Journal of Oncological Science 1 (2016) 17–24

Contents lists available at ScienceDirect

Journal of Oncological Science

journal homepage: www.elsevier.com/locate/JONS

Original article

Thymoquinone enhances cisplatin-induced neprotoxicity in high dose

Ahmet Dirican [a,*], Osman Sahin [b], Funda Tasli [c], Erkan Sogut [d], Yuksel Kucukzeybek [e], Ahmet Alacacioglu [e], Mustafa Gunes [f], Gulcan Saglam [d], Eyup Coban [g], Mustafa Oktay Tarhan [h], Buket Demirci [i]

[a] Celal Bayar University Faculty of Medicine, Department of Medical Oncology, 45030 Manisa, Turkey
[b] Department of Nephrology, Faculty of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
[c] Department of Medical Pathology, Medical Faculty, Sifa University, Izmir, Turkey
[d] Department of Biochemistry, Katip Celebi University of Medicine, Izmir, Turkey
[e] Department of Medical Oncology, Katip Celebi University of Medicine, Izmir, Turkey
[f] Department of Urology, Medical Faculty, Suleyman Demirel University, Isparta, Turkey
[g] Internal Medicine Ataturk Training and Research Hospital, Katip Celebi University, Izmir, Turkey
[h] Institute of Oncology, Dokuz Eylul University, Izmir, Turkey
[i] Department of Medical Pharmacology, Medical Faculty, Adnan Menderes University, Aydin, Turkey

ARTICLE INFO

Article history:
Received 20 October 2015
Received in revised form
1 November 2015
Accepted 9 November 2015
Available online 5 February 2016

Keywords:
Cisplatin
Nephrotoxicity
Thymoquinone
Apoptosis
Rat

ABSTRACT

Background: Cisplatin-induced nephrotoxicity is an important problem of the cancer treatments. The major bioactive component of *Nigella sativa*, thymoquinone (TQ) might limit the nephrotoxic effect of cisplatin in low doses. However, it is not clear how it can affect the kidney as an anti-cytotoxic agent when administered in higher doses or in cisplatin co-treatment. Therefore, we examined the *in vivo* interactions between cisplatin and TQ by measuring serum cystatin C (cys C), creatinine and neutrophil gelatinase-associated lipocalin (NGAL) levels and analyzing the expression status of p53 and NGAL by immunohistochemistry.

Methods: Wistar rats were divided into four groups: Control, TQ treatment (group II; 40 mg/kg i.p. for 5 days), cisplatin treatment (group III; 7 mg/kg, i.p. for at day 3) and TQ and cisplatin co-treatment (group IV).

Results: Administration of 40 mg/kg TQ had no effect on serum kidney parameters. In cisplatin received group's serum creatinine level was insignificant, but serum Csy C and NGAL levels were significantly increased. All serum creatinine, NGAL and Cys C levels were increased in co-treatment of cisplatin and TQ. Additionally, in this group, renal tubular damage was found significantly higher than both control and only cisplatin-treated groups. The kidney immunohistochemistry staining of NGAL and p53 were significantly more intense in group IV rather than the others.

Conclusions: This study showed that the administration of cisplatin and high dose of TQ act synergistically to produce nephrotoxicity and the involvement of apoptotic pathway and proximal tubule damage might be the leading cause of on this effect.

1. Introduction

Cisplatin is a potent chemotherapeutic agent and used for the treatment of a broad spectrum of malignancies. *In vitro* studies show that cisplatin selectively and persistently inhibits the synthesis of deoxyribonucleic acid (DNA) whereas, ribonucleic acid (RNA) and protein synthesis are relatively spared.[1] However, new effects of cisplatin have been discovered with an increasing knowledge in pathological mechanisms of cancer. These effects are related to the inhibition of DNA synthesis and repairment that might result in cell cycle arrest at the G1, S, or G2-M phase, therefore apoptosis is induced.[2] The major limiting factors in the use of cisplatin are its side effects which include general cell-damaging effects, such as nausea and vomiting, myelosuppression

* Corresponding author. Tel.: +90 0505 4790614, +90 236 233 1920.
E-mail address: ahmetdirican@yahoo.com (A. Dirican).
Peer review under responsibility of Turkish Society of Medical Oncology.

http://dx.doi.org/10.1016/j.jons.2015.11.005

(neutropenia and thrombocytopenia) and immunosuppression.[3] Additionally, nephrotoxicity is regarded as the most common and serious side effect of cisplatin.[4] In the rat animal models, cisplatin primarily damages the proximal tubule, especially the S3 segment.[5] The histopathological characteristics of cisplatin-induced nephrotoxicity in rats are massive necrosis and subsequent regeneration of renal proximal tubular cells.[6] The underlying mechanism of cisplatin-induced nephrotoxicity is not completely understood; however, several mechanisms, including hypoxia, free radicals, inflammation, and apoptosis are thought to be involved.[7] The major benefits of the combination therapies are to reduce the development of drug resistance while using the drugs effectively in cancer treatment and each drug can be given at its optimal dose without intolerable side effects. Therefore cisplatin is currently used in combination with other antineoplastic drugs in *in vitro* cancer models and clinical trials.[8]

Thymoquinone (TQ) is one of the major bioactive components (30–48%) of *Nigella sativa* L. plant which is named as black seed or black cumin.[9] It's steam-distillated essential oil was investigated for its antioxidant and anti-inflammatory properties.[10] It has also been shown that TQ have anti-neoplastic activity against a variety of cancer cell lines.[11] TQ has enhanced the cytotoxic effect of cisplatin in cancer. Also, TQ has been reported to protect the normal cells against cisplatin nephrotoxicity when it is administrated orally in small doses (50 mg/lt in drinking water).[12–15] Moreover, Sagit et al. have been reported that 40 mg/kg TQ has a protective effect on female rats with cisplatin-induced hearing dysfunction.[16] However, there are only a few reports in the literature about the toxicity of TQ. Therefore, it is necessary to study the toxicity of this constituent in animal models. It has been reported that when it is given intraperitoneally, the toxic doses of TQ are varied from 10 mg/kg to 57.5 mg/kg in the rats.[17,18] For the aim of evaluating anti-cancer effect, TQ was given 5–20 mg/kg intraperitoneally in the studies.[12,19] But unfortunately these studies have not been focused on the kidney effect of the agent. Furthermore, the adverse effect of anti-cancer doses of TQ on kidney is unknown. In addition, the impact of administration of TQ over the reno-protective doses concomitantly with a nephrotoxic drug, such as cisplatin on kidney is also ambiguous.

We therefore designed this study to investigate the mechanisms of the possible effect of TQ against *in vivo* cisplatin-induced renal damage in a rat nephrotoxicity model. For this purpose, serum creatinine, Cys C and NGAL levels were measured. Histological changes were evaluated and the expression status of p53 and NGAL were analyzed by immunohistochemistry.

2. Experimental methods

2.1. Animals

The study has the permission of Animal Ethical Committee of Adnan Menderes University (ADU-HADYEK 64583101/2013/028) and the guidelines for the Care and Use of Laboratory animals were strictly followed. Female Wistar rats (n = 28, 8–10wk-old) were kept in an environmentally controlled room at constant temperature (21 ± 1 °C) and humidity (75 ± 5%) under a 12 h light/dark cycle. The animals were acclimatized for 1 week before the study and had free access to standard laboratory feed and water ad libitum.

2.2. Experimental protocol

The rats were divided into four groups. The experimental groups were as follows: *Group I (control)*: The rats in this group were administered intraperitoneally (i.p.) %1 ethanol concentration for 5 days and served as a healthy animal group. *Group II*: TQ (Sigma–Aldrich, Alfagen, Izmir) -treated; 40 mg/kg b.w, i.p. for 5 days.[16] *Group III*: Cisplatin (Platinol®, Bristol-Myers Squibb, Istanbul) -treated; 7 mg/kg b.w., i.p.[14] *Group IV*: TQ (40 mg/kg b.w., i.p. for 5 days) and cisplatin (7 mg/kg b. w., i.p.) co-treated. TQ administration was started two days before the single i.p. injection of cisplatin. TQ was dissolved in ethanol. Final ethanol concentration was 1%. On the day 6 (72 h after the cisplatin treatment), anesthesia induced by a single i.p. injection of Ketamine (Ketalar®, Pfizer, Istanbul, Turkey) and Xylasine (Rompun®, Bayer, Istanbul, Turkey) (50 mg/kg and 5 mg/kg, respectively). Blood samples were taken by

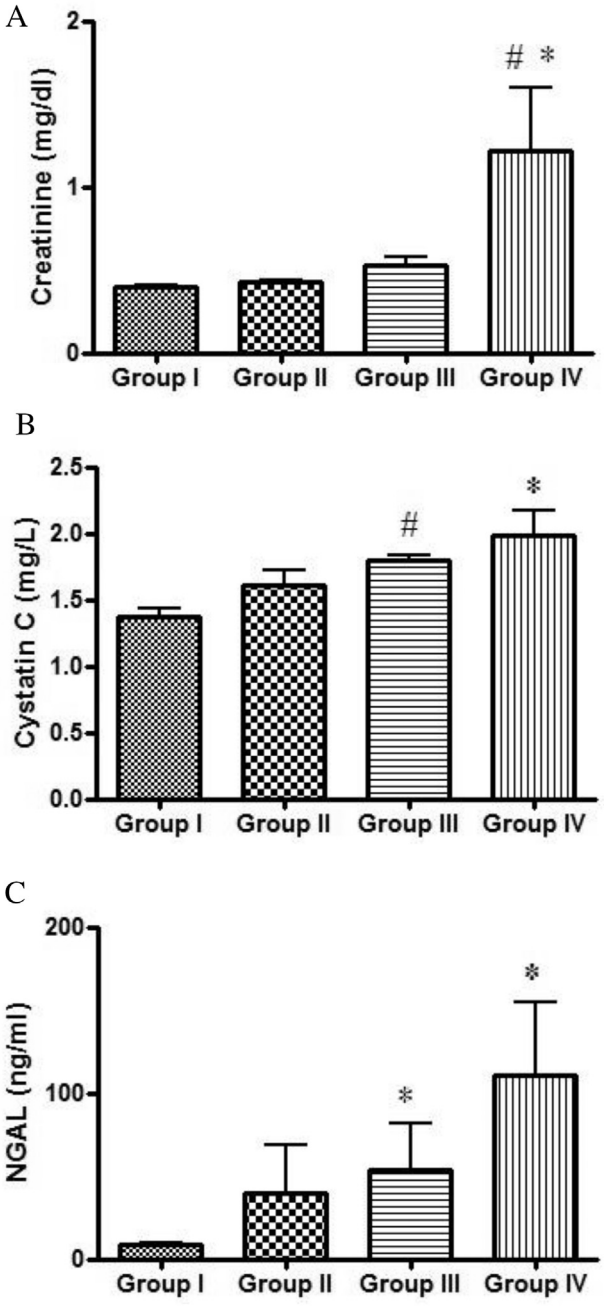

Fig. 1. The effect of thymoquinone on renal functions in cisplatin-induced nephrotoxicity. Values are mean ± SEM (n = 7–8) **A**. Serum creatinine level.*P < 0.01 *vs*. group I (control); #P < 0.05 vs group II (TQ). **B**. Serum CyS C level. *P < 0.01 *vs*. group I; #P < 0.05 *vs*. group I. **C**. Serum NGAL level. *P < 0.01 *vs*. group I (control).

cardiac puncture for serum analyses and the kidneys were harvested for histological studies and immune-histochemical analysis.

2.3. Biochemical analysis

Blood samples were allowed to clot in a serum separator tube (about 1 h) at room temperature and centrifuged at 1000 × g for 15 min. Serum samples were then aliquoted and stored at −20 °C until analyze. Serum creatinine levels were measured by spectrophotometric method with an auto analyzer (Architect c8000; Abbott, IL, U.S.A.). Serum cystatin C and NGAL levels were measured in all groups with using rat cystatin C (Biovendor GmbH, Germany) and rat lipocalin-2/NGAL (Boster Biological Technology, USA) ELISA kits according to the instructions of the manufacturers.

2.4. Histopathological examination of the kidney

The kidney samples of the rats in different groups, fixed in 10% neutral buffered formalin for 24 h. Washing was performed in tap water, and then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness by sledge microtome. Renal tissues were stained with hematoxylin and eosin (H&E) for histological examination. Tissue sections stained with H&E were examined by light microscopy and histopathological assessments made.

Tubular damage was examined under the microscope (200× magnification) and scored based on the percentage of cortical

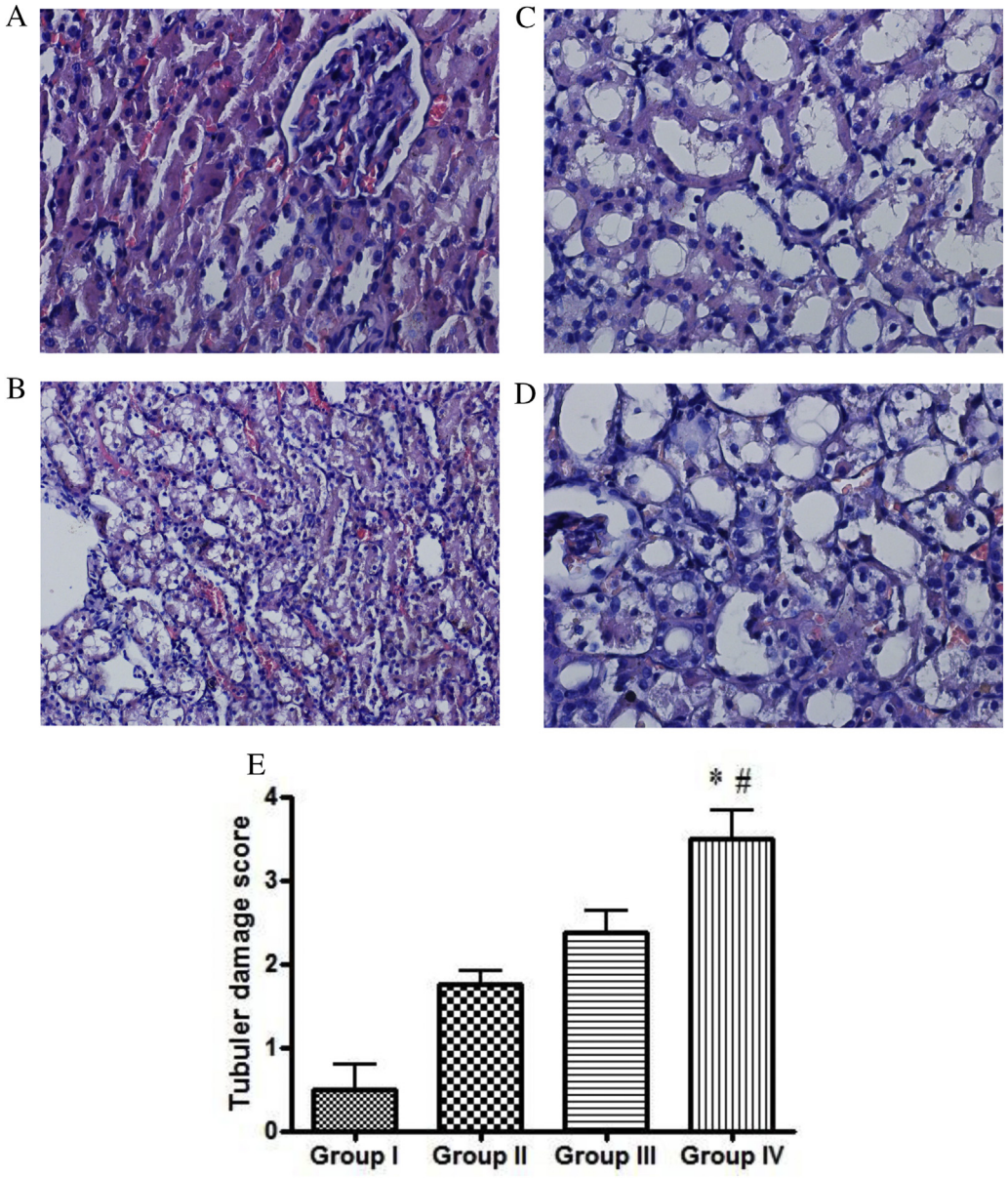

Fig. 2. Histology of the rat kidneys; (**A**) Group I: saline-treated, intact tubular epithelium with is shown (Magnification ×400); (**B**) Group II: slightly vacuolar changes (Magnification ×200); (**C**) Group III: vacuolar changes in the epithelium of tubulin, tubulin minimally dilated, covering large areas of the image focus ATN (Magnification ×400); (**D**) Group IV: significant loss of tubule epithelial cells and loss of epithelial cells, severe ATN (Magnification ×400); (**E**) The calculation of the tubular damage score is described under material and methods. Values are mean ± SEM (n = 7–8). *P < 0.01 *vs.* group I (control); #P < 0.05 *vs.* group II (TQ).

tubules showing epithelial necrosis: 0 = normal; 1: <10%; 2: 10–25%; 3: 26–75%; 4: >75%. Tubular damage was defined as the loss of the proximal tubular brush border, blebbing of apical membranes, tubular epithelial cell detachment from the basement membrane or intraluminal aggregation of cells and proteins as described.[20] To examine apoptosis, the cells were stained with H&E to monitor apoptotic morphology by light microscopy. The typical apoptotic morphology examined included cellular shrinkage, formation of apoptotic bodies, nuclear condensation, and fragmentation. Apoptosis was graded semi-quantitatively as 0 = no apoptosis, 1 = apoptosis positive.[21]

2.5. Immunohistochemistry analysis of p53 protein expression in tissue samples

Immuno-histochemical staining was performed by the standard avidin-biotin-peroxidasecomplex (ABC) method (Vectastain Lab.,Inc., Burlingame, CA, USA). Briefly, each 4-μm tissue section was deparaffinized, rehydrated and incubated with fresh 0.3% H_2O_2 in methanol for 30 min at room temperature. After rehydration through a graded ethanol series, the sections were microwaved in zinc sulfate hepta-hydrate buffer at 90 °C for 10 minutes for anti-p53 Mab and cooled to 30 °C. After incubation with normal horse serum for 30 min, the sections were then incubated with Mabs at their optimum dilution at 4 °C overnight, washed in phosphate buffered saline and incubated with secondary antibody for 30 min at room temperature. p53 protein expression status assessed using a semiquantitative score in as 0 = no staining, 1 = minimal staining, 2 = moderate staining, or 3 = severe findings.

2.6. NGAL staining of kidney tissue by immunohistochemistry analyses

Paraffin sections were deparaffinized through xylene and descending grades of ethanol, fixed with 4% paraformaldehyde in PBS for 30 min at 4 °C, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, and incubated with a polyclonal antibody to NGAL at 1:500 dilution for 1 h at room temperature.[22] Slides were then exposed for 30 min in the dark to secondary antibody conjugated with Cy5 (Amersham, Arlington Heights, IL, USA), and visualized with a fluorescent microscope (Zeiss Axiophot; Carl Zeiss, Inc., Thornwood, NY, USA) equipped with rhodamine filters. As a negative control, the primary antibody was excluded. All images were captured at the same exposure. We used an arbitrary scoring system of 0 (none), 1 (mild), 2 (moderate), and 3 (intense).

2.7. Statistical analysis

All statistical analyses were performed using SPSS version 19.0 for Windows (Statistical Package for Social Sciences, Chicago, IL). Results are expressed as means ± SEM. Statistical significance among groups was determined by Kruskall-Wallis analysis followed by Mann–Whitney U post hoc analysis. P values of ≤ 0.05 were considered as significant.

3. Results

3.1. Biochemical measurement

Serum creatinine, Cys C and NGAL levels were significantly higher in the cisplatin plus TQ treatment group (group IV) than the other groups (Fig. 1A, B, C). In group III (only cisplatin given) serum creatinine levels were slightly higher than group I (control group) ($p > 0.05$), in group IV serum creatinine levels were higher than both group I and group II (only TQ given) and the difference was significant ($p < 0.001$, $p < 0.05$ respectively; Fig. 1A). Besides this, the serum levels of NGAL and Cys C which are the earlier marker of acute kidney injury[23] were increased in group II, group III and group IV but, the differences were only significant in group III and group IV (Fig. 1B,C). Alone TQ treatment was slightly increased the serum creatinine, NGAL and Cys C levels but, remained insignificant (Fig. 1A, B, C).

3.2. Histopathological alterations

The detrimental effects of the combination of cisplatin and TQ were also reflected by histologic changes. As shown in Fig. 2A–D, rats treated with either cisplatin or TQ alone, and sacrificed at 72 h later, displayed slightly abnormal renal histology. In contrast, the rat which received both the cisplatin and TQ had evidence of tubular injury such as the loss of the proximal tubular brush border, blebbing of apical membranes, tubular epithelial cell detachment from the basement membrane or intraluminal aggregation of cells and proteins. The tubular damage score of the group IV rats was significantly higher than group I or group II rats (respectively; $p < 0.01$, $p < 0.05$, Fig. 2E). Also apoptosis semi-quantitative score of group IV animals was significantly higher than group I and group II animals (Fig. 3, $p < 0.01$).

3.3. Increases in expression of p53 protein in kidney tissue

Fig. 4A–D showed by immunohistochemistry of p53 expression in kidney of controls and treated animals. In group III and group IV, p53 expression was found to be significantly increased compared to controls. The TQ treated group did not have any significant effect on the expression of p53 (Fig. 4E). Also immunohistochemistry staining showed p53 induction in the cells of renal cortex and outer medulla, but not in the inner medulla (data not shown). In addition, p53 was not induced in glomeruli (data not shown). Although p53 is induced in both proximal and distal tubular cells during cisplatin-induced nephrotoxicity, proximal tubules are the main site for p53 induction.

3.4. Increased NGAL staining in kidneys

NGAL staining in the group I and group II was minimal to absent (Fig. 5A–B). In contrast, NGAL was easily detected in group III and group IV, primarily in proximal tubules (Fig. 5C–D). The glomeruli

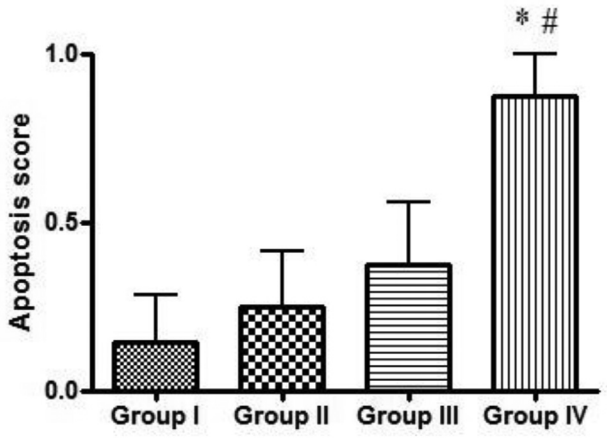

Fig. 3. The apoptosis score of the all experiment groups. The calculation of the apoptosis score is described under material and methods. Values are mean ± SEM (n = 7–8) *P < 0.01 *vs.* group I (control); #P < 0.01 *vs.* group II (TQ).

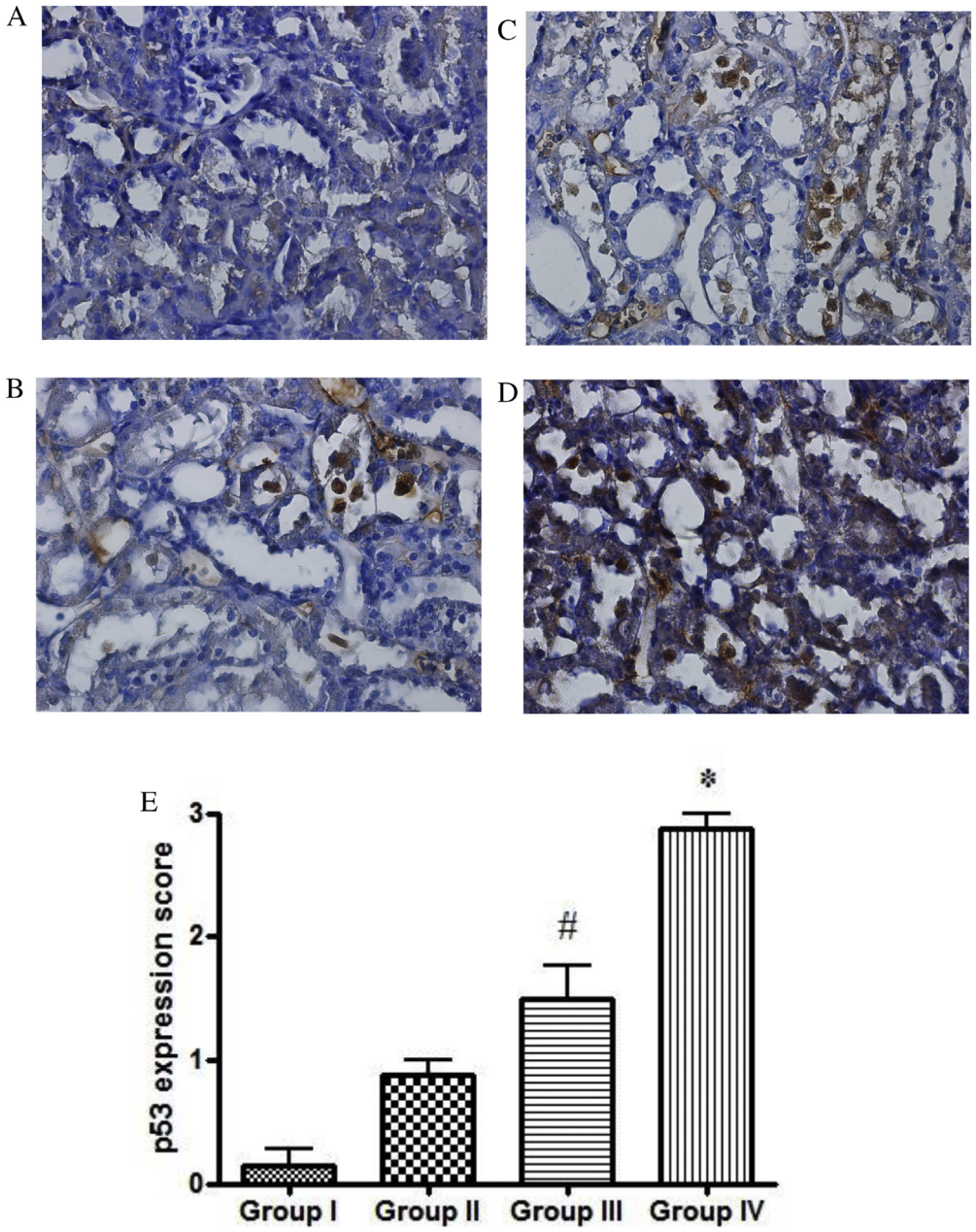

Fig. 4. p53 protein expression in the kidneys was evaluated by immunohistochemistry (**A**–**D**). (**A**) Group I: p53 staining was not detected in the nucleus of tubule epithelial cells (Magnification ×400). (**B**) Group II: p53 staining in epithelial cells shed into the lumen of tubulin staining (score I) (Magnification ×400). (**C**) Group III: nuclear p53 staining in a large number of tubule epithelial cells (score 2) (Magnification ×400). (**D**) Group IV: diffuse nuclear p53 staining (Magnification ×400). The p53 protein expression score is described under material and methods. (**E**) Values are mean ± SEM (n = 7–8)*P < 0.01 *vs.* group I (control); #P < 0.05 *vs.* group I.

were not stained. Using a scoring system of 0 (no staining) to 3 (most intense staining), NGAL expression was significantly increased in group III and group IV specimens. The overall distribution of NGAL staining is shown in Fig. 5E.

4. Discussion

The present study examined the interactions between two diverse renal insults, cisplatin and TQ, on renal function. Our results indicate that cisplatin and TQ exhibit remarkable synergy in producing renal dysfunction. We analyzed staining intensity of NGAL and p53 by immunohistochemistry. Staining intensity of NGAL and p53 were increased significantly in the kidneys of rats treated with cisplatin and cisplatin co-treated with TQ (group IV) compared with the staining intensity in the kidneys from the control group. Histological analysis showed that cisplatin damaged the proximal tubular cells; these changes were increased by TQ co-administration. Serum creatinine levels were increased significantly in group IV but, serum NGAL and Cys C levels which are the early markers of nephrotoxicity increased significantly only the group III and IV. Alone TQ treatment did not show any significant effect on NGAL, p53, histopathological alterations and serum renal markers in the kidneys of animals.

Nephrotoxicity is a major side effect that limits the use of cisplatin in many cancer patients. In previous studies, it has been shown that one of the molecular mechanisms of cisplatin induced

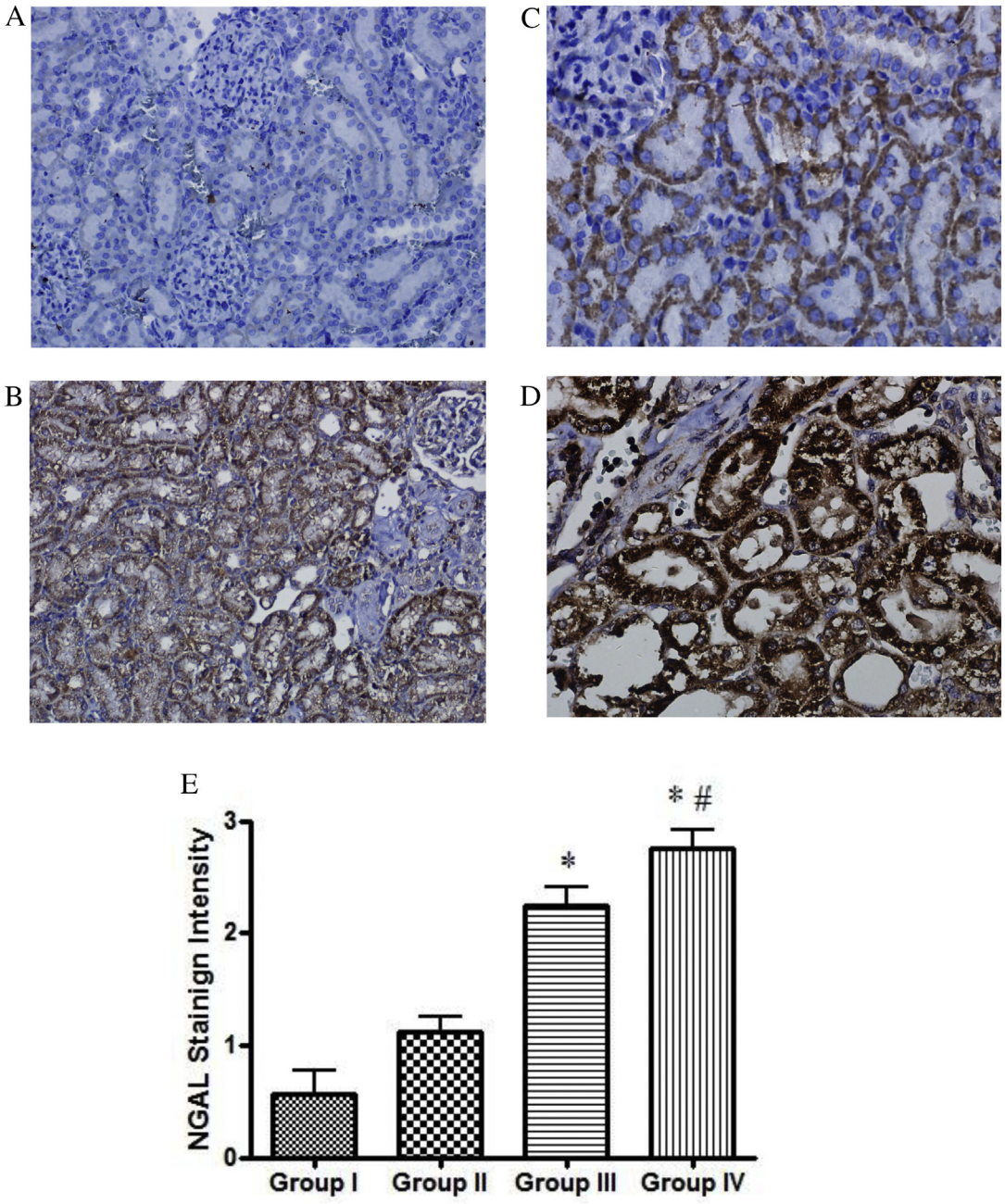

Fig. 5. Representative images of NGAL staining of the kidney tissues. Group III (Magnification ×400) (**C**) and Group IV (Magnification ×400) (**D**) was detected intense staining. Group I (Magnification ×200) (**A**) and group II (Magnification ×400) (**B**) was showed none or mild staining. Distribution of NGAL staining intensity according to groups. (**E**) Values are mean ± SEM (n = 7–8) *P < 0.01 *vs.* group I (control); #P < 0.05 *vs.* group II.

nephrotoxicity is the induction of apoptosis, particularly in renal proximal tubular cells.[24,25] The exact mechanism of cisplatin induced apoptosis is not completely understood, but it has been described that tumor suppressor gene p53 is activated.[26] Neutrophil gelatinase-associated lipocalin (NGAL) is a promising indicator in acute renal failure.[27] Several studies have evaluated serum NGAL as biomarkers of acute kidney toxicity in different patient population. There are some studies regarding to NGAL use as an early indicator in acute renal failure.[23,28] NGAL is increased in experimental cisplatin toxicity, characterized by the injury of the S3 segment of the proximal tubule.[27] It seems to be that NGAL is a common and sensitive response to tubular injury. However, there are limited studies in its use in chemotherapeutic agent-induced acute renal failure.[27]

Herbal medicines such as black seed oil, extracted from the seeds of *Nigella Sativa*, have been used for many years for the treatment of various diseases and to maintain a healthy life. TQ is the bioactive constituent of the volatile oil of black seed.[29] General aspects of TQ are thought to be as antioxidant, anti-inflammatory and antineoplastic, but the molecular pathways of TQ's anticancer effect are not clear. However, TQ is known to induce apoptosis by p53-dependent in cancer cell lines.[30,31] TQ toxicity has been assessed in several animal models. These models, has been done the histopathological analysis of liver, kidney, heart and lungs

of mice and rats; unfortunately given doses were enabled to determine nontoxic doses of TQ. In the same study, it has been reported that the rats LD50 was 794.3 mg/kg and 57.5 mg/kg for oral and i.p. administration, respectively. However this study has not been determined neither nephrotoxic adverse effects nor nephrotoxic doses of TQ. The anticancer properties of TQ are observed at lower doses (5–20 mg/kg) than the corresponding LD50.[12,19]

In our study, the dose of 40 mg/kg/day (five days) of TQ caused no harmful effects on kidney function or morphology (Fig. 1). While the serum creatinine levels did not increase in the cisplatin group (group III), it was found that the levels of creatinine significantly increased in the TQ + ciplatin group (group IV). In addition, we did not observe an increase in tubular injury and apoptosis score in the TQ group (group II) and cisplatin group (group III), but we demonstrated significantly augmentation in tubular injury and apoptosis score in co-treatment group (group IV). To explain the underlying mechanisms of tubular injury, immuno-histochemical staining has been done with p53 and NGAL and showed that both density of p53 and NGAL staining significantly increased in group III and IV (Fig. 4 and 5). Of these results, we showed that TQ did not have nephrotoxic effect even in high doses, but when it was used with a nephrotoxic agent such as cisplatin, TQ had synergistic toxic effect on kidney. Apoptosis was induced synergistically by the combination therapy with TQ and cisplatin and we suggest that nephrotoxic effect of these drugs occurred by this way. Furthermore, on the combination therapy group, NGAL staining was prominently seen in proximal tubules, suggesting that two drugs target proximal tubules synergistically.

Interestingly, alone TQ has been also found more potent than cisplatin in its cytotoxic effect on SiHA human cervical squamous carcinoma cells.[32] The combination of TQ with cisplatin enhanced cell death in lung cancer xenografts in mice.[33] Here, we have shown that coexistence of TQ with cisplatin, potentiates cisplatin's harmful effects on the kidney cells. TQ has favorable effect if the cancer treatment is the issue, but it might be used with caution due to its side effects on the other tissues; pharmaceutical windows of TQ seem to be narrow. Natural products and plant derivatives used in folk medicine are of vast medical importance due to their potential as a source of molecules with pharmacologic properties. Patients use plant remedies to prevent cancer, to treat the symptoms of cancer and side effects of treatment, and even to treat cancer. Plant based therapies include single chemicals extracted from plant, or part of plant. The active ingredients of most plant extracts are unknown. Compounds that are reliably present in the extract serve for quality control, but often there is no evidence that these compounds are responsible for therapeutic or side effects. In some studies, it has been shown that TQ can prevent the nephrotoxic effect of cisplatin, however we found that it enhanced the nephrotoxic effect of cisplatin in contrast to these studies.

Finally, this study, for the first time, demonstrated the synergistic nephrotoxicity by apoptotic pathway of cisplatin and TQ. The increases of p53 and NGAL intensity staining by the combination treatment of cisplatin and TQ might be one of the critical routes underlying the synergistic nephrotoxic effect *in vivo*. Therefore, cancer patients should be told that they should not use plant-derived substances in discriminately and they should also be informed about the serious side effects of using these substances especially in combination with chemotherapy. However, further research is required to confirm our study results.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Williams CJ, Whitehouse JM. Cis-platinum: a new anticancer agent. *Br Med J*. 1979;23:1689–1691.
2. Desoize B, Madoulet C. Particular aspects of platinum compounds used at present in cancer treatment. *Crit Rev Oncol Hematol*. 2002;42:317–325.
3. Tsang RY, Al-Fayea T, Au HJ. Cisplatin overdose: toxicities and management. *Drug Saf*. 2009;32:1109–1122.
4. Safirstein R, Winston J, Goldstein M, Moel D, Dikman S, Guttenplan J. Cisplatin nephrotoxicity. *Am J Kidney Dis*. 1986;8:356–367.
5. Leibbrandt ME, Wolfgang GH, Metz AL, Ozobia AA, Haskins JR. Critical subcellular targets of cisplatin and related platinum analogs in rat renal proximal tubule cells. *Kidney Int*. 1995;48:761–770.
6. Dobyan DC, Levi J, Jacobs C, Kosek J, Weiner MW. Mechanism of cis-platinum nephrotoxicity: II. Morphologic observations. *J Pharmacol Exp Ther*. 1980;213:551–556.
7. Uehara T, Yamate J, Torii M, Maruyama T. Comparative nephrotoxicity of cisplatin and nedaplatin: mechanisms and histopathological characteristics. *J Toxicol Pathol*. 2011;24:87–94.
8. Rodríguez-Enríquez S, Marín-Hernández A, Gallardo-Pérez JC, Carreño-Fuentes L, Moreno-Sánchez R. Targeting of cancer energy metabolism. *Mol Nutr Food Res*. 2009;53:29–48.
9. Hajhashemi V, Ghannadi A, Jafarabadi H. Black cumin seed essential oil, as a potent analgesic and anti-inflammatory drug. *Phytother Res*. 2004;18:195–199.
10. Trang NT, Wanner MJ, Phuong le VN, Koomen GJ, Dung NX. Thymoquinone from Eupatorium ayapana. *Planta Med*. 1993;59:99.
11. Shoieb AM, Elgayyar M, Dudrick PS, Bell JL, Tithof PK. In vitro inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone. *Int J Oncol*. 2003;22:107–113.
12. Jafri SH, Glass J, Shi R, Zhang S, Prince M, Kleiner-Hancock H. Thymoquinone and cisplatin as a therapeutic combination in lung cancer: in vitro and in vivo. *J Exp Clin Cancer Res*. 2010;29:87. http://dx.doi.org/10.1186/1756-9966-29-8.
13. Nessa MU, Beale P, Chan C, Yu JQ, Huq F. Synergism from combinations of cisplatin and oxaliplatin with quercetin and thymoquinone in human ovarian tumour models. *Anticancer Res*. 2011;31:3789–3797.
14. Ulu R, Dogukan A, Tuzcu M, et al. Regulation of renal organic anion and cation transporters by thymoquinone in cisplatin induced kidney injury. *Food Chem Toxicol*. 2012;50:1675–1679.
15. Badary OA, Nagi MN, al-Shabanah OA, al-Sawaf HA, al-Sohaibani MO, al-Bekairi AM. Thymoquinone ameliorates the nephrotoxicity induced by cisplatin in rodents and potentiates its antitumor activity. *Can J Physiol Pharmacol*. 1997;75:1356–1361.
16. Sagit M, Korkmaz F, Akcadag A, Somdas MA. Protective effect of thymoquinone against cisplatin-induced ototoxicity. *Eur Arch Otorhinolaryngol*. 2013;270:2231–2237.
17. El-Dakhakhany M. Studies on the Egyptian *Nigella sativa* L: some pharmacological properties of its seed's active principle in comparison to its dihydro-compound and its polymer. *Arzneim Forsch Drug Res*. 1965;15:1227–1229.
18. Al-Ali A, Alkhawajah AA, Randhawa MA, Shaikh NA. Oral and inraperitoneal LD50 of thymoquinone, an active principle of *Nigella sativa*, in mice and rats. *J Ayub Med Coll Abbotta Bad*. 2008;20:25–27.
19. Gali-Muhtasib H, Ocker M, Kuester D, et al. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. *J Cell Mol Med*. 2008;12:330–342.
20. Mukhopadhyay P, Horvath B, Zsengeller Z, et al. Mitochondrial targeted antioxidants represent a promising approach for prevention of cisplatin-induced nephropathy. *Free Radic Biol Med*. 2012;52:497–506.
21. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007;35:495–516.
22. Mishra J, Ma Q, Prada A, et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol*. 2003;14:2534–2543.
23. Tsigou E, Psallida V, Demponeras C, Boutzouka E, Baltopoulos G. Role of new biomarkers: functional and structural damage. *Crit Care Res Pract*. 2013:361078. http://dx.doi.org/10.1155/2013/361078. Epub 2013 Feb 5.
24. Jiang M, Wei Q, Pabla N, et al. Effects of hydroxyl radical scavenging on cisplatin-induced p53 activation, tubular cell apoptosis and nephrotoxicity. *Biochem Pharmacol*. 2007;73:1499–1510.
25. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney Int*. 2008;73:994–1007.
26. Rjiba-Touati K, Ayed-Boussema I, Bouaziz C, et al. Protective effect of erythropoietin against cisplatin-induced nephrotoxicity in rats: anti genotoxic antiapoptotic effect. *Drug Chem Toxicol*. 2012;35:89–95.
27. Mishra J, Mori K, Ma Q, Kellya C, Baraschb J, Devarajana P. Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. *Am J Nephrol*. 2004;24:307–315.
28. Wagener G, Jan M, Kim M, et al. Association between increases in urinary neutrophil-associated lipocalin and acute renal dysfunction after adult cardiac surgery. *Anesthesiology*. 2006;105:485–491.
29. Gali-Muhtasib H, Roessner A, Schneider-Stock R. Thymoquinone: a promising anti-cancer drug from natural sources. *Int J Biochem Cell Biol*. 2006;38:1249–1253.

30. Gali-Muhtasib H, Diab-Assaf M, Boltze C, et al. Thymoquinone extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism. *Int J Oncol*. 2004;25:857–866.
31. El-Mahdy MA, Zhu Q, Wang QE, Wani G, Wani AA. Thymoquinone induces apoptosis through activation of caspase-8 and mitochondrial events in p53-null myoloblastic leukemia HL-60 cells. *Int J Cancer*. 2005;117:409–417.
32. Woo CC, Kumar AP, Sethi G, Tan KH. Thymoquinone: potential cure for inflammatory disorders and cancer. *Biochem Pharmacol*. 2012;83:443–451.
33. Schneider-Stock R, Fakhoury IH, Zaki AM, El-Baba CO, Gali-Muhtasib HU. Thymoquinone: fifty years of success in the battle against cancer models. *Drug Discov Today*. 2013:1359–6446. http://dx.doi.org/10.1016/j.drudis.2013.08.021 [Epub ahead of print].