

Original Research Article

Genoprotective and Genotoxic Effects of Thymoquinone on Doxorubicin-Induced Damage in Isolated Human Leukocytes

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Abstract

Purpose: To investigate the potential genoprotective effects of thymoquinone (TQ) on the cytotoxicity and genotoxicity-induced by doxorubicin (DXR), a key chemotherapeutic drug.

Methods: Isolated human peripheral leukocytes were treated with varying concentrations of TQ (5.0, 10.0, or 20.0 μ M) alone or in combination with DXR (0.15 μ g/mL). Comet assays and apoptotic cell studies were performed to evaluate the effect of TQ on the cytotoxicity and genotoxicity-induced by DXR.

Results: TQ treatment, alone, (5.0, 10, or 20 μ M) increased DNA damage index (DI) in a concentration-dependent manner (0.64 ± 0.09 , 0.84 ± 0.07 , and 0.93 ± 0.06 , respectively). DXR (0.15 μ g/mL) increased DI (1.67 ± 0.09) compared with no treatment (0.34 ± 0.03). However, when TQ was administered with DXR, DI was significantly reduced (0.96 ± 0.04 , 0.80 ± 0.05 , and 0.79 ± 0.04) compared with DXR alone (1.67 ± 0.09). Similarly, apoptotic cells decreased (10.8, 11.8 and 14.2 %) compared with that induced by DXR alone (27.6 %).

Conclusion: TQ can be used as a genoprotective agent against DXR-induced genotoxicity. The dual behavior of TQ observed in this study is dose-dependent and therefore its mechanism of action needs to be clarified in future studies.

Keywords: Thymoquinone, Genotoxicity, Genoprotection, Doxorubicin, Apoptotic, Oxidative stress, DNA damage index

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INTRODUCTION

Nigella sativa L. seeds (Ranunculaceae), commonly known as black seed, are used as a traditional Arab herbal medicine for treating many diseases and ailments [1, 2]. Thymoquinone (TQ), the main essential oil of *N. sativa* has been intensively studied and is reported to possess antioxidant and anti-inflammatory properties [3,4] and to decrease the nephrotoxicity of some chemotherapeutic agents [5].

The hepatoprotective effect of TQ was reported in a study that used isolated rat hepatocytes in suspension culture that were treated with tert-butylhydroperoxide (TBHP) [6]. The hepatoprotection afforded by TQ was equal to that of silybin, a known hepatoprotective agent with antioxidant properties. Specifically, TQ prevented the leakage of certain hepatic enzymes (e.g., alanine transaminase [ALT], aspartate transaminase [AST], and co-enzyme glutathione [6,7].

There is a wide consensus in cancer research that TQ exerts promising anticancer activity in both *in vitro* and *in vivo* models [8]. It is effective against several types of cancer cell lines, and classical hallmarks of apoptosis such as chromatin condensation, translocation of phosphatidyl serine across the plasma membrane, and DNA fragmentation have been documented in TQ-treated cells [9,10]. Moreover, TQ was found to have anticancer activity against breast cancer cells through its potential effect on the peroxisome proliferator-activated receptors (PPARs) [11].

Cytotoxic and genotoxic effects of TQ have also been demonstrated in primary rat hepatocyte cultures [12]. For example, one study showed that TQ induced significant anti-proliferative effects at 20 μM and acute cytotoxicity at higher concentrations [13]. Moreover, it has been reported that TQ induces p53-independent apoptosis through activation of caspase-8 and other caspases involved in the caspase cascade [14]. Based on the existing evidence, we analyzed the *in vitro* genotoxic and genoprotective properties of TQ on doxorubicin (DXR)-induced genotoxicity in isolated cultured human leukocytes using comet assays.

EXPERIMENTAL

Cells and medium

Human peripheral blood was collected in heparinized vials from five healthy donors (25 – 30 years old). Leukocytes were isolated and cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20 % fetal calf serum (Cultilab, Campinas, SP, Brazil), streptomycin (10 $\mu\text{g}/\text{mL}$) penicillin (5 $\mu\text{g}/\text{mL}$), and 2 % phytohemagglutinin (Life Technologies, Carlsbad, CA, USA). Cells were cultured at 37 °C in culture flasks containing 5 mL complete medium.

The experimental protocols were approved by Research Ethics Committee (REC) of College of Pharmacy, Salman Bin Abdulaziz University, Al-kharj, Saudi Arabia. Written informed consent was obtained from each blood donor prior to their participation.

Chemicals

All chemicals and reagents used in the present study were of the highest analytical grade from Sigma-Aldrich. DXR was purchased as 10-mL vial ready for infusion (Adriamycin® - Pharmacia), with a concentration of 2 mg/mL.

Cell culture treatments

Different concentrations of TQ (Sigma-Aldrich; 5.0, 10.0, or 20.0 μM) were tested in combination with DXR in preliminary experiments; concentrations of TQ above 20 μM were cytotoxic and significantly reduced cell viability. The concentration of DXR (0.15 $\mu\text{g}/\text{mL}$) was similarly defined in preliminary experiments (data not shown) and in our previous study that quantified numbers of chromosomal aberrations [15]. Cytotoxic and cytostatic effect of the combined treatment can mimic antimutagenicity as they interfere with the appearance of mutant cells.

The isolated leukocyte cell cultures were treated either with vehicle (positive control) or DXR (Negative control). Three types of TQ treatment were assessed for their effects on DXR-induced damage in cells. After incubation for 24 h, the cell cultures were treated with TQ either simultaneously, 2 h before, or 2 h after DXR treatment. After each treatment, DXR and TQ remained in the culture media until cell harvest. After 24 h of DXR treatment, cells were harvested and assessed for cell viability, apoptosis, and DNA damage to individual cells (comet assay).

Comet assay

A 300- μL aliquot from each culture was taken 48 hours after incubation to test for cell viability by trypan blue exclusion and for use in the alkaline version of the comet assay [16]. The cell suspension (300 μL) was centrifuged for 5 min at 500 rpm in a refrigerated microcentrifuge. The resulting pellet was homogenized with 80 μL of a low melting point agarose (0.5 %), spread onto microscope slides pre-coated with a normal melting point agarose (1.5 %), and covered with a coverslip. After 5 min at 4 °C, the cover slip was removed and the slides were immersed in cold lysis solution for 24 h. After lysis, the slides were placed in an electrophoresis chamber and covered with electrophoresis buffer for 20 min to facilitate DNA winding. The electrophoresis continued for 20 min (25 V and 300 mA). Next, the slides were submerged for 15 min in a neutralization buffer, dried at room temperature, and fixed in 100 % ethanol for 5 min. Immediately before analysis, slide staining was performed using ethidium bromide (20 $\mu\text{g}/\text{mL}$). Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each slide) using a fluorescent microscope (ZEISS, Oberkochen, Germany). According to fragment migration, the nuclei were visually classified as: 0

(no damage), 1 (minimal damage with a short tail length smaller than the diameter of the nucleus), 2 (moderate damage with a tail length one or two times the diameter of the nucleus), 3 (significant damage with a tail length between two and a half to three times the diameter of the nucleus), and 4 (significant damage with a long tail of damage greater than three times the diameter of the nucleus). DNA damage was assessed using software as described in the comet test manual (Fig 1).

Morphological characterization of normal, apoptotic, and necrotic cells

We determined the frequencies of normal, apoptotic, and necrotic cells in cells treated with TQ 2 h before DXR treatment. We applied 2 μ L of fluorescein diacetate staining solution dissolved in DMSO (15 μ g/mL), propidium iodide (5 μ g/mL), and Hoechst 33342 (2 μ g/mL) (all from Sigma) to a 100 μ L cell suspension. For the three independent experiments, 500 cells per treatment were analyzed with an epifluorescence microscope. An intact and blue-stained nucleus was considered normal, a fragmented and a blue nucleus was considered apoptotic, and a red nucleus was considered necrotic.

Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis one-way method of variance (six independent experiments were analyzed) followed by Student–Newman–Keuls tests with 95 % confidence intervals.

RESULTS

Figure 2 shows the comet assay results and the DNA damage index (DI) findings for human leukocytes treated with DXR (0.15 μ g/mL) and different concentrations of TQ (5.0, 10, and 20 μ M). DXR and TQ (5.0, 10, and 20 μ M) applied separately increased the DI. However, compared to DXR treatment alone, the combined treatment of DXR and TQ (whether before or after) significantly reduced both DI and the frequency of comet-positive cells. The reduction was between 96 % and 97.5 % and was not dose-dependent.

As shown in Table 1, TQ treatment (5, 10, and 20 μ M) significantly reduced the number of apoptotic cells induced by DXR (0.15 μ g/mL) and consequently increased the number of viable cells. The most significantly protective TQ concentration was 5.0 μ M.

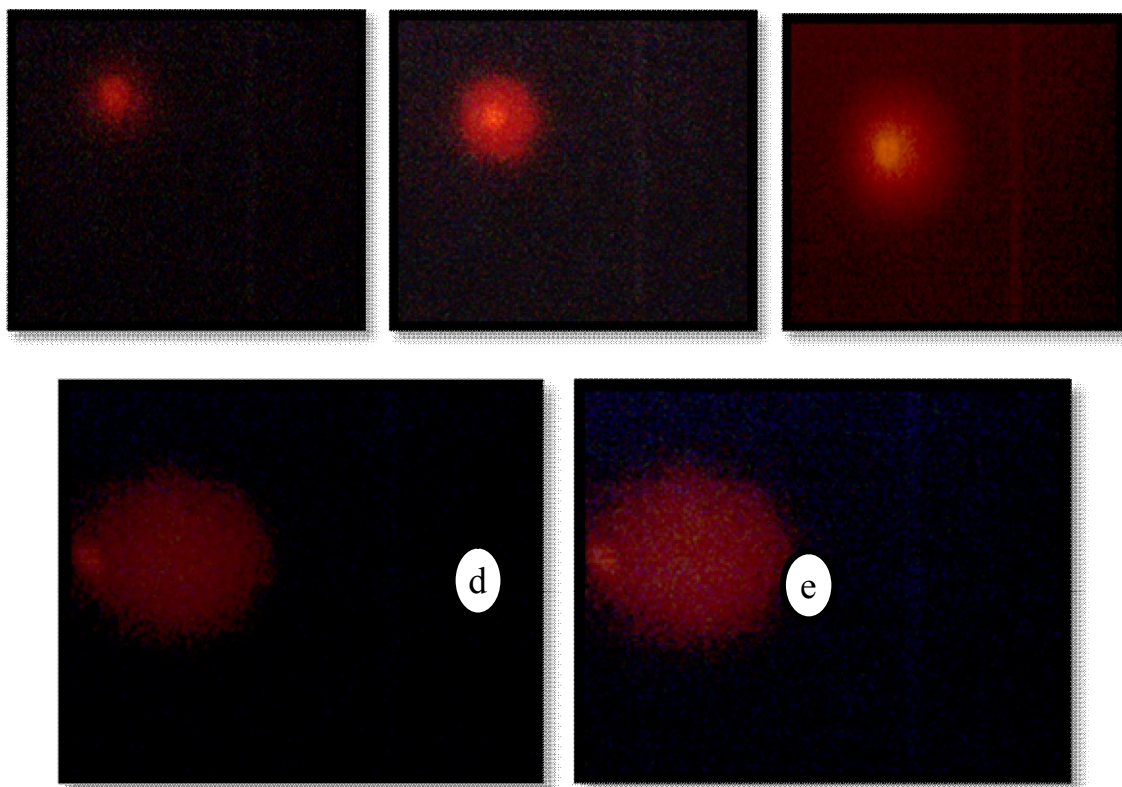


Fig. 1: Different levels of DNA damage in human leukocytes in the comet assay: (a) none, class 0; (b) low, class 1; (c) moderate, class 2; (d) high, class 3; and (e) total, class 4

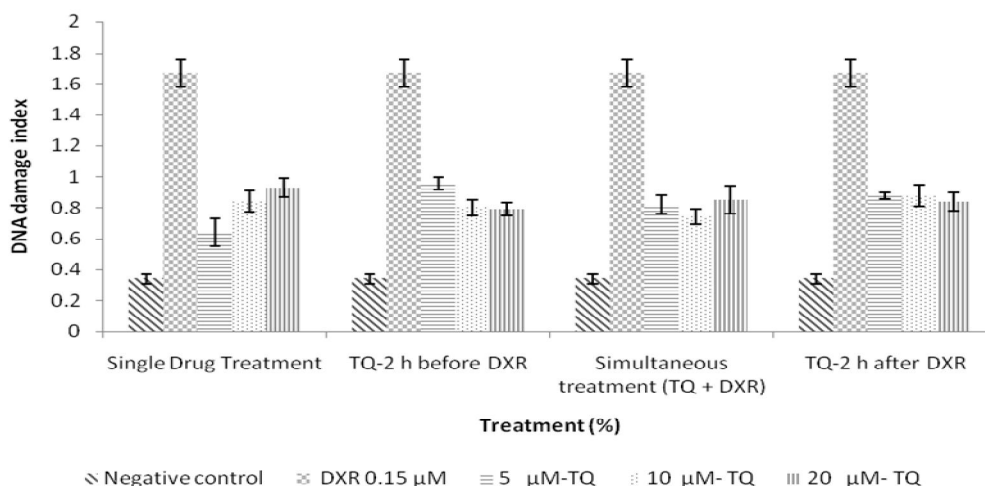


Fig. 2: Results of the comet assay assessing the effect of TQ on human leukocytes treated with DXR

Table 1: Apoptotic cells observed in isolated human leukocytes following different treatments with TQ and/or DXR

Treatment (%)	Scored cells (N)	Viable cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Single drug treatment				
Control	1500	96.9	2	1.1
DXR	1500	69	27.6 ^a	3.4
5 µM TQ	1500	94.2	4.8 ^a	1
10 µM TQ	1500	92.6	5.9 ^a	1.5
20 µM TQ	1500	91.6	7.3 ^a	1.1
Concurrent treatment (TQ + DXR)				
5 µM TQ + DXR	1500	88.4	10.8 ^b	0.8
10 µM TQ + DXR	1500	86.6	11.8 ^b	1.6
20 µM TQ + DXR	1500	84.2	14.2 ^b	1.6

A total of 1500 cells were scored in each one of the three replicates; doxorubicin (DXR) was applied at a concentration of 0.15 µg/mL; ^a*p* < 0.05 compared with negative control (vehicle-treated); ^b*p* < 0.01 compared with positive control (DXR)

DISCUSSION

N. sativa has been traditionally used to treat inflammations, liver disorders, and arthritis. Experimentally, it has been demonstrated that *N. sativa* extracts and TQ, the main constituent of its volatile oil, possess antioxidant, anti-inflammatory, hepatoprotective, and genoprotective properties [12,15].

The present study investigated the genoprotective and genotoxic effects of TQ alone and in combination with DXR. DXR is a potent genotoxic anticancer drug that generates undesirable effects in healthy cells. We observed that cells treated with TQ alone exhibited cytotoxic effects in a concentration-dependent manner; with a significant increase in the percentage of necrotic cells at 5.0 µM TQ. Furthermore, TQ also induced concentration-dependent genotoxic effects in leukocyte cultures, including an increase in DNA DI and a greater percentage of apoptotic cells, which was

concentration-dependent. We found that 40 µM TQ and higher concentrations (data not shown) caused severe cytotoxic effects in the cultures and were lethal to leukocytes.

These results are consistent with previous reports that showed that high TQ doses deplete cellular GSH [4]. Similarly, TQ has been reported to be a pro-apoptotic agent in variety of cell types, including cells of hematopoietic origin [17]. It has been suggested that TQ activates p53, and reduces anti-apoptotic protein concentration of Bcl-2 [18].

In this study, we employed DXR as the DNA damaging agent. DXR is a recognized topoisomerase II poison that generates reactive oxygen species (ROS) via two different routes of free radical formation. The first involves a non-enzymatic reaction of DXR with iron to form a stable complex, which reacts with oxygen to form superoxide anions, hydrogen peroxide, and hydroxyl radicals. The second involves formation

of highly reactive semiquinone intermediates through redox modifications [19]. Cell cultures were treated with DXR after 24 h, when most of the stimulated leukocytes were in the middle of S phase. It is noteworthy that TQ treatment alone (5.0, 10.0, and 20.0 μM) increased DNA damage index (DI) and apoptotic cells. Despite these significant genotoxic effects, all concentrations of TQ effectively reduced the genotoxic and clastogenic effects of DXR.

Whereas most of the comets observed following DXR treatment belonged to classes 2, 3, and 4, the TQ-treated cultures exhibited predominantly class 1 comets. It is noteworthy that the protective effects of TQ demonstrated here did not occur in a dose-dependent manner. TQ equally reduced the DXR-induced damage at the lowest and highest concentrations tested.

A similar "dual behavior" was also observed with vitamin C, which exhibited both pro- and anti-oxidative activities by decreasing cell death, membrane damage, and lipid peroxidation subsequent to oxygen exposure. This dual role is probably due to its opposing action on two types of oxidative stress [20]. TQ is metabolized *in vivo* by cellular oxidoreductases to hydroquinones or semiquinones radicals leading to production of ROS. These radicals may be responsible for the adverse effect of aqueous *N. sativa* extract on liver hepatocytes [20].

TQ also partially prevented DXR-induced oxidative damage in the cardiac tissue of rats supplemented with a TQ-enriched diet [21]. It has been suggested that superoxide scavenging and anti-lipid peroxidation partially explain the protective effect of TQ against DXR-induced cardiotoxicity [22]. Recently, other mechanisms related to TQ antioxidant properties have been proposed to explain the chemo-protective effects of TQ. TQ attenuated the secretion of pro-inflammatory cytokine, TNF- α in rat serum of cyclophosphamide-induced pulmonary injury [23,24].

The genoprotective effect and genotoxic effect of TQ may be related to TQ quinone structure. TQ undergoes one or two electron reductions by cellular reductases. One-electron reduction results in the formation of semiquinones, which are converted to ROS when they react with molecular oxygen [20]. Two-electron reductions produce the antioxidant hydroquinone [23]. This obligatory two-electron reduction competes with the one-electron reduction of quinones and protects cells against oxidative stress [25]. Both TQ and dihydrothymoquinone (DHTQ) are potent superoxide anion scavengers and general free

radical scavengers and prevent lipid peroxidation [26]. Interestingly, the *in vitro* antioxidant protection afforded by DHTQ may be greater with low TQ concentrations [27]. In contrast to the antimutagenic anti-oxidant effects reported in the present study in DXR- and TQ-treated lymphocytes, other researchers found that TQ increased necrotic cells at concentrations between 2.5 and 20 μM and caused genotoxicity at concentrations more than 1.25 μM [15]. However, we did observe this genotoxic effect in the present study when TQ was administered alone.

Regarding the timing of the supplementation with TQ, the present results showed that pre-, post-, or simultaneous treatment equally reduced the DXR-induced genotoxic effects at all tested concentrations of TQ. Similar results were obtained in lymphocyte cultures treated with vitamin C, a potent antioxidant, before, simultaneously, or after DXR treatment [28]. TQ exerts protective effect based on different mechanisms. TQ is involved in antioxidant defense and modulates the response of DNA repair factors and p53 expression. In the present work, only the lowest concentration of TQ was clastogenic and able to reduce DXR-induced damage. The other tested TQ concentrations exhibited some genotoxic effects. However, when applied alone without genotoxic agents, they also reduced the DNA damage induced by DXR.

CONCLUSION

TQ has genoprotective effects in low dose but can be genotoxic at high concentrations. Therefore, it is recommended that TQ dose should be modulated to achieve the lowest dose that protects against genetic damage with the least toxicity.

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