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EXPERIMENTAL STUDY

Protective effect of thymoquinone against cyclophosphamide-induced genotoxic damage in human lymphocytes

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ABSTRACT
OBJECTIVE: Protective effect of thymoquinone (TQ) against the cytotoxic and genotoxic effects of cyclophosphamide (CP) was assessed in human peripheral blood lymphocyte culture.
METHODS: Mitotic indices were determined as endpoints of cytotoxicity, while sister chromatid exchanges (SCE) served as endpoints of genotoxicity. Firstly, the genotoxic effect of 0.16 μg/ml of CP was tested and CP was detected as genotoxic. In the second set, CP group was treated with 20 μM and 40 μM TQ.
RESULTS: TQ reduced the SCE frequencies, suggesting its protective action on human lymphocytes *in vitro* against the CP induced genotoxic damage.
CONCLUSIONS: Our results suggest that TQ produces a protective mechanism against CP-induced genetic damage, and suggest a role of DNA strand breaks in the genotoxicity *(Tab. 1, Fig. 1, Ref. 19)*. Text in PDF *www.elis.sk.*
KEY WORDS: thymoquinone, cyclophosphamide, human lymphocytes, genotoxicity.

Introduction

Phytotherapy has attracted considerable attention in the recent years and is increasingly used as an alternative to chemical drugs (1, 2). Thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone; TQ) is a bioactive ingredient derived from *Nigella sativa* L. commonly known as black seed (Fig. 1A). TQ exhibits pleiotropic pharmacological activities including antioxidant, anti-inflammatory, immunomodulatory, anti-microbial, antidiabetic and antitumor effects (3–5). TQ has been demonstrated as a cytotoxic agent in several multi-drug resistant human tumor cell lines. Molecular mechanisms underlying these anticancer effects were attributed to inductions of cell cycle arrest, apoptosis, oxidative damage of cellular macromolecules, blockade of tumor angiogenesis and inhibitions in migration, invasion and metastasis of cancer cells and TQ induces DNA damage, telomere attrition by inhibiting telomerase and cell death in cancer cells (6, 7). Also, the combination of TQ and conventional chemotherapeutic agents could produce greater therapeutic effect as well as enhance cytotoxic effect in tumor cells (8).

Chromatid breaks, chromosome breaks, sister union and chromatid exchanges were the most common chromosomal abnormalities. Mutagens may induce single- and double-strand breaks and these breaks are subsequently converted in to chromosome fragments and finally to micronuclei or sister chromatid exchange (SCE) after one cell division. The frequency of SCE and the mitotic index (MI) have been used extensively for cytogenetic examination of peripheral lymphocytes in determining the mutagenic effects of chemotherapeutic drugs (9). These measures are indicators of exposure to genotoxic chemicals and markers of genome instability and disease states (10).

Cyclophosphamide (CP), the alkylating agent is widely used as an anti-tumor and immunosuppressive agent. However, it is known that many anticancer drugs have been found to be mutagenic, teratogenic, and carcinogenic in *in vitro* and *in vivo* test systems, and secondary malignancies are known to be associated with several therapeutic treatments (11). Although CP has been shown to induce genotoxic effects in a number of assay systems, limited experimental data are available on their clastogenic potential in human lymphocytes *in vitro*. CP may cause several side effects after treatment in the human body including the heart, liver, kidney of which mostly due to its structural properties prone to induce oxidative stress (12).

In this study by using SCE assays method, the modulatory effects exerted by the extract of TQ against the CP induced genotoxicity in the human lymphocyte cultures *in vitro* were tested.

Materials and methods

Chemicals

Thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone), Cyclophosphamide (CAS No.6055-19-2), 5-Bromo-2-deoxyuridine

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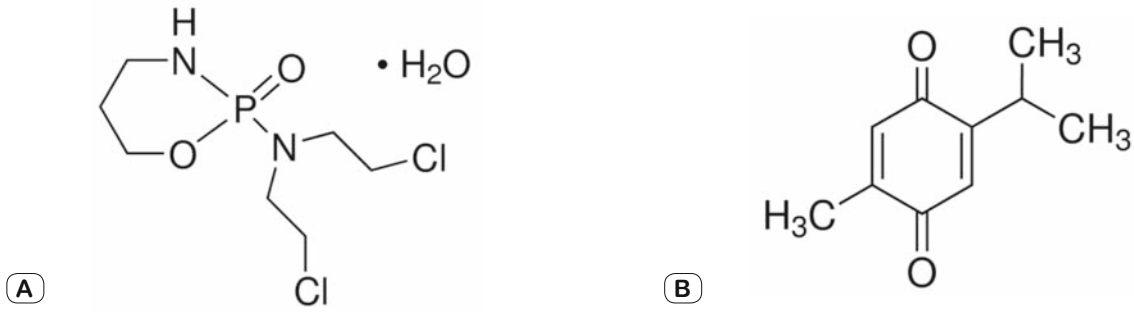

Fig. 1. A) Structure of Cyclophosphamid, B) Structure of Thymoquinone.

(CAS No.59-14-3) and colchicine (CAS No.477-30-5) were purchased from Sigma Chemicals. Peripheral Blood Karyotyping Medium (01-201-1B) was purchased from Biological Industries. Giemsa solution from Merck, India. The chemical structures of TQ and CP are shown in Figure 1.

SCE assay

For duplicate peripheral blood cultures, briefly 0.2 ml of heparinized whole blood samples from two healthy donors (one male, one female, non-smokers, age: 18–25) were added to 5 ml Chromosome Medium B supplemented with 10 µg/ml BrdUrd. The tubes were separated to six groups. For a group, the cells treated with 0.16 µg/ml Cyclophosphamide. The protective effect of TQ on cultures treated with CP was investigated by adding 20 and 40 µM TQ. The effect of TQ without CP was also tested. An untreated control was also established for each experiment. Then the culture tubes were incubated at 37 °C for 72 h. The cells were exposed to colchicine (0.06 µg/ml) 2 h before harvesting. The cells were harvested by 0.4 % KCl as hypotonic solution and methanol with glacial acetic acid (3 : 1) as fixative. The staining of air-dried slides was modified fluorescence plus Giemsa method. The slides were irradiated with 30 W, 254 nm UV lamp at 15 cm distance in Sorensen buffer, then incubated with 1×SSC at 60 °C for 45–60 min and stained with 5% Giemsa prepared with Sorensen buffer. The slides were coded before scoring. In order to score SCE, 25 second-division metaphases were analyzed for each donor at 1000x magnification using Olympus BH2 oil immersion lens, and the frequency of SCE per cell was recorded.

Cell cycle kinetics

The MI explained the effects of the chemicals on G2 stage of cell cycle. Cells undergoing metaphase divisions were detected with BrdU-Harlequin technique for differential staining of metaphase chromosomes. The MI was calculated by applying the following formula:

MI= Metaphase divisions (M) x 100 / N

A total of 3000 cells per donor were scored for the determination of MI.

Statistical analysis

Data were analyzed using Windows for SPSS version 21 statistical software program. The data are expressed as arithmetic mean ($\bar{X}$) ± standard deviation (SD). The normality assumption was tested with the Shapiro-Wilk test. Continuous variables show a normal distribution. The differences in the marker means, MI and MN frequencies among the experiment groups, and control groups were analyzed using the One-way Analysis of Variance test. Multiple comparisons were done by the Least Significant Difference test. All p-values were two-tailed and a value of < 0.05 was accepted as significant.

Results

Data on the mean frequency of SCEs and MI for cells after treatment with CP and TQ alone, as well as those with combined treatment by these chemicals are presented in Table 1. Control value of mean SCE frequency was determined as 6.48. CP treatment increased SCE rate to 14.32. This value was highly more significant than the value of control ($p < 0.05$). The mean SCE frequency at 20 and 40 µM TQ concentrations were not significant compared to the control. In addition, 40 µM TQ decreased the SCE frequency in CP-treated group. The MI showed reduction in CP and mixture groups except in alone TQ treatment groups when compared with control.

Tab. 1. Frequency of SCE, and MI in cultured human lymphocytes treated with cyclophosphamide and thymoquinone.

Test substance	concentration	SCE (X±SD)	MI (X ±SD)
Control	-	6.48±2.34	4.97±2.11
CP	0,16 µg/ml	14.32±3.62 [a]	2.38±0.94 [a]
TQ	20 µM	6.44±2.43 [b]	4.73±0.89 [b]
	40 µM	6.56±2.38 [b]	4.62±1.58 [b]
TQ + CP	20 µM+0,16 µg/ml	14.28±3.83 [ac]	3.08±1.02 [ac]
	40 µM+0,16 µg/ml	10.56±2.53 [abcd]	2.74±0.81 [ac]

SCE – sister chromatid exchange; MI – mitotic index; SD – standard deviation; CP – cyclophosphamide; TQ – thymoquinone, [+]A total 50 cells were scored for the SCE assay and 3000 cells were scored for the MI

[++] $p < 0.001$, [+++ a] significant from the control; [b] significant from CP; [c] significant from TQ; [d] significant between the cultures treated with 20 µM TQ and 40 µM TQ

Discussion

Genetic mutations causing abnormal cell growth and differentiation are the basis for all types of cancer. Recent studies showed that chromosomal aberrations positively correlate with cancer risk (10). Structural or numerical chromosomal abnormalities can be assessed reliably by evaluating the frequency of SCE in dividing cells8. SCEs in lymphocytes are among the most widely used and well established assays for the detection of early biological effects induced by DNA-damaging agents (13,14).

The safety of a drug product is dependent not only on the toxicological properties of the active drug substance itself, but also on the impurities it contains. These components that have the potential to induce genetic mutations, chromosomal breaks and rearrangements are considered as potential genotoxic impurities, which may cause cancer in humans. Some researchers suggested that CP generates active metabolites, like 4-hydroxycyclophosphamide, phosphoramide mustard and acrolein. These metabolites preferably alkylate N^7 position of the guanine residue of DNA and this leads to inter- and intra-strand cross-links, DNA strand breaks, cessation of DNA synthesis, DNA-protein cross-links and DNA adduct formation (15). There are a lot of studies on genotoxicity of CP. In these studies, CP has been reported to induce structural chromosomal aberrations, SCEs and MN frequency in cultured cells (12). Cyclophosphamide-mediated genotoxicity either occurrs induction nondisjunction and aneuploidy via microtubule alterations or induction of mutations following activation to DNA-reactive intermediates or modulation of endogenous or dietary mutagenic agents (16). The clastogenic potential of CP was confirmed in this study by observing high incidence of SCE formation in the lymphocyte cells. In contrast, TQ exhibited protective and anti-genotoxic effects on DNA damage in this study and this may be attributed to its antioxidant properties.

To determine possible cytotoxic, cytostatic, or mitogenic effects of CP, we analyzed the MI. The results showed that CP caused significant departures of MI values in relation to the control. In cells with a relatively high level of genetic damage, cytotoxic effects occur. CP decreased MI by ~ 48 % compared to negative control, possibly due to arrest of mitosis because of repair of genetic material.

Numerous studies were performed to assess the protective effects of plant extracts and/or their isolated bioactive components against genotoxicity induced by mutagenic agents using the CA, SCE, and MN tests in human lymphocyte cells (1, 12). However, to our knowledge, no studies are available in the scientific literature on the combined effects of TQ with any known mutagenic substance for determining the protective effects of this essential oil component. Any reduction in the frequency of SCE gives an indication of the protective effect of a particular substance (8, 17). In our study, 40 μM concentration of TQ decreased the SCE frequency at the CP treatment group, but MI frequency statistically did not alter in CP + TQ groups compared to CP alone. The molecular mechanisms behind of TQ's anti-cancer role are still not clearly understood. The protection afforded by TQ against the CP-induced genotoxicity and DNA damage may be due to free radical scavenging, increased antioxidant status and apoptosis. *In vitro* studies show that TQ can inhibit growth of several types of cancer cells. Such anti-proliferative activities exhibited by TQ involve inhibition of cancer cell signal transduction and apoptosis, which consequently leads to reduced DNA oxidative damage (4, 7, 8). It also controls the Akt pathway, which means it controls the process that manages cell survival for both normal and cancer cells (18).

The combination therapy has provided a better way to counteract cancer, specifically against resistant tumors. A large number of studies have been performed that indicate that TQ improves the therapeutic potency of various chemotherapeutic drugs increasing their antitumor activity and by decreasing their toxicity to normal cells (19). Our present work demonstrated that *TQ* has not shown any genotoxicity, and it also reduces the genotoxicity caused by reactive metabolites of CP. It is shown for the first time that TQ has a protective effect against genotoxicity induced by CP in lymphocytes. Therefore, it could be provided as one of the herbal supplementation in chemoprevention of CP to ameliorate the side effects of it. To draw conclusions for potential cellular protective effects associated with the medicinal use of TQ, further studies may validate this finding among larger groups of CP and TQ doses *in vitro* and *in vivo*.

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