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Research Article
Evaluating the Protective Effects of Amniotic Fluid-Derived Culture Medium on Cisplatin-Induced Nephrotoxicity in a Rat Model

[1]Mehmet Zeynel Keskin, [2]Nilsen Erdoğan, [3]Aykut Sarıtaş, [4]Ömer Faruk Özer, [5]Yaren Ece Aydoğdu, [6]Yusuf Özlem İlbey, [7]Mümin Alper Erdoğan, [1]Yusuf Arıkan and [8]İbrahim Aydoğdu

[1]Department of Urology, Tepecik Training and Research Hospital, Saglik Bilimleri University, Izmir, Turkey
[2]Department of Pathology, Bağcılar Training and Research Hospital, Istanbul, Turkey
[3]Izmir City Hospital Anesthesiology Clinic, Izmir, Turkey
[4]Department of Biochemistry, School of Medicine, Bezmialem Vakif University, Istanbul, Turkey
[5]School of Medicine, Bezmialem Vakif University, Istanbul, Turkey
[6]Department of Urology, School of Medicine, Bezmialem Vakif University, Istanbul, Turkey
[7]Department of Physiology, Faculty of Medicine, Izmir Katip Çelebi University, Izmir, Turkey
[8]Department of Pediatric Surgery, School of Medicine, Bezmialem Vakif University, Istanbul, Turkey

Abstract

Background and Objective: Cisplatin (CP) is a chemotherapy drug extensively utilized for its efficacy in treating a variety of cancer types. Despite its therapeutic benefits, CP is notably associated with renal toxicity as a side effect. The ongoing research into mitigating agents against CP-induced renal toxicity focuses on substances with antioxidant and anti-inflammatory characteristics. This investigation aims to explore the potential protective properties of a cell culture medium derived from human amniotic fluid (AMX) against renal toxicity prompted by CP in a rodent model. **Materials and Methods:** Fifty-five male Wistar albino rats were divided into five groups: Control, AMX, CP, CP+AMX and AMX+CP. All agents were administered intraperitoneally. The study assessed serum parameters, histopathological changes and oxidative/antioxidative markers in the kidneys. Blood urea nitrogen (BUN) and creatinine (Cre) levels were measured to evaluate renal function, while total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) were analyzed for oxidative damage. **Results:** The AMX pre-treatment group showed a significant decrease in BUN and Cre levels, indicating a reduction in CP-induced renal damage. Histopathologically, AMX pre-treatment reduced inflammation, congestion and edema in the kidneys. Although TAS levels increased post-CP administration, no significant changes were observed in TOS and OSI values. The study demonstrates that AMX can mitigate CP-induced nephrotoxicity, as evidenced by improved renal function markers and reduced histopathological damage. **Conclusion:** The protective effects of AMX may be attributed to its anti-inflammatory properties and the presence of growth factors and glutamine. While promising, further research is required to fully elucidate AMX's protective mechanisms against CP-induced renal damage.

Key words: Cisplatin, nephrotoxicity, amniotic fluid, rats, antioxidant, anti-inflammatory, renal protection

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Corresponding Author: Mehmet Zeynel Keskin, Saglik Bilimleri University Tepecik Training and Research Hospital, Yenisehir Mah, Gaziler Cad. No. 468, 35180, Konak, Izmir, Turkey Tel: +90530118458

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

INTRODUCTION

Cisplatin (CP), also known as cis-diamminedichloroplatinum (II), is an exceptionally effective anti-cancer agent utilized in treating various cancers, including those of the lung, uterus, rectum and testis[1]. As a DNA alkylator, CP functions by generating crosslinks within DNA, leading to double helix breakage, thereby exerting its antitumor effects[2]. However, the clinical application of CP requires careful consideration due to its severe side effects. Notably, approximately one-third of patients receiving CP treatment unfortunately develop treatment-related nephrotoxicity[3]. This nephrotoxicity is attributed to tubulopathy resulting from the accumulation of CP in the epithelial cells of the proximal tubule, which is five times higher than its plasma concentration[4].

The need for effective measures to counteract CP-induced nephrotoxicity is an unmet clinical requirement, vital for improving its pharmacotoxicological profile and maximizing its benefits[5]. Nevertheless, no effective method has yet been identified to protect the kidneys during chemotherapy[6]. The pathogenesis of CP-induced nephrotoxicity remains not fully understood, but oxidative stress and reactive oxygen species (ROS) are considered among the most critical mechanisms in its pathogenesis[7]. Similarly, inflammation is also reported to play a significant role in CP nephrotoxicity[8]. In light of this information, research has focused on protective agents, particularly those with antioxidant and anti-inflammatory properties, to minimize nephrotoxicity associated with CP[9].

The medium used in this study, specifically designed for *in vitro* proliferation of primary cultures of human amniotic fluid cells and chorionic villus samples, is utilized in prenatal diagnostic testing. This medium, which is augmented with bovine fetal serum, an antibiotic (gentamicin) and an amino acid (L-glutamine), promotes optimal cell adhesion and proliferation. Thus far, the potential advantages of this human amniotic fluid-derived cell culture medium (AMX) against cisplatin (CP)-induced nephrotoxicity have not been assessed.

This research aimed to evaluate the possible protective properties of the amniotic fluid-derived cell culture medium against CP-induced nephrotoxicity. The study assessed serum parameters, histopathological alterations and oxidative/antioxidative biomarkers within the renal tissue of rats.

MATERIALS AND METHODS

Study area: This examination was carried out in Istanbul, Turkey from September, 2019 to March, 2020 at the Experimental Application and Research Center of Bezmialem Vakif University, Turkey.

Ethical approval: The Bezmialem Vakif University's local ethics committee for animal studies gave its clearance (2019/99) before the procedures started and the whole study complied with the Research Council's Handbook for the Care and Use of Laboratory Animals.

Animals: The study was conducted at the Bezmialem Vakıf University, Experimental Animal Laboratory. It involved 55 male Wistar albino rats aged 10-12 weeks, weighing 250-300 g. The rats were housed at room temperature (25°C) with a relative humidity of 50-55% and a 12:12 hrs light-dark cycle in well-ventilated, clean plastic cages, starting one week before the study commenced. Animals had *ad libitum* access to feed and water.

Experimental protocol: The rats were divided into five groups of 11 each: Control (C), AMX, CP, CP+AMX and AMX+CP. All agents were administered intraperitoneally. The first day of treatment was considered day 1. The control group received 1 mL/kg physiological saline (PS) for the first four days. The AMX group received 1 mg/kg PS on the first day, followed by 0.3 cc AMX for the next three days. The CP group was given a single 7 mg/kg dose of cisplatin on the first day, followed by 1 mL/kg PS for the next three days. The CP+AMX group received a single 7 mg/kg dose of cisplatin on the first day, followed by 0.3 cc AMX for the subsequent three days. The AMX+CP group received 0.3 cc AMX for the first three days, followed by 7 mg/kg CP on the fourth day. At the end of the experiment, rats were anesthetized with an i.p., injection of 50 mg/kg ketamine and 5 mg/kg xylazine. The experiment was concluded at the end of the fourth week, with the collection of tissue and blood samples for biochemical and histopathological analyses. On day 28, all rats underwent bilateral nephrectomy under anesthesia, followed by serum collection and sacrifice.

For histopathological analysis, right kidneys were immediately preserved in 10% buffered formaldehyde. The right kidneys were evaluated histopathologically for renal tubular damage. The left kidneys were cleansed of blood with ice-cold 0.9% NaCl solution, blotted and stored at -40°C for biochemical analyses. The left kidneys were analyzed for total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI). Serum levels of blood urea nitrogen (BUN) and creatinine (Cre) were also measured and compared across groups.

Blood analysis: Blood samples were collected from each rat post-sacrifice and centrifuged at 400xg for 15 min at 4°C. The serum was then stored at -85°C for kidney function tests. Serum BUN and Cre levels were measured using commercial kits (Roche Diagnostic, Mannheim, Germany) on automated devices (COBAS c 501; Hitachi Ltd., Tokyo, Japan).

Biochemical analysis: Each rat's frozen tissue, weighing approximately 100 mg, was homogenized in a 1 mL solution of phosphate-buffered saline in an Eppendorf tube using a Tissue Lyser II grinding jar set. Subsequently, each homogenate underwent centrifugation at a force of 1,200 times the acceleration due to gravity for 15 min. The total antioxidant status (TAS) of kidney tissue was quantified using an autoanalyzer (AU5800; Beckman Coulter Inc., Brea, California, USA) and a Rel Assay kit (Rel Assay Diagnostics, Gaziantep, Türkiye)[10]. This method is based on the ability of antioxidant molecules to convert the blue-green ABTS or ABTS radical into a colorless form. The variations in absorbance at a wavelength of 660 nm are directly related to the overall quantities of antioxidants in the sample. Trolox was employed as a calibrator and the outcomes are denoted as micromoles of Trolox equivalents per liter per milligram of protein. The TOS in renal tissue was quantified using an autoanalyzer and a Rel Assay kit (Rel Assay Diagnostics, Gaziantep, Türkiye)[11]. This kit operates based on the idea that oxidant molecules convert iron ions from their ferrous to ferric state, resulting in the formation of a chromogenic-colored complex in an acidic setting. The color intensity, as determined by a spectrophotometer, is directly proportional to the concentration of oxidant molecules present in the sample. The calibrator utilized in this study was hydrogen peroxide and the results are reported as micromoles of H_2O_2 equivalents per liter per milligram of protein. The OSI or the oxygen saturation index, is calculated by dividing the total oxygen saturation (TOS) by the total arterial saturation (TAS). The resulting value is reported in arbitrary units (AU).

Histopathological examination: Tissue samples were fixed in 10% formalin and then embedded in paraffin blocks. Sections of 4 µm thickness were prepared from these blocks, deparaffinized, hydrated and stained with Hematoxylin and Eosin (H&E). All sections were evaluated under a light microscope (Olympus CX51, Tokyo, Japan) for inflammation, congestion of Red Blood Cells (RBCs) and necrosis. The assessment was performed blindly by a single pathologist. Scoring was categorized as 0: Normal, 1: Mild, 2: Moderate and 3: Severe.

Statistical analysis: Power analyses were conducted prior to the study, determining that eleven rats per group would suffice to interpret the results (86% actual power at a 95% confidence interval, with $\eta 2 = 0.35$ and effect size $f = 0.73$). Statistical analysis was performed using SPSS version 22.0 software. The Shapiro-Wilks test assessed the normality of variable distributions. Statistical data were expressed as Mean±Standard Deviation (SD) or median and interquartile range for normally and non-normally distributed variables, respectively. A paired t-test was used for comparisons between two dependent groups. The One-way Analysis of Variance (ANOVA) tested differences between groups. Where significant differences were found, the Tukey multiple comparison test was applied for further analysis. Significance was set at $p<0.005$ in the Tukey's test. The Kruskal-Wallis test evaluated differences among groups for histological parameters and the Mann-Whitney U test compared each pair of groups. Significance was set at $p<0.05$.

RESULTS

Blood parameters: The average values and standard deviations of blood urea nitrogen (BUN) and creatinine (Cre) for all rats included in the study were presented in Table 1. When analyzing BUN across the five groups, significant differences necessitated further pairwise analysis ($p<0.001$). This revealed a significant decrease in BUN in the AMX group compared to the control group ($p = 0.003$). In pairwise analyses related to total antioxidant status (TAS), a significant difference was observed between the control and CP groups ($p = 0.034$). The CP group showed a significant increase in BUN compared to the AMX group ($p = 0.002$), while the AMX+CP group exhibited a significant decrease in BUN compared to both the CP group ($p<0.001$) and CP+AMX group ($p = 0.002$) (Table 2).

For Cre levels, parametric tests were used. After confirming the homogeneity of distribution according to Levene's test, ANOVA was performed, revealing significant differences between groups ($p<0.001$). Subsequent *post hoc* Tukey's tests found significant increases in Cre levels in the CP ($p = 0.001$) and CP+AMX ($p = 0.002$) groups compared to the AMX group. However, no significant increase was observed in the AMX+CP group compared to the AMX group ($p = 0.75$) (Table 2).

Table 1: Average results of blood parameters in experimental groups

Blood parameter (n = 55)	Mean	Standard deviation	Minimum	Maximum
BUN (mg/dL)	50.4	18.81	33.0	115
Cre (mg/dL)	0.53	0.04	0.45	0.65

BUN: Blood urea nitrogen and Cre: Creatinine

Table 2: Pairwise analysis of blood parameters in the experimental groups

Blood parameter	Control (n = 11)	AMX (n = 11)	CP (n = 11)	CP+AMX (n = 11)	AMX+CP (n = 11)
BUN (mg/dL)					
Mean (min-max)	46.64 (41-51)	41 (33-47)[a]	54.73 (40-72)[b]	70.55 (36-115)[ab]	39.09 (36-43)[acd]
Cre (mg/dL)					
Mean (min-max)	0.531 (0.50-0.56)	0.505 (0.45-0.54)	0.57 (0.47-0.65)[T]	0.567 (0.51-0.64)[T]	0.523 (0.49-0.58)

AMX: Amniotic fluid, BUN: Blood urea nitrogen, Cre: Creatinine, CP: Cisplatin, [a]p<0.05 compared with control group in Mann-Whitney U test, [b]p<0.05 compared with AMX group in Mann-Whitney U test, [c]p<0.05 compared with CP group in Mann-Whitney U test, [d]p<0.05 compared with CP+AMX group in Mann-Whitney U test and [T]p<0.005 compared with AMX group in Tukey's test

Table 3: Results of biochemical findings of experimental groups

Biochemical variables (n = 55)	Mean	Standard deviation	Minimum	Maximum
TAS (μmol Trolox equivalent/L)	1.05	0.07	0.87	1.32
TOS (μmol H_2O_2 equivalent/L)	26.21	25.57	6.00	146
OSI (AU)	24.07	20.95	5.71	110.61

TAS: Total antioxidant status, TOS: Total oxidant status and OSI: Oxidative stress index

Table 4: Pairwise biochemical findings in the experimental groups

Biochemical variables	Control (n = 11)	AMX (n = 11)	CP (n = 11)	CP+AMX (n = 11)	AMX+CP (n = 11)
TAS (μmol Trolox equivalent/L)					
Mean (min-max)	1.02 (0.87-1.32)	1.01 (0.99-1.08)	1.08 (0.99-1.14)[ab]	1.06 (0.99-1.14)[b]	1.06 (1.02-1.23)[b]
TOS (μmol H_2O_2 equivalent/L)					
Mean (min-max)	40.54 (7-146)	18.36 (6-39)	23.18 (9-33)	16.27 (7-45)	32.72 (9-91)
OSI (AU)					
Mean (min-max)	36.09 (7.53-110.61)	18.04 (5.71-36.11)	21.26 (8.33-29.73)	14.98 (6.48- 39.47)	29.96 (8.57- 73.98)

AMX: Amniotic fluid, CP: Cisplatin, TAS: Total antioxidant status, TOS: Total oxidant status, OSI: Oxidative stress index, [a]p<0.05 compared with control group in Mann-Whitney U test and [b]p<0.05 compared with AMX group in Mann-Whitney U test

Table 5: Histopathological findings in the experimental groups

Histopathological variables	Control (n = 11)	AMX (n = 11)	CP (n = 11)	CP+AMX (n = 11)	AMX+CP (n = 11)
Inflammation					
Mean (min-max)	0	0	1.54 (0-2)[ab]	1.09 (0-2)[ab]	0.9 (0-2)[abc]
Congestion					
Mean (min-max)	0.45 (0-1)	0.72 (0-1)	1.36 (1-2)[ab]	1.27 (1-2)[ab]	1.18 (1-2)[ab]
Necrosis					
Mean (min-max)	0	0	1.81 (1-3)[ab]	1.36 (0-2)[ab]	1.09 (0-2)[ab]

AMX: Amniotic fluid, CP: Cisplatin, [a]p<0.05 compared with control group in Mann-Whitney U test, [b]p<0.05 compared with AMX group in Mann-Whitney U test and [c]p<0.05 compared with CP group in Mann-Whitney U test

Biochemical results: Average values and standard deviations for total oxidant status (TOS), TAS and oxidative stress index (OSI) for all rats included in the study are presented in Table 3. Biochemical analysis of TOS, TAS and OSI across the five groups found no significant differences in TOS (p = 0.191) and OSI (p = 0.19), while significant differences were observed in TAS levels (p = 0.008). Pairwise analysis of TAS showed significant differences between the control and CP groups (p = 0.034). Both the CP+AMX (p = 0.019) and AMX+CP (p = 0.019) groups had higher TAS values compared to the AMX group (Table 4).

Histopathological investigation: Histopathological evaluation in our study was based on inflammation, congestion and edema. When comparing these parameters across the five groups, significant differences were observed (p<0.001). Subsequent pairwise analyses revealed significant increases in inflammation (p<0.001), congestion (p = 0.003) and necrosis (p<0.001) in the CP group compared to the control group. In the CP+AMX group, significant increases in inflammation (p = 0.001), congestion (p = 0.007) and necrosis (p<0.001) were observed compared to the control group. Similarly, the AMX+CP group also showed significant increases

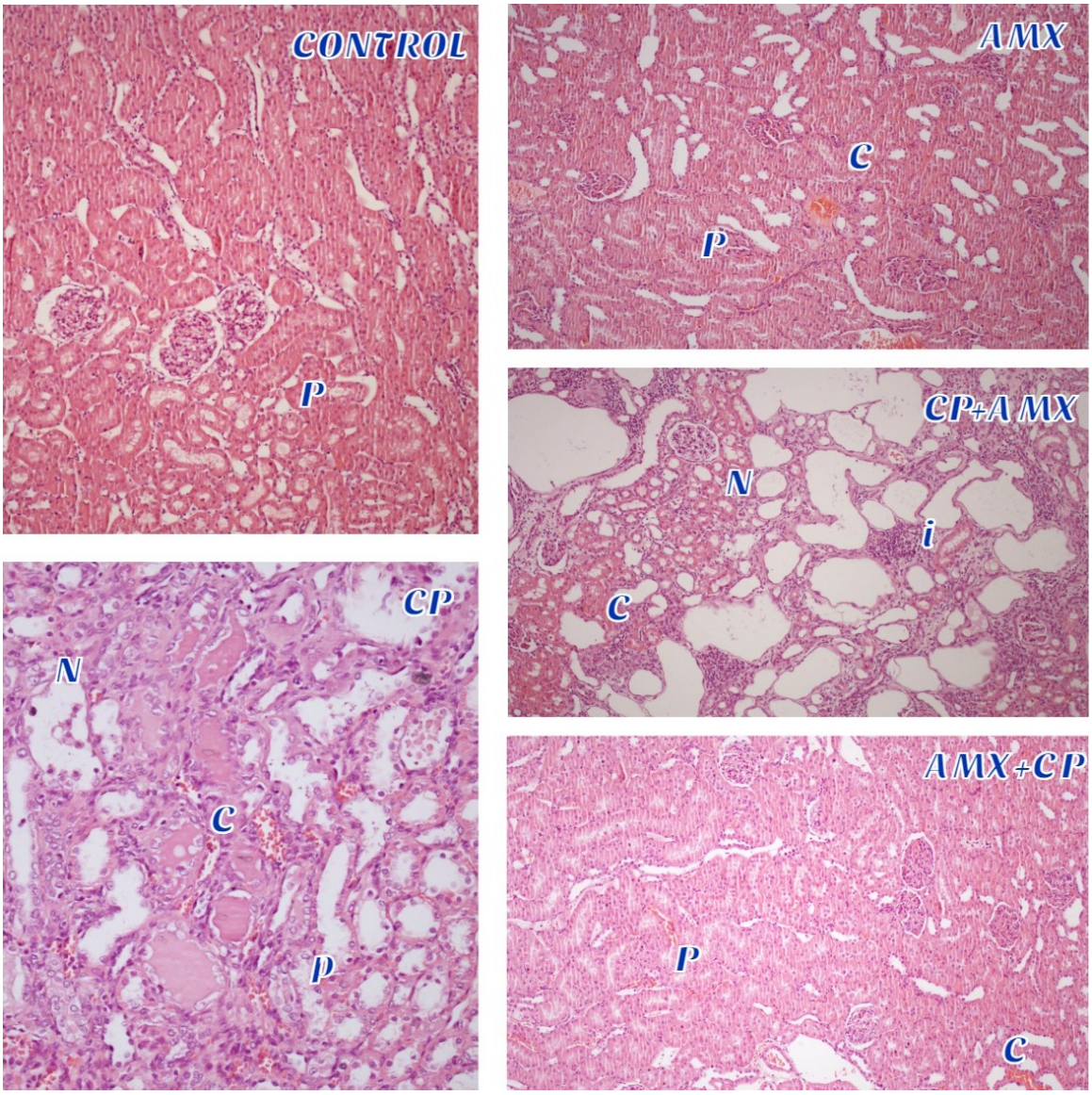

Fig. 1: Light microscopy images of H&E stained sections of kidney samples from control and experimental groups
P: Proximal tubules, I: Inflammation, C: Congestion of RBCs and N: Necrosis. Control group H&E x100, AMX group H&E x40, CP group H&E x400, CP+AMX group H&E x40 and AMX+CP group H&E x40

in all three parameters compared to the control group ($p = 0.001$, $p = 0.007$ and $p = 0.001$). No significant histopathological differences were observed between the CP and CP+AMX groups, but inflammation was significantly reduced in the AMX+CP group compared to the CP group ($p = 0.034$) (Table 5) (Fig. 1).

DISCUSSION

Cisplatin (CP) is one of the widely used chemotherapeutic agents for various types of cancer. The most crucial aspect of utilizing CP in cancer chemotherapy is protecting against the side effects it may cause[12]. The CP alkylates DNA by creating intra-strand crosslinks, thereby intervening in DNA repair mechanisms and leading to p53 activation and cell cycle cessation, which induces apoptosis[1]. Since there is currently no approved treatment protocol or specific antidote against the possible toxic effects of cisplatin, different toxicity prevention strategies are being rigorously explored[13].

To achieve maximum growth, it is essential to develop amniotic fluid cells in a culture medium that is filled with nutrients. The AMX is a full-support medium developed for short-term culture of human amniotic fluid cells for cytogenetic studies and *in vitro* diagnostic procedures.

The AMX, which contains fetal bovine serum (FBS), gentamicin and L-glutamine, ensures high levels of cell adhesion and growth. This optimized medium has a buffering system developed to ensure greater pH stability during culture manipulations[14]. Its efficacy has been shown in various studies[15-18]. One study demonstrated that AMX injection suppressed oxidative stress and stimulated the antioxidant system, thereby preventing ischemia and reperfusion injury in rat testes[19]. Another study showed that AMX, an amniotic fluid-derived cell culture medium, reduced oxidative stress and inflammation and was effective in tracheal healing[20]. This study investigated the effects of AMX on cisplatin-induced renal injury and examined the potential mechanisms underlying this effect.

Glutamine in AMX is known to be a substrate used for the synthesis of the antioxidant Glutathione (GSH)[21]. Thus, glutamine's antioxidant properties can play a role experimentally in cells[22]. The first animal study in the literature evaluating glutamine against CP-induced nephrotoxicity found that orally administered glutamine was not effective enough to protect the kidneys from CP-induced nephrotoxicity[23]. However, another study showed that parenterally administered glutamine reduced renal function impairment, tissue damage and tubular cell death caused by CP[24]. Subsequent studies have supported the protective effect of glutamine[25,26].

Serum BUN and creatinine levels are known markers of glomerular damage and their increase indicates impaired kidney function[27]. The CP-induced nephrotoxicity damages proximal and distal tubules, leading to suppressed reabsorption, increased BUN and creatinine levels and elevated vascular resistance[28]. In the current study, an increase in serum BUN and creatinine levels was observed after CP administration, consistent with similar findings in the literature[29,30]. Pre-administration of AMX before cisplatin led to a decrease in both BUN and creatinine levels, indicating an improvement in kidney functions.

Reactive oxygen species (ROS) are oxygen and nitrogen-based reactive molecules produced in mitochondria during normal cellular activities[31]. In excessive amounts, ROS can lead to alterations in the mitochondrial membrane, which in turn results in the production of even more ROS[32]. In normal cells, a balance is maintained between prooxidants that generate ROS and antioxidants that scavenge them[33]. In the present study, total oxidant status (TOS) was utilized as a marker for prooxidant molecules, while total antioxidant status (TAS) served as a marker for antioxidant molecules. Concurrently, the oxidative stress index (OSI), calculated as TOS/TAS, was also assessed. Mirapoglu *et al.*[20] identified significant variations in TAS, TOS and OSI values compared to a control group in their study on the effects of AMX administration on tracheal healing in rats. In the current study, however, no significant changes were noted in TOS and OSI values following CP administration. Nonetheless, a marked increase in TAS was observed after CP administration. These findings in biochemical analysis are in contrast with results reported in the literature[34-36]. It is also noteworthy that neither pre- nor post-CP administration of AMX resulted in any significant alterations in TAS values.

In this study, significant increases in inflammation, congestion and edema at the histopathological level in the kidneys were observed after CP administration compared to the control group. Oxidative molecules are implicated in the histopathological damage that follows CP administration[37]. The inflammatory response developed against these oxidative molecules contributes to this histopathological damage. The histopathological changes induced by CP in our study align with those reported in similar studies in the literature[34,38,39]. Additionally, our study demonstrated that pre-administration of AMX significantly reduced tissue inflammation compared to CP. This improvement is thought to stem from the anti-inflammatory properties of AMX.

This study, primarily a pilot investigation into the protective effects of AMX against CP-induced nephrotoxicity, is subject to several limitations. To evaluate oxidant and antioxidant effects, other tests such as malondialdehyde (MDA), Glutathione (GSH) or superoxide dismutase (SOD) could be utilized. The topic investigated in this study could be further detailed by administering different doses of AMX or by creating an experimental tumor model. Another limitation is the lack of support for the study's findings with immunohistochemical and electron microscopic evidence.

In conclusion, cisplatin treatment causes tissue damage and increases the levels of urea and creatinine in the blood. This study is the first in the literature to demonstrate that pre-administration of AMX can prevent the rise in BUN and creatinine levels in the blood and mitigate histopathological tissue damage caused by cisplatin. These characteristics may be attributed to the antioxidant and anti-inflammatory properties of AMX, as well as to the abundance of growth factors and the presence of glutamine in AMX. In this context, AMX emerges as a promising agent in combating CP-induced nephrotoxicity.

Despite these promising results, the study recognizes certain limitations, such as the absence of more detailed oxidative and antioxidative balance assays and the lack of immunohistochemical and electron microscopic evidence. Future research should aim to explore these avenues, possibly involving different AMX dosages or experimental tumor models, to fully understand AMX's protective mechanism. Nonetheless, this study marks a significant step forward in identifying AMX as a potential therapeutic agent for reducing CP-induced nephrotoxicity, encouraging further exploration in this vital area of cancer treatment.

CONCLUSION

This study has provided valuable insights into the protective effects of AMX against cisplatin (CP)-induced nephrotoxicity in rats. Our findings indicate that pre-administration of AMX can significantly reduce CP-induced renal damage, as evidenced by lowered blood urea nitrogen (BUN) and creatinine levels. This is a crucial development in the quest for mitigating CP's nephrotoxic side effects, which currently pose a significant challenge in clinical settings. Furthermore, the histopathological analysis showing reduced inflammation, congestion and edema in renal tissues with AMX pre-treatment highlights its potential therapeutic benefits.

SIGNIFICANCE STATEMENT

This study provides critical insights into the protective effects of AMX against nephrotoxicity induced by cisplatin (CP) in rats, highlighting its potential role as a renal-protective agent for cancer patients undergoing chemotherapy. The results demonstrate that AMX administration, particularly when given prior to CP treatment, significantly enhances antioxidant activity, reduces inflammation and mitigates the detrimental impact of CP on renal tissue. These findings contribute to the growing body of research on nephroprotection strategies for cancer patients and emphasize the potential therapeutic value of AMX in safeguarding renal function during chemotherapy involving CP.

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