolenic acid, known as omega 3 fatty acid, is a protective nutrient for the heart and cardiovascular system and is the precursor of docosahexaenoic acid (DHA) and pentanoic acid (EPA), which are added to the arachidonate structure[3-5]. Spontaneously hypertensive rats fed an alpha-linolenic acid diet have been shown to reduce blood pressure. A 12-week alpha-linolenic acid diet has been reported to reduce the systolic and diastolic blood pressures of Japanese people with high, moderate and normal blood pressure[6]. Alpha linolenic acid has strong antioxidant activity and reduces oxidative stress, which also contributes to the prevention of inflammation[7]. Alpha-linolenic acid was shown to reduce inflammation caused by lipoprotein saccharide (LPS). The ALA inhibits translocation of Nuclear Factor Kappa-β (NF-κβ) and Mitogen-Activated Protein Kinase (MAPK) phosphorylation. Inhibition of these nuclear factors reduces iNOS, COX-2, TNF-alpha which are inflammatory mediators[8].

Rotarod performance testing is used to evaluate the effect of drugs on motor coordination or fatigue resistance in mice and rats in a safe and humane manner using a single unit. It is a performance test based on a rotating rod with forced motor activity performed by a mouse or rat. After the drug is administered, the animal is placed in the device and the time until it falls is recorded as fall latency[9].

This study was planned to investigate whether alpha-linolenic acid, which has anti-inflammatory and antioxidant properties, would be protective on the balance disorder, which is an indicator of ototoxicity caused by cisplatin.

MATERIALS AND METHODS

This *in vitro* study was performed at Cukurova University, Faculty of Medicine, Department of Pharmacology in October, 2021.

Chemicals: The CIS (100 mg kg^{-1}) and alpha-linolenic acid (all-cis-9,12,15-octadecatrienoic acid) (ALA) were purchased from Sigma Aldrich (St. Louis, Missouri, United States of America).

Experimental process: In this study, mice were obtained (weighing 30 g, 32 male BALB/c albino) from Çukurova University Experimental Research Animal Ethics Center (TIBDAM) and informed Cukurova University Committee of Ethics about the protocol of our study. Animals were kept in a 12 hrs light and12 hrs dark cycle at 20-22°C and 50-55% humidity. Food and water were provided *ad libitum*. Then the 32 mice were randomly divided into 4 groups. control group 0.09% NaCl (physiological saline) solution for, ALA treated group 200 mg kg^{-1} alpha-linolenic acid by gavage for, CIS treated group 100 mg kg^{-1} intraperitoneal (i.p.) CIS and ALA+CIS treated group 100 mg kg^{-1} i.p. CIS and ALA (200 mg kg^{-1}). All of drugs administered a time in a day for 9 days.

The control group was also administered physiological serum for 9 days under the same experimental conditions. Rotarod motor coordination test was performed on the 9th and 10th day of the experiments after the rotarod test mice are killed by cervical dislocation to dissect of organ of corti from mouse then it was stored in Eppendorf tubes to use in ELISA Test. The levels of Bcl-2, Bax, Gpx, CAT, activated (cleaved) caspase-3 were examined in organ of Corti explants.

Homogenization of tissue: The 3 mL radio-immunoprecipitation assay (RIPA) buffer, 30 µL Phenylmethanesulfonyl Fluoride (PMSF), 30 µL sodium vanadate, protease inhibitor of 30 µL were added to the tissues in Eppendorf tubes then lysed the tissues on ice by using an ultrasonic disintegrator (SONOPULS, HD 2070 and Germany). The homogenates were then centrifuged at 10,000 rpm for 10 min, the supernatants were removed and the remaining pellets discarded frozen at -20°C.

Rotarod test was performed to evaluate the effect of CIS and the effect of CIS together with alpha-linolenic acid on motor coordination. The rotarod apparatus was set to rotate at 12 revolutions per min. Mice were placed one by one on the rotating drum. The time in seconds that each animal fell from the drum was recorded using a stopwatch for up to 120 sec and displayed as the performance time.

Biochemical analyzes: Bradford method was used for total protein of samples. Procedures described in previous work of Stanely *et al.*[9].

Enzyme Linked Immunosorbent Assay (ELISA) Test: The determination of Bcl-2, Bax, cyclooxygenase-2, cleaved caspase-3, iNOS and phospholipase A_2 enzymes were tested by ELISA according to the manufacturer's test protocol. The ELISA kits purchased from R and D Systems, Inc. (Minneapolis, Minnesota).

Statistical analysis: Statistical analysis was carried out by using Graph Pad Prism 4.0 (Graph Pad Software, San Diego, United States of America). Data were stated as Mean±Standard error (SE) comparisons of Group were made using One-way Analysis of Variance (ANOVA) (Bonferonni *post hoc*) test. The p-values considered significantly less than 0.05.

RESULTS

On the 9th day of CIS application, the falling times of mice treated with CIS in the rotarod test decreased statistically significantly. The ALA application significantly increased this period (Fig. 1). On the 10th day of CIS application, the falling times of the mice to which CIS was applied in the rotarod test decreased statistically significantly. Alpha-linolenic acid application significantly increased this period (Fig. 2).

While gentamicin application caused an increase in the inflammatory mediators (COX-2, PLA_2 and iNOS) application of ALA decreased this increase significantly (Fig. 3-5).

The Bax and cleaved-caspase-3 levels were found significantly higher in the CIS treated group than in the control group ($p<0.05$), However, Bax and activated caspase-3 levels were significantly lower in the ALA+CIS treated group than in the CIS treated group (Fig. 6-7).

The Bcl-2 levels were significantly decreased in the CIS treated group compared to the control group ($p<0.05$) and

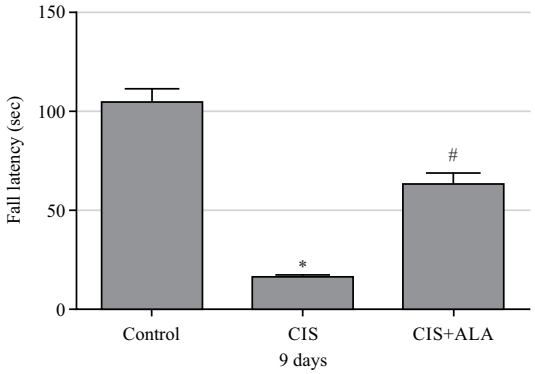

Fig. 1: Effect of alpha-linolenic acid on motor coordination of the 9 days' gentamicin applied mice (n = 12)
Statistical analysis: ANOVA, *post hoc*: Bonferroni, *Control $p< 0.05$ and #Gentamicin $p<0.05$

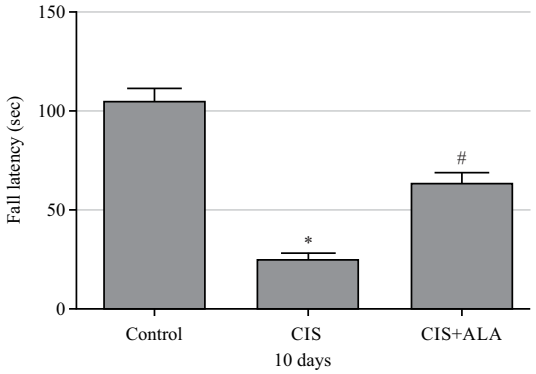

Fig. 2: Effect of alpha-linolenic acid on motor coordination of the 10 days gentamicin applied mice (n = 12)
Statistical analysis: ANOVA, *post hoc*: Bonferroni *Control $p<0.05$ and #Gentamicin $p<0.05$

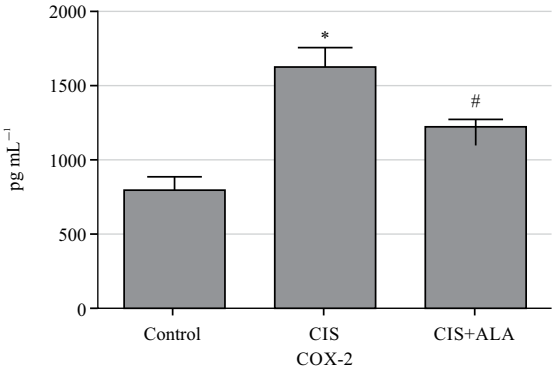

Fig. 3: Effect of alpha-linolenic acid on Cyclooxygenase-2 enzyme of the gentamicin applied mice (n = 12)
Amount of COX-2 in the homogenates is shown as pg mL^{-1}, Statistical analysis: ANOVA, *post hoc*: Bonferroni, *Control $p<0.05$ and #Gentamicin $p<0.05$

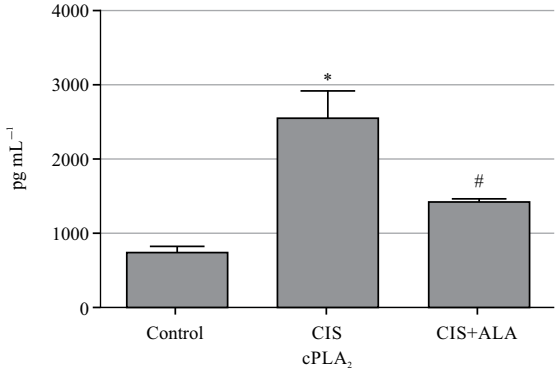

Fig. 4: Effect of alpha-linolenic acid on Phospholipase A_2 enzyme of the gentamicin applied mice (n = 12)
Amount of $cPLA_2$ in the homogenates is shown as pg mL^{-1}, Statistical analysis: ANOVA, *post hoc*: Bonferroni, *Control $p<0.05$ and #Gentamicin $p<0.05$

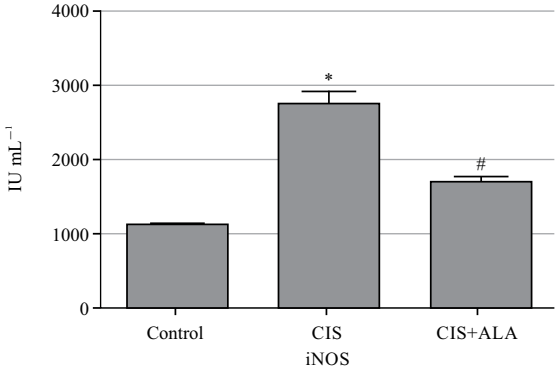

Fig. 5: Effect of alpha-linolenic acid on iNOS enzyme of the gentamicin applied mice (n = 12)
Amount of iNOS in the homogenates is shown as pg mL^{-1}, Statistical analysis: ANOVA, *post hoc*: Bonferroni, *Control p<0.05 and #Gentamicin p<0.05

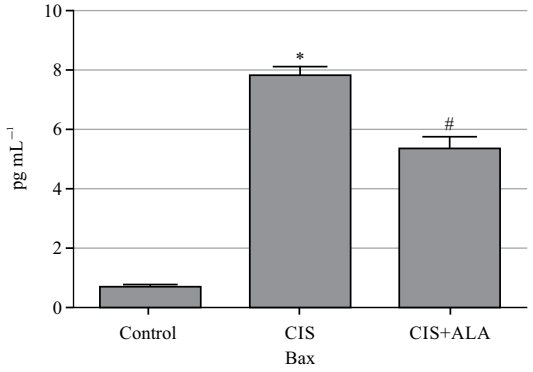

Fig. 6: Effect of alpha-linolenic acid on bax of the gentamicin applied mice (n = 12)
Amount of bax in the homogenates is shown as pg mL^{-1}, Statistical analysis: ANOVA, *post hoc*: Bonferroni, *Control p<0.05, #Gentamicin p<0.05

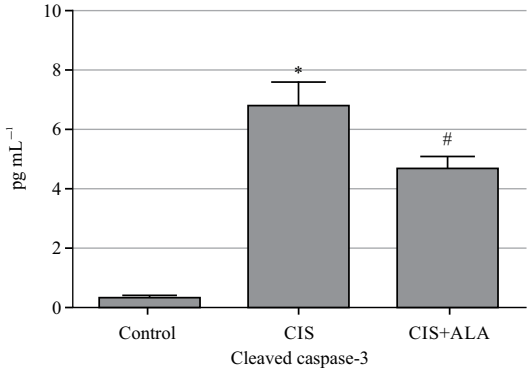

Fig. 7: Effect of alpha-linolenic acid on caspase-3 of the gentamicin applied mice (n = 12)
Amount of caspase-3 in the homogenates is shown as pg mL^{-1}, Statistical analysis: ANOVA, *post hoc*: Bonferroni, *Control p<0.05 and #Gentamicin p<0.05

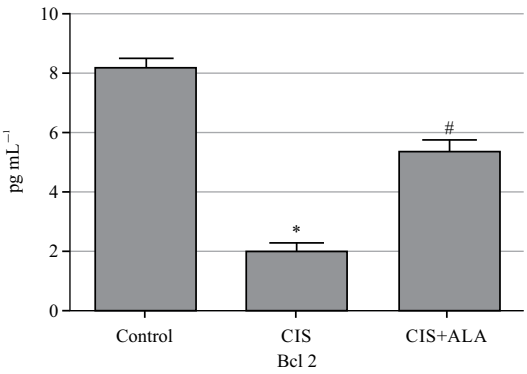

Fig. 8: Effect of alpha-linolenic acid on bcl-2 of the gentamicin applied mice (n = 12)
Amount of bcl-2 in the homogenates is shown as pg mL^{-1}, Statistical analysis: ANOVA, *post hoc*: Bonferroni, *Control p<0.05 and #Gentamicin p<0.05

were significantly increased in the ALA+CIS treated group than in the CIS treated group (p<0.05) (Fig. 8).

DISCUSSION

In this study, the rotarod test was used to evaluate the motor coordination of mice. The Cis application to mice shortened the fall time of the animals in the rotarod test. Alpha-linolenic acid reversed this situation. Cisplatin-induced ototoxic damage has been shown to occur especially in the outer hair cells and inner hair cells in the basal fold of the cochlea[10]. The rotarod test we used showed that alpha-linolenic acid has a protective role against CIS-induced ototoxicity. A study has shown that ALA reduces the expression of inflammation mediators such as iNOS and Cox-2[7,8]. Since this will reduce inflammation, the migration of monocytes and macrophages will be prevented and cochlear damage will be prevented.

Cisplatin causes its ototoxicity through two separate mechanisms, these are ion channel blockade and lipid peroxidation. In the study conducted by Peters *et al.*[11] they showed that cisplatin causes hyperpolarization by blocking the ion transfer channels in the membrane of outer hair cells.

Increased intracellular oxidative stress causes the initiation of apoptosis[12,13]. Although reactive oxygen products, which increase with oxidative stress, serve as secondary messengers in the signal transduction system at low concentrations, they damage the vital structures of the cell when produced in excessive amounts[14]. Transcription factors such as NF-κβ, p53 and AP-1 are also modulated by these reactive oxygen products[15]. Additionally, ROS serve as secondary messengers in the physiological stimulation of

angiotensin and inflammatory cytokines. However, super oxide radicals are produced by neutrophils and phagocytes in the immunological defense system against bacteria. In short, there is an intracellular balance between intracellular reactive oxygen products[16]. This study suggested that CIS application causes cochlear damage by increasing oxidative stress and inflammation. Alpha-linolenic acid has a protective effect by reducing CIS-induced oxidative stress and inflammation due to its antioxidant and anti-inflamatory properties. Our study showed that ALA protects against cisplatin-induced ototoxicity. Cisplatin is widely used in cancer treatment and the most important effect of this substance is its toxic effects on many organs, such as ototoxicity and nephrotoxicity. Since ALA is a natural and economical substance, it is important that it reduces the side effects of cisplatin. In light of the data obtained from this study, it is clear that ALA will prevent the side effects of cisplatin because it reduces both inflammatory mediators and apoptotic mediators. Based on previous studies with ALA, it is predicted that ALA will prevent the side effects of many toxic drugs and will lead by Kaplan *et al.*[17].

CONCLUSION

Cisplatin is one of the antineoplastic agents that can be used frequently at all ages. For this reason, many agents have been tried to prevent ototoxicity caused by cisplatin. Since there is no molecule that will contain all the sites of action of cisplatin ototoxicity, there is no curative treatment yet. Additionally, other studies need to show whether the agents used to prevent ototoxicity will reduce the antineoplastic effect of cisplatin. More studies are needed to prevent and treat cisplatin ototoxicity. In conclusion, our study showed that dietary intake of alpha-linolenic acid would be beneficial as it prevents cis-dependent motor coordination impairment.

SIGNIFICANCE STATEMENT

Cytotoxicity is one of the most important problems of cisplatin use. Radical chemotherapeutics such as cisplatin are the main treatment for cancer. However, chemotherapeutics are toxic to cancer cells as well as normal cells. In this study cisplatin treatment increased inflammatory and apoptotic mediators alpha-linolenic acid decreased these mediators in cochlear cell. These findings suggest that alpha-linolenic acid attenuate cytotoxicity of cisplatin. Cisplatin is the definitive cure for cancer when the patient survives and ALA can increase patient survival.

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