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The therapeutic effect of Cilastatin on drug-induced nephrotoxicity: a new perspective

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Abstract. – OBJECTIVE: By creating nephrotoxicity models with cisplatin, vancomycin, and gentamicin in HK-2 (human renal proximal tubule cell) and HEK293T (human embryonic kidney epithelial cells) cell lines, we aimed to evaluate the effect of cilastatin on recovery of cell damage after toxicity had occurred.

MATERIALS AND METHODS: In the first phase of the study, the doses of cisplatin, vancomycin, and gentamicin (50% inhibitive concentration; IC50) were determined. In the second phase, the effective dose of cilastatin against these drugs was determined, and IC50 doses of nephrotoxic agents were administered simultaneously. In the third phase of our study, to evaluate the possible therapeutic effect of cilastatin after toxicity had occurred, the analyses of cell viability, apoptosis, oxidative stress, expression of kidney injury molecule-1 (KIM-1), and neutrophil gelatinase-associated lipocalin (NGAL) were performed.

RESULTS: In the second phase of the study, it was observed that cilastatin increased cell viability when treated simultaneously with a nephrotoxic agent. In the third phase, cilastatin provided a significant increase in cell viability. After treatment with each agent for 24 hours, we determined that adding cilastatin to the medium had an effect on the recovery of cell damage by increasing cell viability and reducing apoptosis and oxidative stress. The expression of KIM-1 and NGAL increased when nephrotoxicity occurred and decreased with the addition of cilastatin to the medium.

CONCLUSIONS: The findings of the study suggest that cilastatin may have a healing effect after the development of nephrotoxicity.

Key Words:
In vitro, Cilastatin, KIM-1, NGAL, Nephrotoxicity.

Introduction

It has been reported that drug-induced nephrotoxicities account for about 20% of acute kidney injury (AKI) occurring during hospitalization. Nephrotoxicity limits the effective use of drugs and increases morbidity and mortality by causing serious kidney damage. During drug development studies, most of the preclinical projects fail due to safety problems. Studies on animal models have led the studies to *in vitro* modeling due to the differences between species and the high cost and limitations placed on animal studies. For this purpose, *in vitro* kidney cell culture models developed to evaluate kidney damage have been evolved. These models have been shown to be an indispensable tool for evaluating the functionality of these cells[1-3].

Cilastatin is a specific renal dehydropeptidase-I (DHP-I) inhibitor located on the brush-border zone of proximal tubular epithelial cells (PTECs). DHP-I is an enzyme responsible for both the metabolism of thienamycin beta-lactam antibiotics and the conversion of leukotriene D4 into leukotriene E4. Cilastatin prevents imipenem from being metabolized by DHP-I in the proximal renal tubules and increases its stability. It has been used for a long time to increase the effectiveness of imipenem and to prevent its nephrotoxic effect[4]. The protective effect of cilastatin on this imipenem-induced nephrotoxicity has also been investigated on other drugs, such as antibiotics, immunosuppressants, chemotherapeutics, and analgesics. These studies[5-16] have shown that it has a protective effect on drug-induced nephrotoxicity without altering their therapeutic activity in target cells.

Cisplatin, vancomycin, and gentamicin are among the drugs that are known to be nephrotoxic

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and are commonly used in clinical practice. The effect of cilastatin has also been studied on these drugs and it has been shown in various studies that, when administered in combination, it prevents the nephrotoxic effects of these drugs. To date, the protective effect of cilastatin has been studied in many studies[5-12]; however, its possible effectiveness after toxicity has not been investigated. In this study, we aimed to evaluate the effect of cilastatin on the repair of cell damage after creating nephrotoxicity models with cisplatin, vancomycin, and gentamicin in HK-2 (human renal proximal tubule cell) and HEK293T (human embryonic kidney epithelial cells) cell lines.

Materials and Methods

Cell Culture

Hk-2 human kidney proximal tubule cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was cultured in Dulbecco's Modified Eagle's and Ham's F-12 Medium (DMEM/F12, GIBCO, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. HEK293T cell line was cultured in T75 flask in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium by adding 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO_2 and at 95% humidity.

Determination of Appropriate IC50 Doses of Cisplatin, Vancomycin, and Gentamicin in Nephrotoxicity Model and Also Regenerative Effective Doses of Cilastatin Against Nephrotoxicity

After the achievement of appropriate proliferation dynamics in HEK293T and HK-2 cell lines, cell proliferation assays were performed using a CCK-8 kit (Abbkine, China, item No: KTC011001). Cells were washed with phosphate-buffered saline, trypsinized, counted with a hemocytometer, and seeded into 96-well plates ($1x10^4$ cells/mL). After a 24 h incubation at 37°C in a 5% CO_2 incubator, the medium was removed, and cells were treated with drugs in different concentrations in order to determine the 50% inhibitive concentration (IC_{50}) of cisplatin (TRC, Canada, CAT:C499500), vancomycin (Vancomax, TURKEY) and gentamicin (Wisent Bioproducts, CANADA,CAT:400-135), and to determine the appropriate nephroprotective doses of cilastatin (BLD Pharm., CHINA, CAT:BD121289) on nephrotoxicity model induced by these drugs.

Determination of IC50 Doses for Cisplatin, Vancomycin, and Gentamicin

In the HK-2 and HEK293cell lines, 1 x 10^4 cells/well (100 ml) were cultivated into 96-well plates. Twenty-four hours after the cells were cultivated, culture media were changed and incubated with cisplatin (Toronto research chemical) at doses of 1 µM, 10 µM, 50 µM, 100 µM, and 200 µM; vancomycin (Vancomax) at doses of 10 µM, 100 µM, 1000 µM, 2000 µM, 4000 µM, and 5000 µM, and gentamicin at doses of 1000 µM, 5000 µM, 10000 µM, and 20000 µM. Then, the cells were cultivated for 48 hours at 37°C, 5% CO_2, and 95% humidity. At the end of the incubation period, cell viability in the wells was analyzed using a CCK-8 kit (Abbkine, China, item no: KTC011001). In line with the kit protocol, 5 µl of the kit chemical was added to the wells after 48 hours of incubation. After 3 hours of incubation at 37°C, absorbance values were measured in Promega Glomax (Promega, Madison, WI, USA) device at 450 nm.

Determination of Appropriate Dose, Duration of Treatment, and Effective Combinations of Cilastatin Against Nephrotoxic Drugs With Established IC50 Doses

To determine the most effective dose and length of treatment of cilastatin against nephrotoxic drugs, we treated the cells with cilastatin at doses of 50, 100, 250, and 500 µg/ml after the IC50 doses of cisplatin, vancomycin, and gentamicin. For this purpose, the cell lines were seeded in 96-well plates at 1 x 10^4 cells/well (100 ml), and cilastatin at doses of 50, 100, 250, and 500 µg/ml was applied together with the IC50 doses of each nephrotoxic agents for 24 and 48 hours. At the end of the incubation periods, cellular viability was analyzed in accordance with the CCK-8 kit (Abbkine, China, item no: KTC011001) protocol. All of the treatments were performed in triplicate. Three groups of experiments were designed to evaluate the effect of cilastatin after nephrotoxicity occurred in HEK293T and HK-2 cell lines.

Group 1. Cilastatin was added to the medium 16 hours after the application of the nephrotoxic agent, and evaluation was made after 24 hours:

- Cisplatin at the IC_{50} dose for 16 hours + cilastatin at a dose of 100 µg/µl for 8 hours.
- Vancomycin at the IC_{50} dose for 16 hours + cilastatin at a dose of 100 µg/µl for 8 hours.
- Gentamicin at the IC_{50} dose for 16 hours + cilastatin at a dose of 100 µg/µl for 8 hours.

Group 2. Cilastatin was added to the medium 24 hours after the application of the nephrotoxic agent, and evaluation was made after a total of 48 hours:

- Cisplatin at theIC_{50} dose for 24 hours + cilastatin at a dose of 100 μg/μl for 24 hours
- Vancomycin at the IC_{50} dose for 24 hours + cilastatin at a dose of 100 μg/μl for 24 hours
- Gentamicin at the IC_{50} dose for 24 hours + cilastatin at a dose of 100 μg/μl for 24 hours

Group 3. Cilastatin was added to the medium 48 hours after the application of the nephrotoxic agent, and evaluation was made after 72 hours:

- Cisplatin at the IC_{50} dose for 48 hours + cilastatin at a dose of 100 μg/μl for 24 hours
- Vancomycin at the IC_{50} dose for 48 hours + cilastatin at a dose of 100 μg/μl for 24 hours
- Gentamicin at the IC_{50} dose for 48 hours + cilastatin at a dose of 100 μg/μl for 24 hours

Each group was treated in 3 replicates. Viability in the wells was analyzed using the Abbkine CCK-8 kit protocol. According to the results of the experiments, group 2 was determined as the most suitable group to evaluate our hypothesis.

Apoptosis Assay

TUNEL chromogenic Apoptosis detection kit (ABP Bioscience TUNEL, USA) was used to analyze total apoptotic cells in cells after treatment with nephrotoxic agent and cilastatin. For this purpose, HEK293T and HK-2 cell lines were inoculated in 24-well plates with 2.5 x 104 cells/well as determined at the previous experimental stage (Group 2). Cells were removed after 48 hours of total incubation with existing media containing 0.05% trypsin-0.53 mM EDTA (for HK-2 cell line) (Thermo Fisher Scientific, Waltham, MA, USA) and 0.25% trypsin-0.53 mM EDTA (for HEK293T cell line) (Thermo Scientific) in Eppendorf tubes. Viable and non-viable cells were precipitated as a result of centrifugation according to the kit protocol as follows: Cell pellets were fixed with formaldehyde and triton-x-100, dropped on the slide, and pre-preparation was made by incubation processes at 37°C. The double-stranded broken DNA fragments in the apoptotic cells in the prepared samples were passed through the kit solutions and labeled with biotin-11-dUTP by means of the TdT enzyme. Marked cells were stained with methyl green dye, and dark-colored apoptotic cells were analyzed under a light microscope (OLYMPUS CX41, Tokyo, Japan).

Oxidative Stress Analysis

Intracellular ROS/RNS levels were evaluated by using the Cellular ROS/RNS Detection Assay Kit (Abcam, Cambridge, MA, USA; catalog number: ab139473) according to the manufacturer's protocol. The kit includes fluorescent reagents as major components, such as nitric oxide (NO) Detection Reagent (red fluorescent) and Oxidative Stress Detection Reagent (green fluorescent) as determined at the previous experimental stage (Group 2). Briefly, cells were plated on 24 wells, and following the drug treatments, cells were captured under the fluorescence microscope (Olympus CX41, Tokyo, Japan). Fluorescent positive cells were visualized using a wide-field fluorescence microscope equipped with standard green (Ex/Em = 490/525 nm) and red (Ex/Em = 650/670 nm) fluorescent cubes. The positive fluorescence cells were evaluated by the use of the ImageJ program.

Biomarker Analysis

To isolate total RNA, HEK293T and HK-2 cell lines were seeded on 6-well plates at 2.5 x 10^5 cells/well. Cells were treated with the drugs as determined at the previous experimental stage (Group 2). After 48 hours, the nutrient media were vacuumed into the cells, placed on ice, and collected with 500 μl TRIzol (Qiazol, Qiagen, Hilden, Germany) and put into 1.5-ml Eppendorf (EP) tubes, and their RNA isolations were performed. Briefly, a TRIzol reagent was used to lyse the cells. Chloroform was added to the lysate for phase separation. The aqueous phase (RNA) was transferred to a clean Eppendorf tube, and RNA was precipitated by 2-propanol. After a quick wash with 70% ethanol, the extracted RNA was dissolved in 30 μl nuclease-free water. After RNA was harvested from the cells, total RNA samples were converted into cDNA using the Hi-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The qRT-PCR reaction was performed on the Bio-Rad CFX-96 (Hercules, CA,USA) device using 2X SYBR green (Quantitech, Qaigen, Hilden, Germany) master mix to analyze the expression of KIM1 and NGAL on the samples transformed into cDNA. The sequence and specification of the used primers are presented in Table I.

Before preparing the reaction mixture, cDNA samples were diluted to 1/5 using nuclease-free water. Then, the reaction mixture was prepared with 2 μl cDNA sample, 10 μl 2X master mix, and 0.3 μM forward and reverse primers, amounting to a total volume of 20 μl. GAPDH was used as an endogenous control for the analysis of the results. The reaction cycle was performed in 3 replicates for each sample as described below.

Statistical Analysis

For the analysis of the results obtained from the experiments performed to determine the IC_{50} doses

Table I. Primer sequences for each primer.

	Sequence (5'->3')
KIM-1 Forward	CTGCAGGGAGCAATAAGGAG
KIM-1 Reverse	ACCCAAAAGAGCAAGAAGCA
NGAL Forward	TGAGCACCAACTACAACCAG
NGAL Reverse	AGAGATTTGGAGAAGCGGATG
GAPDH Forward	TGAACGGGAAGCTCACTGG
GAPDH Reverse	TCCACCACCCTGTTGCTGTA

of the nephrotoxic drugs: cisplatin, vancomycin, and gentamicin, applied to the cells, and also the most appropriate doses of cilastatin for nephroprotective effect, Graphpad Prism (version 8.0.1., La Jolla, CA, USA) program was used. * corresponds to $p<0,05$.

Results

Determination of the Nephrotoxic IC50 Doses Of Cisplatin, Vancomycin, and Gentamicin

Both cell lines were incubated with nephrotoxic agents at various doses. IC_{50} values were determined for cisplatin (9.98 µM), gentamicin (2757 µM), and vancomycin (1815 µM) in the HK-2 cell line. IC_{50} values were also determined for cisplatin (10.2 µM), gentamicin (1165 µM), and vancomycin (1545 µM) in the HEK293T cell line (Figure 1). The most prominent toxic effect was observed for cisplatin on both cell lines.

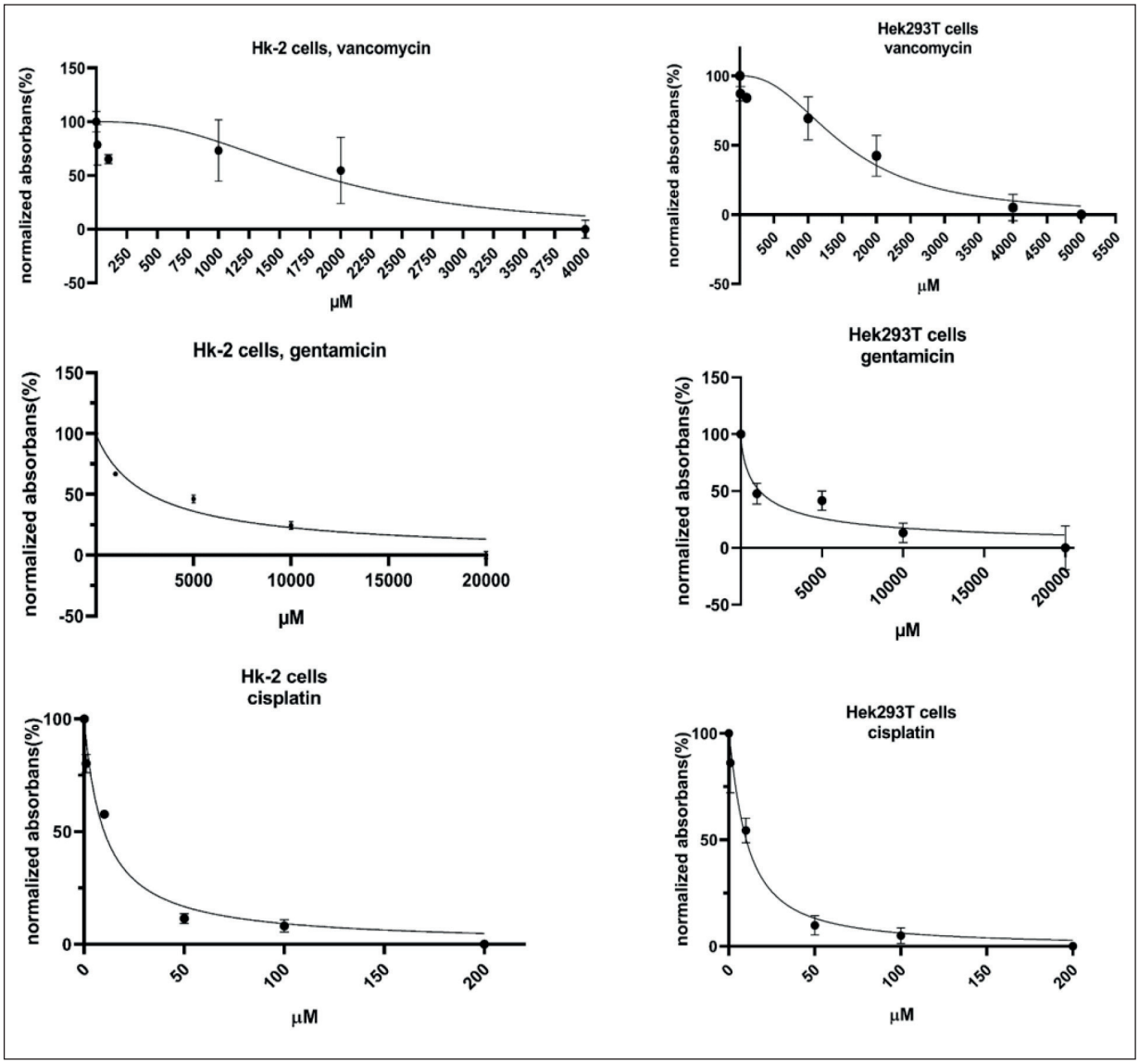

Figure 1. The effects of cisplatin, vancomycin, and gentamicin on cellular toxicity in HEK293T and HK-2 cell lines

Table II. The 24-hour cell survival results after simultaneous administration of nephrotoxic agents and cilastatin.

Hk-2 cells	Cisplatin	Vancomycin	Gentamicin
w/o cilastatin	54.4505 ±2.01	58.1868 ±2.67	60.43956 ±3.12
Cilastatin 50 µg/ml	64.9267 ±3.54	64.91758242 ±3.39	63.956044 ±4.01
Cilastatin 100 µg/ml	69.5971 ±1.92	68.18681319 ±2.61	65.989011 ±3.62
Cilastatin 250 µg/ml	62.967 ±4.21	60.82417582 ±2.91	61.428571 ±2.11
Cilastatin 500 µg/ml	57.4176 ±5.02	57.82967033 ±3.83	59.89011 ±4.32
Hk-2 cells	**Cisplatin**	**Vancomycin**	**Gentamicin**
w/o cilastatin	57.06422018 ±2.12	59.35779817 ±3.41	55.2293578 ±2.25
Cilastatin 50 µg/ml	61.78899083 ±3.14	70.04587156 ±3.59	69.44954128 ±3.26
Cilastatin 100 µg/ml	69.35779817 ±2.67	73.57798165 ±3.87	76.55963303 ±2.65
Cilastatin 250 µg/ml	67.24770642 ±4.35	69.12844037 ±3.98	62.43119266 ±3.46
Cilastatin 500 µg/ml	54.81651376 ±2.31	59.26605505 ±3.54	61.97247706 ±4.48

Determination of the Appropriate Nephroprotective Dose of Cilastatin

As a result of simultaneous analysis of different concentrations of cilastatin with nephrotoxic agents for 24 and 48 hours in both cell lines, the nephroprotective dose of cilastatin was determined as 100 µg / ml for both 24 and 48 hours applications (Tables II and III).

Analysis of Possible Therapeutic Efficacy of Cilastatin After the Development of Toxicity

After 16, 24, and 48 hours of exposure to toxic agents, cilastatin added to the medium at a dose of 100 µg / ml resulted in a significant increase in cell viability in all three groups compared to the control group (Figure 2 and **Supplementary Materials**) ($p<0.05$). The cell viability enhancing effect of cilastatin in the groups treated with cisplatin in HK-2 cell lines was more significant compared to the groups treated with other drugs ($p<0.05$).

Cilastatin Use Reduces the Number of Apoptotic Cells That Increase After Nephrotoxicity

TUNEL assay was carried out on the samples of Group 2, in which the cell viability enhancing effect of cilastatin was best observed. Cilastatin added to the medium 24 hours after the development of drug toxicity significantly reduced apoptosis in both cell lines and all drug applications ($p<0.05$) (Figure 3 and **Supplementary Materials**).

Cilastatin Administration Reduces Oxidative Stress That Occurs After Nephrotoxicity

Oxidative stress analysis was carried out on Group 2 samples, where the cell viability enhanc-

Table III. The 48-hour cell viability results after simultaneous administration of nephrotoxic agents and cilastatin.

Hk-2 cells	Cisplatin	Vancomycin	Gentamicin
w/o cilastatin	48.2511 ±1.82	52.24215247 ±1.97	44.439462 ±1.79
Cilastatin 50 µg/ml	69.0135 ±2.03	59.28251121 ±2.32	51.255605 ±3.03
Cilastatin 100 µg/ml	82.5112 ±2.31	66.27802691 ±2.41	59.506726 ±2.45
Cilastatin 250 µg/ml	76.9955 ±3.27	53.0044843 ±3.9	50.672646 ±3.41
Cilastatin 500 µg/ml	51.435 ±2.81	47.62331839 ±2.88	49.641256 ±3.67
Hek293 cells	**Cisplatin**	**Vancomycin**	**Gentamicin**
w/o cilastatin	44.93629533 ±3.15	68.36001307 ±2.96	66.05684417 ±2.89
Cilastatin 50 µg/ml	56.30512904 ±1.91	71.59425025 ±2.61	67.82097354 ±3.24
Cilastatin 100 µg/ml	72.57432212 ±1.79	74.14243711 ±2.59	72.47631493 ±3.43
Cilastatin 250 µg/ml	66.5468801 ±3.86	61.00947403 ±3.41	64.14570402 ±2.97
Cilastatin 500 µg/ml	57.62822607 ±2.89	55.81509311 ±3.17	57.3832081 ±2.91

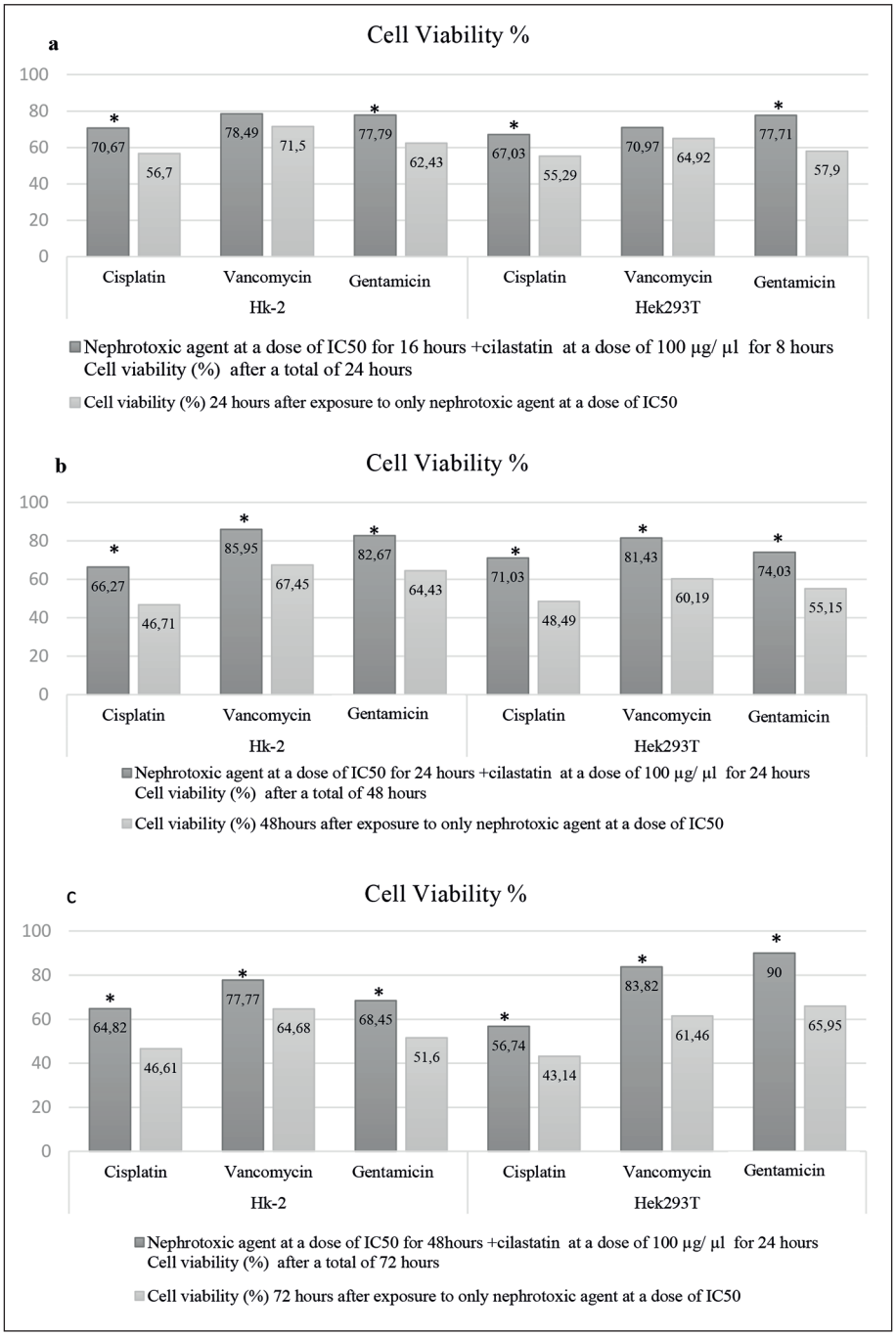

Figure 2. Measurement of cell viability. **A,** 8 hours after addition of cilastatin following 16 hours of exposure to nephrotoxic drug (a total of 24 hours measurement of cell viability). **B,** 24 hours after addition of cilastatin following 16 hours of exposure to nephrotoxic drug (a total of 48 hours). **C,** 48 hours after addition of cilastatin following 24 hours of exposure to nephrotoxic drug (a total of 72 hours) (* $p<0.05$).

ing effect of cilastatin was best observed. After 24 hours of exposure to drug toxicity, cilastatin added to the medium was observed to significantly reduce the levels of reactive oxygen and nitrogen species (ROS and RNS) in both cell lines and all drug applications after 24 hours (Figures 4 and 5).

Cilastatin Administration Affects KIM1 and NGAL Expressions

Biomarker analyses were performed on Group 2 samples, in which the cell viability enhancing effect of cilastatin was best observed. After 48 hours of exposure with only one nephrotoxic

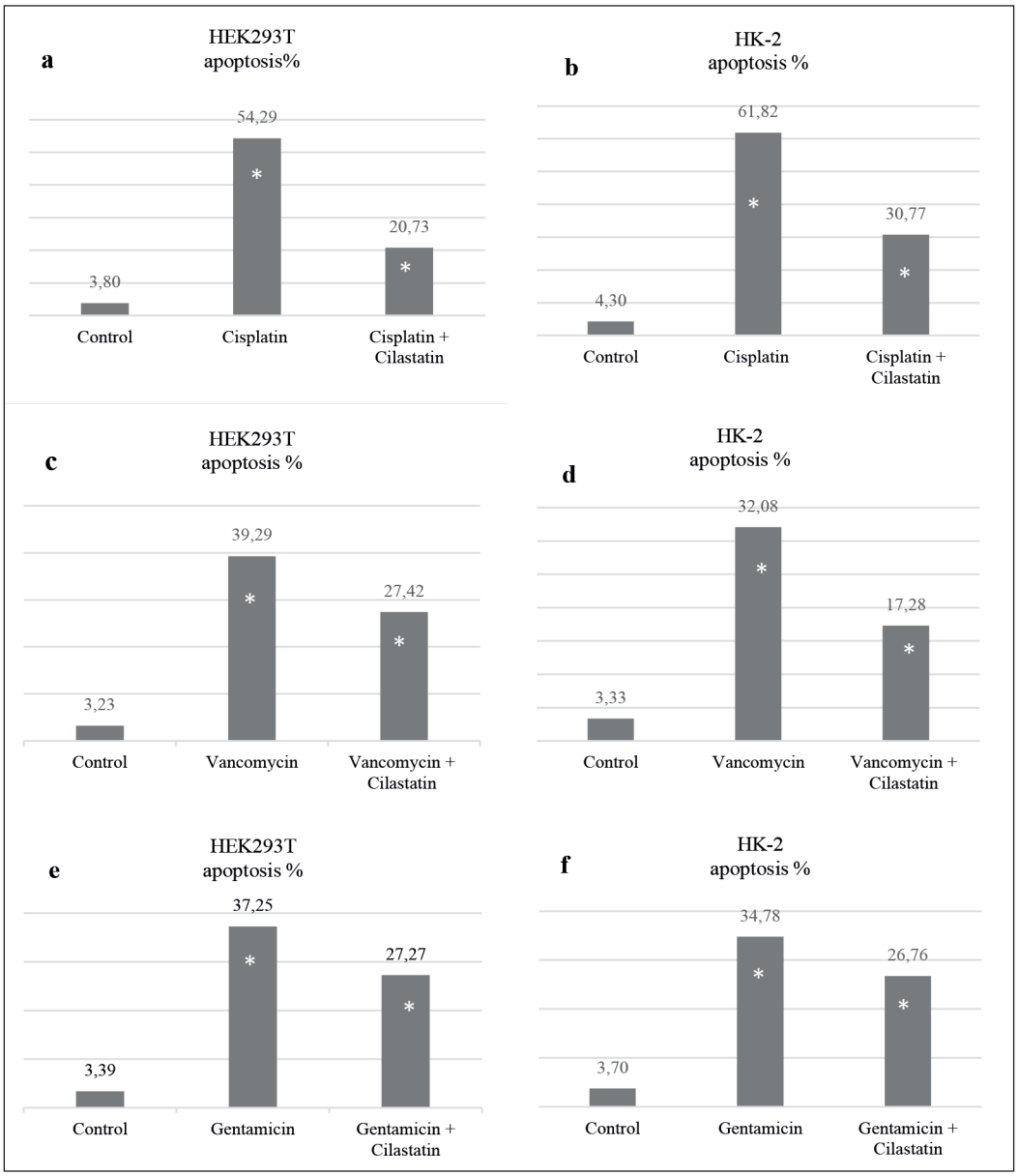

Figure 3. The application of cilastatin reduces the number of apoptotic cells. Results of the analysis of apoptosis after 24 hours of cisplatin exposure, then cilastatin added in Hek293T (**A**) and HK-2 (**B**) cell lines for 24 hours (a total of 48 hours of exposure); Results of the analysis of apoptosis after 24 hours of vancomycin exposure, then cilastatin added in Hek293T (**C**) and HK-2 (**D**) cell lines for 24 hours (a total of 48 hours of exposure). Results of the analysis of apoptosis after 24 hours of gentamicin exposure, then cilastatin added in Hek293T (**E**) and HK-2 (**F**) cell lines for 24 hours (a total of 48 hours of exposure) (*$p<0.05$).

agent, NGAL and KIM1 gene expressions for all three toxic agents and both cell lines were observed to increase significantly compared to the control group ($p<0.05$). After treatment with a nephrotoxic agent for 24 hours, the addition of cilastatin to the medium revealed that the expression of KIM1 and NGAl was less than that observed in the nephrotoxicity group at 48 hours (Figure 6 and **Supplementary Materials**). Expressions of KIM1 and NGAL were 2-fold higher in both cell lines in the cisplatin group compared to the other two drugs.

Discussion

In this study, we hypothesized that, in addition to its preventive effect on drug-induced nephro-

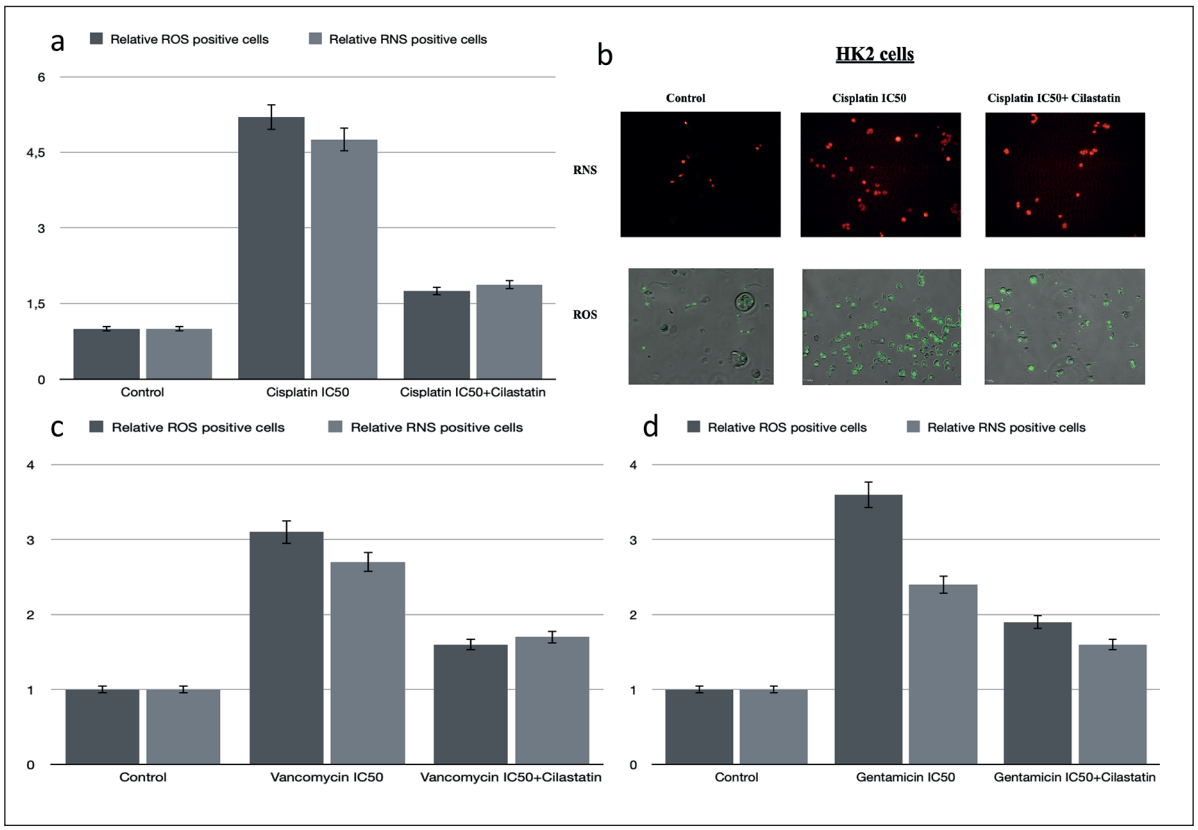

Figure 4. In the HK-2 cell line, after 24 hours of exposure to cisplatin (**A**), vancomycin (**C**), and gentamicin (**D**), cilastatin was added to the medium, and reactive oxygen and nitrogen species (ROS and RNS) analysis results were obtained at 48 hours. Representative graph of ROS and RNS (**B**).

toxicity, cilastatin might also have an impact on recovery after cell damage had occurred. In the *in vitro* nephrotoxicity models that were created, cilastatin increased cell viability when applied simultaneously with all three nephrotoxic agents. After 24 hours of exposure to the nephrotoxic drugs, the addition of cilastatin to the media increased the cell viability, decreased apoptosis and oxidative stress, and affected the recovery of cell damage. In addition, in the *in vitro* setting, the expression of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) increased when nephrotoxicity occurred, and their expression decreased when cilastatin was added to the medium after nephrotoxicity. To the best of our knowledge, this late effect of cilastatin was evaluated for the first time in this study.

Immortal human renal cell lines can be obtained easily and are used in drug studies, particularly in preclinical evaluations, as they can remain stable for a long time[1-3]. It is essential to use the appropriate cell type in the evaluation of nephrotoxicity using *in vitro* modeling. Renal PTECs is the most common cell type used for *in vitro* evaluation of nephrotoxicity. It is the main site of drug-related side effects in the kidney. PTECs contain various drug carriers and metabolic enzymes that can affect the effectiveness of drugs[1-3,6,12]. Therefore, both HK-2 and HEK293T cell lines were used in this study.

Cell viability and cell necrosis-apoptosis are being used as endpoints in understanding the occurrence of toxicity in drug-induced nephrotoxicity assessment in *in vitro* models. In the present study, the development of nephrotoxicity was shown through these evaluations[1,2,12,13]. Cytotoxicity was observed in both cell lines with vancomycin, gentamicin, and cisplatin. In this study, similar to other studies, cisplatin was more cytotoxic in both cell lines than the other two drugs.

It has been reported in various studies that cilastatin has protective effects against drug-induced nephrotoxicity by reducing oxidative stress,

inflammation and apoptosis, and also it prevents the accumulation of nephrotoxic agents in the tubule cell by limiting the transport of nephrotoxic agents into the tubule cell with megaline blockade[5]. In studies conducted in animal and *in vitro* models, inflammation, apoptotic cell death, and oxidative stress played a role in the toxicity mechanism in nephrotoxicity induced by vancomycin, gentamicin, and cisplatin[5-12]. Hori et al[6] reported that cilastatin suppresses nephrotoxicity induced by vancomycin, gentamicin, cisplatin, and colistin through megaline blockade.

Studies conducted with calcineurin inhibitors in which cilastatin inhibited inflammation by suppressing macrophage infiltration, resulting in the release of inflammatory mediators, indicated a decrease in the development of renal fibrosis[15,16]. Nephrotoxic agents and cilastatin were administered simultaneously in clinical, animal, and *in vitro* studies, and most of the studies demonstrated that cilastatin is nephroprotective against toxicity[5-12,17]. In our study, we also showed this protective effect. In addition, we found that cell proliferation increased, and apoptosis and oxidative stress decreased when cilastatin was added to the culture media 24 hours after the toxicity had occurred in the cell lines. This finding indicates that cilastatin has positive effects on recovery from nephrotoxicity after toxicity has occurred.

This effect may occur by preventing the intake of the agent, which is still toxic, into the cell, as well as reducing the ongoing intracellular toxic effect, thereby reducing cell damage or accelerating cell recovery. The general approach in the treatment of AKI due to drug toxicity is the discontinuation of the nephrotoxic agent, hydration, and supportive therapy. The result we obtained reminds us of the question of whether cilastatin can be used to accelerate the recovery of AKI caused by the effect of nephrotoxic drugs, particularly

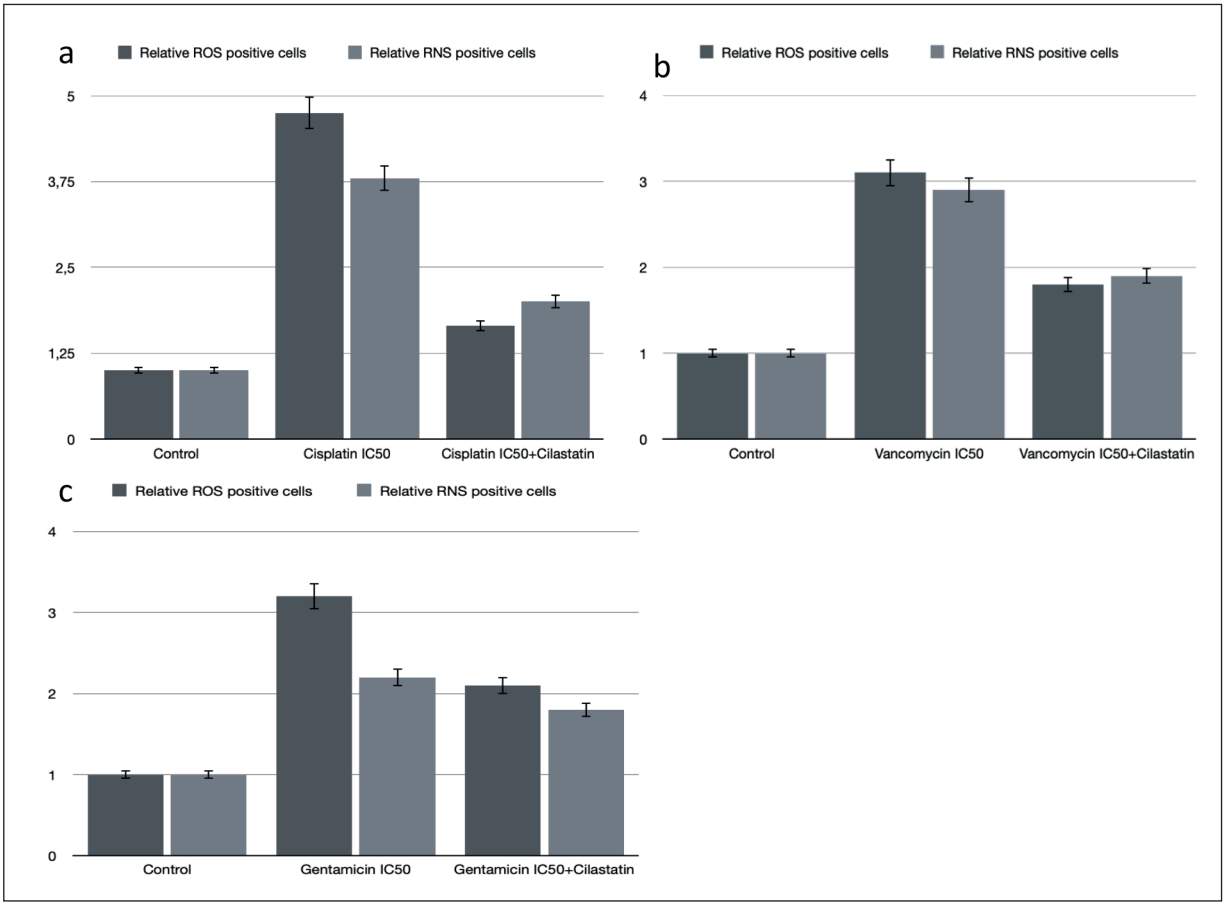

Figure 5. In Hek293T cell line, results of reactive oxygen and nitrogen species (ROS and RNS) analysis at a total of 48 hours, including 24 hours of exposure to cisplatin (**A**), vancomycin (**B**), and gentamicin (**C**), and then 24 hours after addition of cilastatin to the medium.

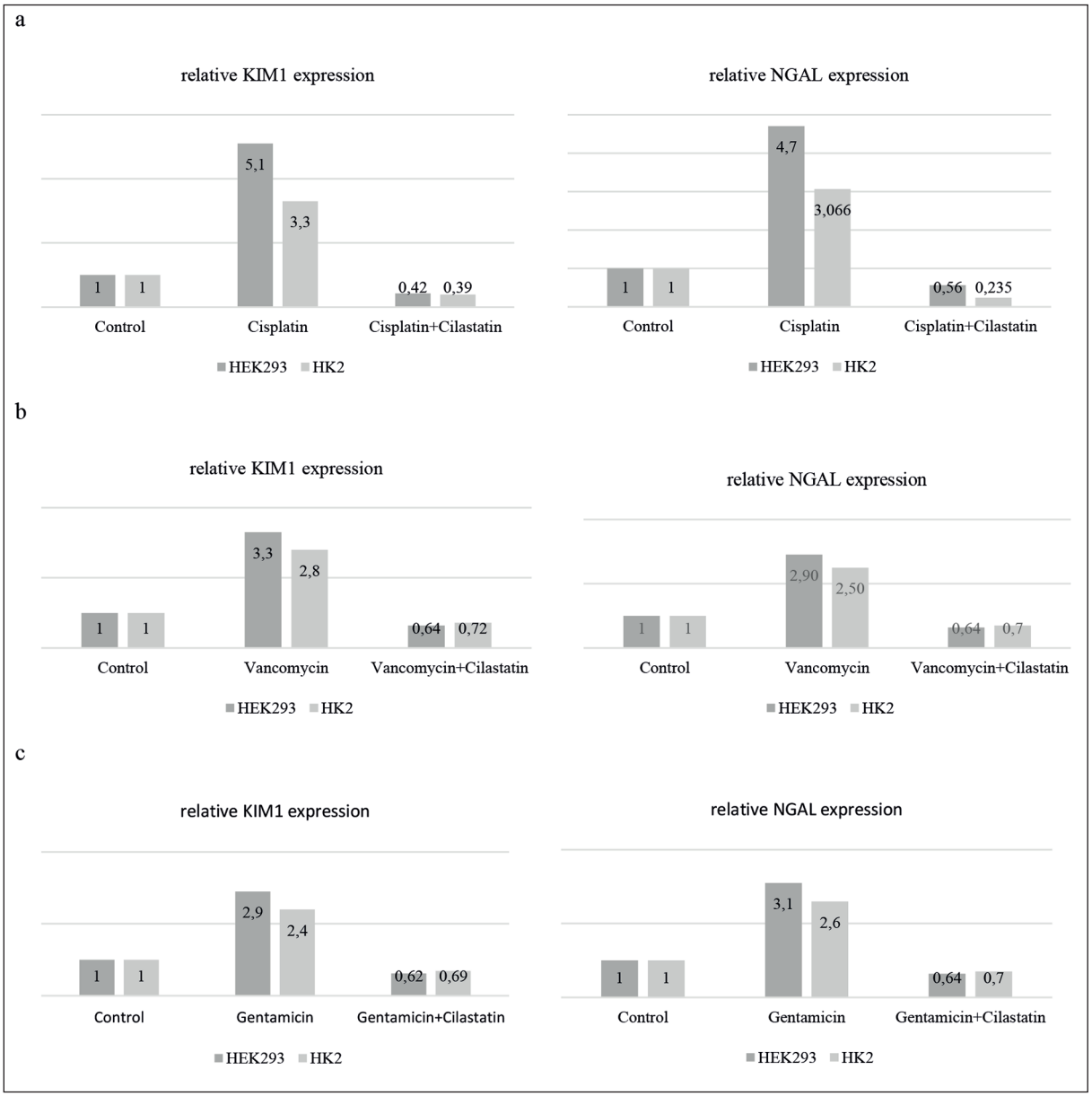

Figure 6. Results of kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL) analysis in Hek293T and HK2 cell lines 24 hours after exposure to cisplatin (**A**), vancomycin (**B**), and gentamicin for 24 hours (**C**), and then 24 hours after addition of cilastatin to the medium (a total of 48 hours).

in intensive care patients. In this context, cilastatin may have a place in the treatment besides its preventive effect on AKI. However, this study is quite insufficient to make such an inference, and this finding should be supported by clinical studies. However, this study may provide a foundation for future studies on drug-induced AKI.

Despite their widespread use, traditional markers such as serum creatinine and blood urea nitrogen are insufficient for the early detection of AKI. Various biomarkers are sought to detect AKI at an early stage. In this context, several studies have suggested that biomarkers, such as kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 clusterin, osteopontin, and cystatin-c, can show kidney damage in the early period[1,2,18-21]. Although these biomarkers have been evaluated in many clinical and preclinical studies, it has not been a long time since investigating these markers in *in vitro* studies. It

has been suggested that biomarker analysis is more reliable than other parameters (cell viability and apoptosis) used to determine toxicity in the evaluation of nephrotoxicity in *in vitro* settings[1,2,20,21]. Although most of the *in vitro* studies are performed by evaluating apoptosis and cell viability, the sensitivity and specificity of these evaluations are still limited[1,19]. In our study, we took advantage of the analysis of these biomarkers and evaluated KIM-1 and NGAL in nephrotoxicity models. We found that the expression of these two biomarkers increased, compared to the control group, only in the setting where nephrotoxic agents were given. Previous studies have shown KIM-1 to be effective in evaluating nephrotoxicity in *in vitro* settings, but variable results related to NGAL have been reported. In our study, we observed that the expression of NGAL increased in toxicity groups compared to the control group. The expression of these markers was lower in both cell lines and in all three drug toxicities, when cilastatin was added to the medium 24 hours after exposure to the nephrotoxic agent, compared to the group treated only with nephrotoxic agent. These findings support the other results of our study.

The main limitation of the present study is that the research was conducted only in the *in vitro* setting. Therefore, there is a need for clinical studies on both aspects, including the protective effect of cilastatin on drug-induced nephrotoxicity and its effect on reducing cytotoxicity. We recommend further studies to gain a better understanding of the mechanism of the regenerative effect of cilastatin on cell damage.

Conclusions

The results of our study suggest that cilastatin has a protective effect on drug-induced nephrotoxicity. Moreover, it is also effective in recovery by reducing apoptosis and oxidative stress and increasing cell proliferation. These findings may shed light on the wider use of cilastatin in clinical trials.

Competing Interest for all Authors

No financial or nonfinancial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethical Approval

Not applicable.

Authors' Contribution

TB conceptualized and designed the study and drafted the initial manuscript. *In vitro* experiments were conducted by OT. TB and OT carried out the initial analyses and reviewed and revised the manuscript. OT supervised the statistics. SY made a critical revision of the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. The authors declare that all data were generated in-house and that no paper mill was used.

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