Celal Bayar University Journal of Science

Attenuation of Docetaxel-induced Oxidative Stress and Apoptosis in HEK 293 Human Embryonic Kidney Cells by Curcumin Treatment

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Received: 13June 2020
Accepted: 20 February 2021
DOI: 10.18466/cbayarfbe.752495

Abstract

Docetaxel (DOC) is a chemotherapeutic that induces microtubule stabilization. It is often used in breast, prostate, lung and gastric cancers but severe side effects such as cardiotoxicity, neurotoxicity, hepatotoxicity, and nephrotoxicity limit its usage. Curcumin (CUR), a natural bioactive compound derived from turmeric. Here, the possible preventive effect of CUR against DOC-induced oxidative stress and apoptosis on HEK-293 immortalized human embryonic kidney cells. Viability was assessed via MTT assay. The generation of ROS was measured by CM-H2DCFDA dye. Phosphatidylserine externalization and caspase 3/7 activity were used to measure apoptosis. CUR pretreatment remarkably inhibited DOC-induced cell viability reduction, ROS generation, and cell apoptosis in HEK-293 cells. Moreover, this study revealed that CUR pretreatment decreased caspase-3 activity. Thus, this study highlights the novel pharmacological mechanisms of CUR and understanding the detailed mechanisms of CUR action.

Keywords: Apoptosis, curcumin, docetaxel, HEK-293, oxidative stress.

1. Introduction

Despite the novel technological and scientific developments, cancer is still one of the biggest threats to humanity for decades. Many strategies are being done to struggle with cancer [1]. Among them, chemotherapy is the most effective one and considered the backbone of the treatment. However, serious side-effects in normal tissues such as cardiotoxicity, neurotoxicity, hepatotoxicity, and nephrotoxicity limit their usages [2-5]. Thus, there is an instant need to find new adjuvant compounds to diminish the chemotherapy-induced severe side effects.

Taxanes that promote microtubule stabilization are critical chemotherapy drugs in the treatment of a wide range of solid tumors [6-9]. Of taxanes, Docetaxel (DOC) is often used in breast, lung, prostate and gastric cancers, but its frequent use often leads to nephrotoxicity, especially in lung cancer patients [10]. Chronic nephrotoxicity of anticancer drugs may have multiple causes and are mediated by various mechanisms. In this study, the effects of DOC were investigated on human kidney embryonic cells (HEK-293) which could represent the possible target tissue and widely used in *in vitro* toxicology studies. One of the most important mechanisms is reactive oxygen species (ROS) formation and subsequently induction of hepatocyte apoptosis [11]. The induction of ROS also decreases endogenous antioxidants via activation of caspases. To avoid these harmful effects of oxidative stress, it is essential to increase the cell's antioxidant defenses with natural compounds having antioxidant activity.

Curcumin (CUR), known as diferuloylmethane, is originated from *Curcuma longa* rhizome. It has been shown to have several biological activities [12-15]. CUR is a potent oxygen free radical scavenger and can inhibit the production of ROS and prevent oxidative stress both *in vitro* and *in vivo* [16]. Previous studies demonstrated that CUR treatment can ameliorate nephrotoxicity by preventing oxidative stress caused by cisplatin, gentamicin and cyclosporine and paracetamol in rats [17-19].

Taken together, the current study was aimed to investigate the protective effect of the CUR against DOC-induced oxidative stress and apoptotic cell death on HEK-293 immortalized human embryonic kidney cells.

2. Materials and Methods

2.1. Materials

CUR was provided by Sigma Chemical Co (USA). To prepare a stock solution, CUR was dissolved in 1 mg/mL ethanol (EtOH) and stored at 4ºC. DOC was purchased from Sigma Chemical Co and dissolved in dimethyl sulfoxide (DMSO). The EtOH and DMSO concentrations used in this analysis were lower than 0.1% and were not cytotoxic. All other chemicals were obtained from Sigma.

2.2. Cell culture conditions

The human embryonic kidney cell line HEK-293 (CRL-11268) was purchased from ATCC (American Type Culture Collection, USA). The cells were cultured by using Eagle's Minimum Essential Medium including 10% fetal bovine serum and 1% penicillin/streptomycin in 75 cm^2 polystyrene cell culture flasks (Cellstar, UK). Cell culture was maintained in a standard incubator containing 5% CO_2 at 37°C.

2.3. Cell viability and morphology

MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was conducted to evaluate cell viability. Disaggregation of cells was carried out with trypsin/EDTA. A total of 10^4 cells were propagated onto 96-well plates in 100 µL medium per well. After 24 h cell attachment, cells were exposed to increasing concentrations of DOC, CUR, or sequentially. MTT (20 µL) was added at 24, 48 and 72 h time points. The dye was carefully drained and 200 µL DMSO was added to dissolve formed formazan crystals. The optic densities were recorded by a microplate reader at 570 nm (Tecan Infinite 200 PRO, Switzerland) [20]. Olympus IX53 inverted microscope was used to monitor HEK-293 cell morphology after DOC, CUR or sequential treatment (Tokyo, Japan).

2.4. Analysis of ROS generation

The formation of ROS in HEK-293 cells was measured by cell-permeable indicator dye [5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester dye (CM-H_2DCFDA, MW 577.8 Da, Molecular Probes, Eugene, OR)]. The CM-H_2DCFDA passively enters the cells and remains non-fluorescent until oxidation occurs in the cell. After oxidation, it fluoresces in the cell. The cell suspension was (10^5 cells/mL) transferred on 96-wells in 200 µL medium containing CM-H_2DCFDA dye. The plate was then incubated for 45 min. at 37 °C. As a control, cells were also exposed to 10 mM N-acetylcysteine (NAC) which is a ROS scavenger. The amount of fluorescence was then measured at 485-520 nm by a microplate reader (Tecan Infinite 200 PRO, Switzerland) and also observed with fluorescence microscopy (Olympus, Tokyo, Japan).

2.5. Flow cytometric analysis of apoptosis

For analyzing apoptosis, phosphatidylserine externalization was detected via Muse™ Annexin V and Dead Cell kit (Millipore, USA). HEK-293 cells in a density of 4×10^5 were seeded in 2 mL medium and incubated 24 h for attachment. Then, attached cells were exposed to compounds alone or sequentially for 48 h. After incubation, centrifugation was performed at 1000×g for 10 min and supernatants were removed. The remaining pellets were suspended with a culture medium. Equal amounts of cell suspension and Muse™ Annexin-V & Dead Cell kit solution were mixed and incubated for 20 min in the dark. The analysis was performed using Muse™ Cell Analyzer (Millipore, USA). For each condition at least 10,000 cells were analyzed.

2.6. Caspase 3/7 activity assay

Caspase-Glo 3/7 Assay (Promega, USA). Cells were (10^4 cells per well) treated with the desired concentrations of the compounds. After incubation periods, CaspaseGlo 3/7 reagent (100 µL) was added and incubated for 1 h in the dark. The formed luminescence was detected by a microplate reader (Tecan Infinite 200 PRO, Switzerland).

2.7. Statistical Analysis

Each experiment was done in duplicate, and three experiments were performed for each condition. Results were presented as mean ± SD. The *Student's t-test* was performed to analyze the differences between the two groups. For analysis of three or more groups, the first one-way analysis of variance test was done and then Dunnett's t-test was performed. The half-maximal inhibitory concentration (IC_{50}) was calculated via Graph Pad Prism 5.0 (San Diego, USA). A p-value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Preventive effect of CUR on DOC-induced cytotoxicity in HEK-293 cells

Today, CUR has attracted intense interest in the protection of normal tissues from chemotherapy-induced toxicity. Before determining the preventive effect of CUR on DOC-induced cytotoxicity in HEK-293 cells, first, the effects of single CUR or DOC on the viability of cells were investigated. For this aim, HEK-293 cells were exposed to increasing concentrations of CUR (5-100 µg/mL) or DOC (0.1-1000 nM) for different time points and viability was assessed via MTT assay. As seen in Fig. 1, the increasing concentrations of single CUR or DOC induced a time-

dependent reduction in cell viability ($p<0.05$). The IC_{50} value of CUR calculated from cell viability data was found to be 12.5 ± 0.52 µg/mL at 48 h ($p<0.05$). The cell viability was 91% at 5 µg/mL CUR concentration but significantly decreased by 25 µg/mL CUR concentration (17% viability) at 48 h. Therefore, 5, 10 and 25 µg/mL concentrations were chosen as a low-, medium- and high-concentration groups, respectively.
There are limited studies demonstrating the effects of CUR on HEK-293 cells in the literature. Concentration-dependent inhibition in cell viability by different incubation times was reported for HEK-293 cells and calculated IC_{50} values were quite different in these studies. Rao et al. calculated the IC_{50} value of CUR on HEK-293 cells as 5.0 ± 0.6 µM at 72 h [21]. In different studies, the IC_{50} value of CUR was calculated at 24 h and found to be 29.8 µM and 458.14 µM, respectively [22, 23]. Only Zhao et al. calculated the IC_{50} value of CUR at 48 h and found it as 11 µM which was similar to the results of the current study [24]. The IC_{50} value of DOC was also calculated in HEK-293 cells and found to be 5.5 ± 2.5 nM at 48 h. Contrary to CUR, there is no study investigating the effect of DOC on HEK-293 cells. Thus, this study is the first study revealing the effect of DOC on HEK-293 cells.

To screen the possible preventive effect of CUR on DOC-induced cytotoxic effects, cells were pretreated with 5, 10 or 25 µg/mL CUR for 48 h and then treated for an additional 48 h with 10 nM DOC. As demonstrated in Fig. 2, 5 and 10 µg/mL CUR pretreatment significantly increased cell viability compared with the 10 nM DOC treated group ($p<0.05$). However, in the 25 µg/mL CUR pretreated group, cell viability decreased as compared to the 10 nM DOC treated group ($p<0.05$). From these data, it can be concluded that CUR pretreatment with low and medium concentrations prevented DOC-induced cytotoxicity and cell damage in HEK-293 cells. However, this effect was not observed at high concentrations of CUR.

Nephrotoxicity is a serious and well described chemotherapy-associated side-effect limiting its clinical use. Ortega-Domínguez et al. revealed that CUR treatment prevented cisplatin-induced nephrotoxicity by diminishing mitochondrial abnormalities and necrosis [25]. *In vivo* part of the study was also revealed that pretreatment of CUR prevented all the histological abnormalities in the kidney as compared to only the cisplatin received group. In another study, mitomycin-induced kidney damage was attenuated by CUR pretreatment in mice [26]. Their results further indicate that CUR pretreatment could reduce chemotherapy-induced cytotoxicity and nephrotoxicity.

To investigate the changes in cell morphology, HEK-293 cells were also analyzed by an inverted microscope. Observations supported the cell viability results and showed that DOC treatment of HEK-293 cells led to rounding of cells indicating cell death. Moreover, loss of cell attachment to the well plates was observed, however, the number of non-adherent cells was decreased by CUR pretreatment (Figure 3).

3.2. CUR suppresses the production of ROS in DOC-treated HEK-293 cells

Oxidative stress and ROS accepted as the possible mechanisms responsible for DOC induced cytotoxicity and scavenging of ROS in normal tissues are known to be effective for protecting cells from chemotherapy-induced toxicity. In many studies, it was shown that CUR could inhibit cytotoxicity via diminishing cellular ROS [27, 28]. Thus, to enlighten the role of CUR in preventing DOC-induced cytotoxicity in HEK-293 cells, cellular ROS levels were measured after staining with CM-H2DCFDA and also observed with fluorescence microscopy. As shown in Fig. 4, cellular ROS was significantly induced after exposure to DOC treatment in HEK-293 cells. ROS levels were significantly diminished in CUR pretreated and NAC treated cells ($p<0.05$).

In the study of Ortega-Domínguez et al., it has been shown that CUR can inhibit ROS as strongly as NAC. In the same study, it was also revealed that CUR attenuates cisplatin-induced mitochondrial damage via diminishing ROS production [25]. CUR not only reduces ROS levels but induces cellular defense machinery such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GTP) activities [29]. Sheu et al. showed that CUR pretreatment increased the SOD activity and, in this way, enhanced the antioxidant capacity of normal tissues [30]. From these results, it can be concluded that the possible mechanism of ROS decreasing effect in HEK-293 cells pretreated with CUR might be associated with the direct free radical scavenging activity or indirect induction of antioxidant defense systems.

3.3. The effects of CUR pretreatment on cell apoptosis in DOC-treated HEK-293 cells

Growing evidence shows that chemotherapy-induced cytotoxicity is mediated by apoptotic induction following oxidative stress. [31]. If ROS levels excessively increase in the cell, redox imbalanced cells become more vulnerable to apoptosis. To elucidate whether the preventive effects of CUR were related to the prevention of cell apoptosis, flow cytometric analysis was done. Cells undergoing early and late apoptosis after exposure to DOC were stained by Annexin V-FITC and PI and found that DOC treatment induced apoptotic cell death in HEK-293 cells.

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Volume 17, Issue 1, 2021, p 73-78
Doi: 10.18466/cbayarfbe.752495

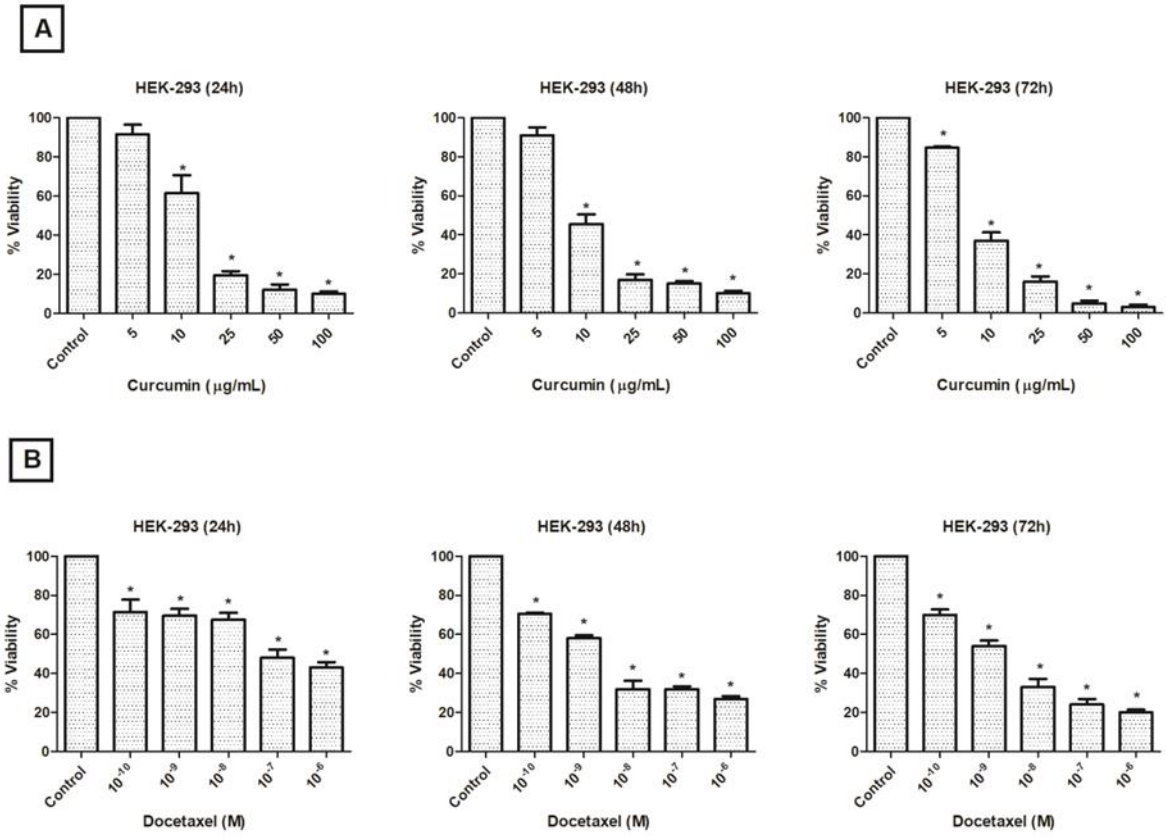

Figure 1: The effect of CUR (A) and DOC (B) on the viability of HEK-293 cells at 24, 48 and 72 h (*p<0.05 compared with the untreated control group).

However, the percentage of stained cells was significantly decreased in CUR pretreated group (p<0.05) (Figure 5).

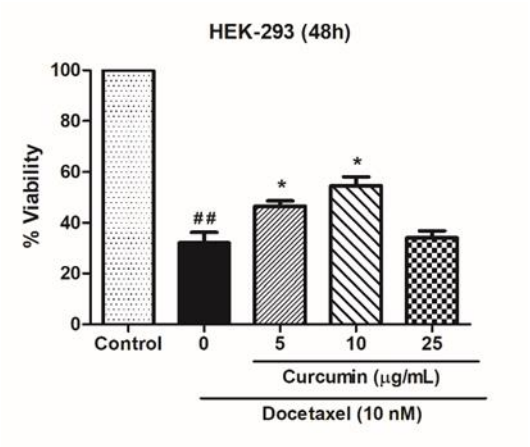

Figure 2: The effect of CUR pretreatment on the viability of HEK-293 cells under DOC exposure. Cells were treated with 5, 10 or 25 µg/mL CUR for 48 h then treated with 10 nM DOC for 48 h (##p<0.05 as compared to untreated control group, *p<0.05 as compared to 10 nM DOC treatment group).

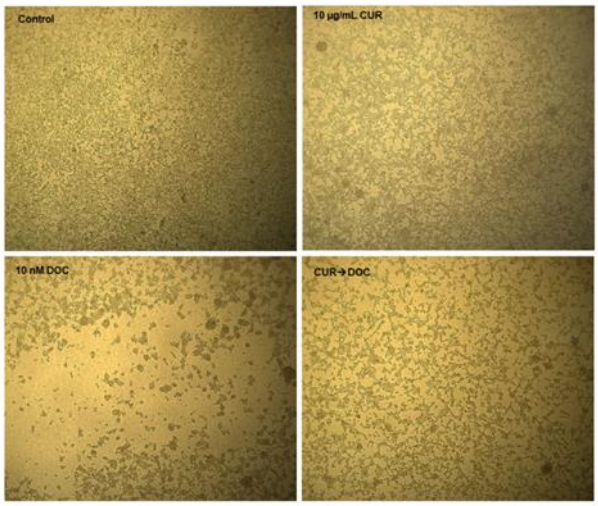

Figure 3: The effect of CUR pretreatment on the cell morphology of DOC-treated HEK-293 cells (20X).

After triggering apoptosis, the process is carried out by an enzyme family namely caspases. Among the caspase family, initiator caspases trigger effector caspases (caspases 3, 6, and 7) which are the final players that execute apoptosis [32].

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Volume 17, Issue 1, 2021, p 73-78
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After demonstrating the preventive effects of CUR pretreatment on apoptosis, caspase 3/7 activity was performed to confirm the data. According to results, pretreatment with CUR decreased the activity as compared to single DOC treatment ($p<0.05$) (Figure 5). These observations are parallel with the previous studies that investigate the preventive effect of CUR on cell apoptosis. Benzer et al. investigated the preventive effect of CUR on doxorubicin-induced apoptosis and demonstrated that pretreatment with CUR decreased the caspase-3 activity [33]. Dai et al., demonstrated that CUR pretreatment protected cells from caspase activation and following apoptosis which was triggered by colistin [34].

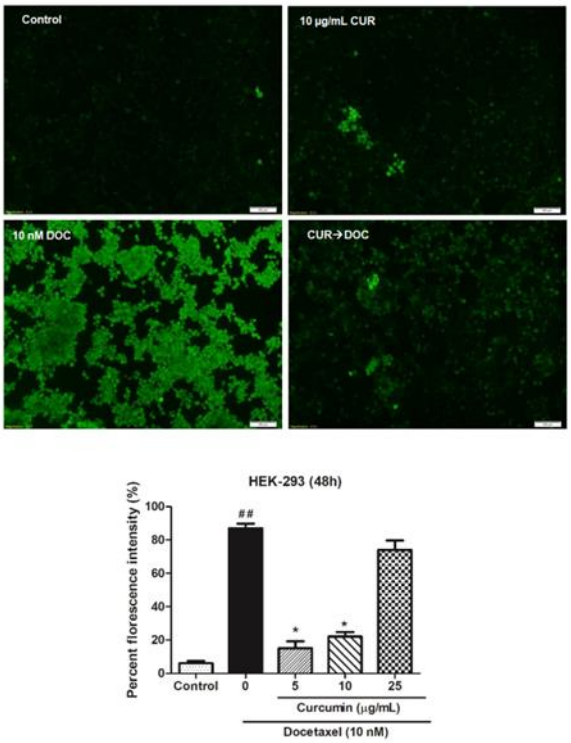

Figure 4: CUR ameliorates DOC-induced ROS in HEK-293 cells.

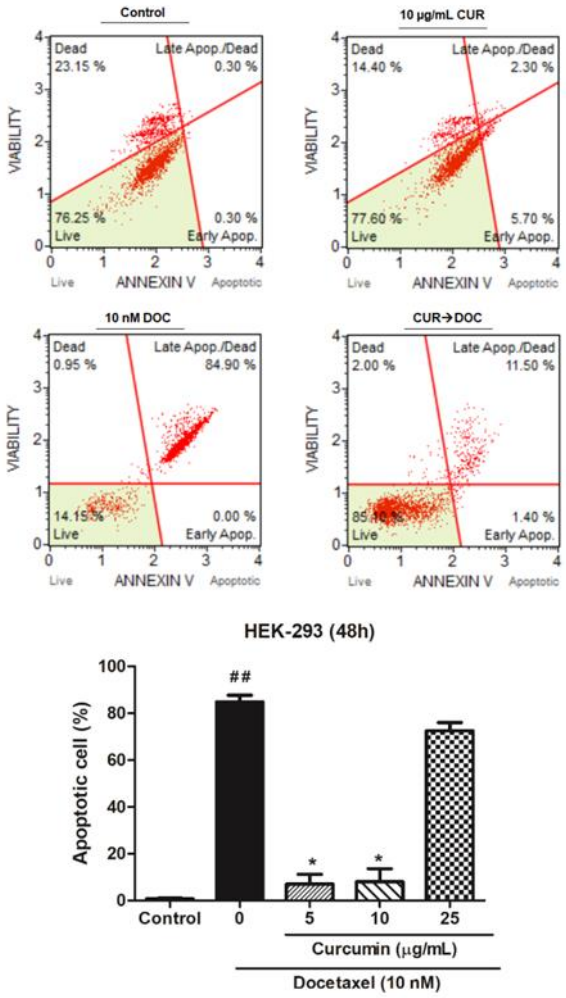

Figure 5: CUR pretreatment inhibited DOC-induced apoptosis in HEK-293 cells at 48 h (##$p<0.05$ as compared to the untreated control group, *$p<0.05$ as compared to 10 nM DOC treatment group).

4. Conclusion

In summary, pretreatment with low concentrations of CUR can alleviate DOC-induced ROS levels in HEK-293 human kidney embryonic cells. The CUR treatment also protects the cell from DOC-induced apoptotic cell death through inhibition of caspases 3 and 7 and increases the viability of cells. However, the detailed protection mechanisms of the CUR in HEK-293 cells require further investigations *in vitro* and *in vivo*. Understanding the detailed mechanisms of CUR action could lead to novel renoprotective interventions.

Author's Contributions

Süleyman İlhan: Drafted and wrote the manuscript, performed the experiment and result analysis.

Ethics

There are no ethical issues after the publication of this manuscript.

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