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Chemoprotective effect of thymol against genotoxicity induced by bleomycin in human lymphocytes

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Abstract

Bleomycin (BLM) as an anti-cancer agent causes tissue toxicities through DNA damaging and cell deaths. The aim of this study was to investigate the effects of thymol against genotoxicity and anti-proliferation induced by BLM in normal human lymphocytes and ovarian cancer cells. Peripheral blood samples were collected from human volunteers and were incubated with thymol at different concentrations at 50, 100, and 150 μM. After 2 h incubation, the whole blood was treated with BLM. Then the lymphocytes were cultured with mitogenic stimulation to determine the micronuclei in cytokinesis blocked binucleated lymphocyte. Human ovarian cancer cells (SKOV-3) were treated with thymol at various concentrations and/or BLM with their combinations and then cell viability were evaluated. Incubation of whole blood with thymol exhibited a significant decrease in the incidence of micronuclei in lymphocytes caused by BLM, as compared with similarly BLM-treated lymphocytes without thymol. Neither enhanced cell death nor cell protective effect was observed using thymol pre-treatment of SKOV-3 cells. This study showed that thymol selectively protects human lymphocytes against DNA damage induced by BLM without any protection on cancer cell. This result is promising for using this natural product in treatment of ovarian cancer with BLM.

Keywords: Bleomycin, genotoxicity, thymol, micronuclei, anti-proliferation

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Introduction

Most anticancer drugs are known to induce DNA damage and apoptosis and cause side sever effects on normal tissues. Anticancer agents elevate oxidative stress in the cells through production of free radicals and other reactive oxygen species (ROS). ROS can attack to critical macromolecules such as DNA resulting in DNA breaks (1, 2). ROS may contribute to side effects that occur with anticancer agents such as bleomycin (3-5). Bleomycin (BLM) is an anticancer agent widely uses in treatment of various cancers (6-9). BLM is able to induce DNA double strand beaks is results in apoptosis and cell damage, also causes carcinogenic and clastogenic effects. BLM specifically oxidizes sugar moiety of DNA, with similar effect to ionizing radiation (10). Treatment of human lymphocytes with BLM causes an increasing in the frequency of chromosome aberrations and micronuclei (11-13). Because BLM causes side effects on cells through oxidative stress, antioxidants might be expected to protect cells against BLM toxicity and DNA damages. Although BLM has high efficiency as an anticancer agent against various cancers, the use of BLM is limited by the development of resistance in some tumor cells and by normal tissues toxicity. Elevated of dose BLM is associated to more affect on tumor cell, however, it increased side effects on normal tissues (14, 15). It is interesting to find a protective agent against BLM-toxicity on normal cells, without any protection on killing effect of BLM on cancer cells.

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Thymol is a natural phenolic compound that presents in various plants, such as thyme (Lamiaceae) and Zataria (16-18). Several biological properties were reported for thymol that has anti-inflammatory activity (19) and protective effects against toxicity are via antioxidant effects in liver and lymphocytes (20-22). Antioxidant, anti-inflammatory effect, and anti-lipid peroxidation of thymol are contributed to protective effects against toxicity related to oxidative stress in cells (20, 23).

The present study was investigated the protective effect of thymol against genotoxicity induced by BLM in human normal lymphocytes and anti-proliferative effect on human ovarian cancer cells.

Materials and methods

Chemicals

Bleomycin (LYOBLE, India) was dissolved in sterile water at stock solution and diluted with cell culture medium. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) was purchased from Sigma (USA). Thymol was from Merck (Germany).

Cell culture

After obtaining permission from medical research committee of the university, this study was performed. Non-smoking healthy male volunteers, ages between 20 to 25 years were enrolled in this study. Human ovarian cancer (SKOV-3) cells were got from the Pasture Institute of Iran and cultured at 37 °C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin–streptomycin (Gibco, UK). Experiments on cells were performed in the exponential growth phase.

Micronuclei assay

Ten mL whole blood was collected in heparinized tube and divided in 1.5 mL tube at 0.9 mL. Blood samples were treated with 100 µL solution of thymol at the concentrations 50, 100, or 150 µM (final concentrations). These samples were incubated for two hours at 37 °C. Thymol was dissolved in ethanol and diluted in cultural medium. Ethanol concentration was same in control and thymol solutions (0.2%). At each concentration and for each volunteer, tubes were treatment with bleomycin (10 µg/ml) and then incubated for 3 h. After incubation, 5 mL RPMI medium was added to each blood sample tubes and then centrifuged for 1500 g for 8 min. After washing cells, 0.5 mL of each sample (control and BLM samples in duplicate) was added to 4.4 mL of RPMI 1640 culture medium (Gibco, USA), which contained 10% fetal calf serum. Then phytohemagglutinin (100 µl/5ml, Gibco, USA) was added to cultures as lymphocyte stimulator. All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cytochalasin B (Sigma, USA) was added after 44 h of culture. Following 72 h of incubation, the cells were collected by centrifugation for 7 min at 3500 rpm, re-suspended in cold potassium chloride. Cells immediately fixed in a fixative solution as methanol: acetic acid two times. After air-dried, cells were stained with Giemsa solution (10%). All slides were evaluated at 100× magnification in order to determine the frequency of micronuclei in the cytokinesis-blocked binucleated cells with a well-preserved cytoplasm (24). At each concentration and for each volunteer, 1000 binucleated lymphocyte cells were examined from the BLM, BLM+thymol, thymol, and control cultures to record the frequency of micronuclei.

Cell anti-proliferation assay

Untreated and treated SKOV-3 cells were subjected to cell proliferation assay using MTT to quantify the metabolic activity to cleave tetrazolium salts (25, 26). Cells (20,000) were seeded in 96-well plates and incubated for 24 h. Cells were treated with thymol at various concentrations (50, 100 and 150 µM) and incubated for 2 h at 37 °C and 5% CO_2. After incubation, BLM was added at concentration 10 µg/mL to each well. All testing and control groups were in four independent wells. At 48 hours of culture, 20 µL MTT (5 mg/ml in phosphate buffer saline) was added to each well, and culturing was continued for 4 hours. Then, culture supernatant was discarded and replaced by 150 µL isopropanol (0.1% HCl), and the cell plates were shaken for 30 minutes. Finally, the absorbance of every culture well was read on an ELISA Reader (Bioteck, USA).

Statistical analysis

Data were presented as mean ± standard deviation (SD) of three or four independent experiments. Data were compared with Student T-test and the differences were considered significant if the p value < 0.05.

Results

Genotoxicity assay

A typical micronucleus in binucleoutided human lymphocyte is shown in figure 1.

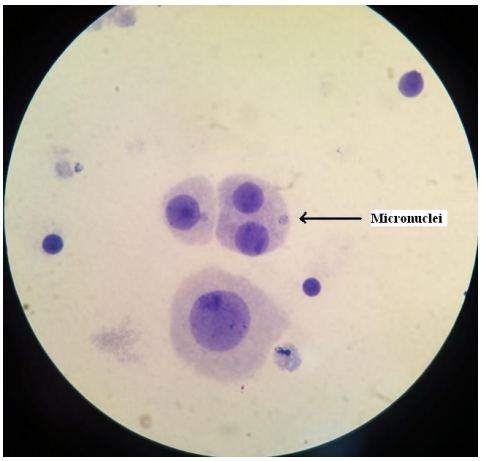

Figure 1 A typical binucleated lymphocyte with micronuclei in our study.

The percentage of micronuclei in binucleated lymphocytes in human bloods treated with BLM was 7.79 ± 2.0, while the percentage in non-treated control lymphocytes was 0.23 ± 0.21. It was showed a significantly increasing of 34-fold in frequency of micronuclei in samples treated with BLM ($p < 0.01$) (Table 1).

Table 1 The frequency of micronuclei induced *in vitro* by bleomycin (BLM) in cultured blood lymphocytes from human volunteers pre-treated with different doses of thymol (T)

Group	Mean ± SD
Control	0.23 ± 0.21
BLM	7.79 ± 2.00[a]
T50 + BLM	2.81 ± 0.99[b]
T100 + BLM	2.42 ± 0.16[c]
T150 + BLM	5.24 ± 1.26[d]
T100	0.37 ± 0.29[e]
T150	0.35 ± 0.48[f]

* 1000 BN cells were examined in each sample.
Comparison between groups: [a] $p < 0.01$ between control and BLM, [b] $p < 0.05$ between T50 + BLM and BLM, [c] $p < 0.05$ between T100+BLM and BLM, [d] non-significant between T150 + BLM and BLM, [e] non-significant between T100 and control, [f] non-significant between T150 and control.

The frequency of micronuclei after pre-treatment with thymol at doses of 50, 100 and 150 µM were 2.81 ± 0.99, 2.42 ± 0.16 and 5.24 ± 0.16, respectively (Table 1).

The data demonstrate that human blood incubated with thymol, and then treated in vitro to BLM, exhibited a significant reduction in micronuclei frequency compared to blood samples incubated with BLM alone. Thymol significantly mitigated frequency of micronuclei at doses 50 and 100 µM in lymphocytes treated with BLM ($p < 0.05$). Total micronuclei values were increased by 64% and 69% in samples treated with thymol and BLM at concentrations of 50 and 100 µM, respectively, compared to BLM-treated control. Thymol was not exhibited any genotoxicity in cultured lymphocytes at concentration of 150 µM without treatment with BLM.

Anti-proliferation on cancer cell

Cell viability of SKOV-3 cells treated with thymol in combination with BLM or alone was determined by MTT colorimetric assay. To examine the proliferation effect of thymol on ovarian cancer, SKOV-3 cells were treated with thymol at various concentrations (50, 100 and 150 µM) for 48 h. Thymol alone was not showed any inhibitory effect on SKOV-3 cells. BLM alone exhibited significantly inhibition effect on growth of SKOV-3 cells. Thymol in combination with BLM was not shown any reduced growth inhibition on ovarian cancer cells (Fig. 2). Thymol was not shown any protection against cytotoxicity in combination with BLM on SKOV-3.

Discussion

In this study we showed thymol has a chemoprotective effect against genotoxicity induced by bleomycin in human lymphocytes. Thymol reduced the frequency of micronuclei in binucleated lymphocytes which was treated with BLM. Thymol exhibited a protective effect at concentrations of 50, 100 and 150 µM by factors 2.7, 3.2 and 1.5, respectively. Thymol was not showed any protection on ovarian cancer cells treated with BLM. In this study, thymol exhibited a selective protective effect against DNA damage induced by BLM on human lymphocyte. It is interest to combine thymol with chemotherapy agent; it is possible to improve the effectiveness of the cancer treatment and minimizing toxicity on normal cells. Oxidative stress and inflammation are the main reasons for cell

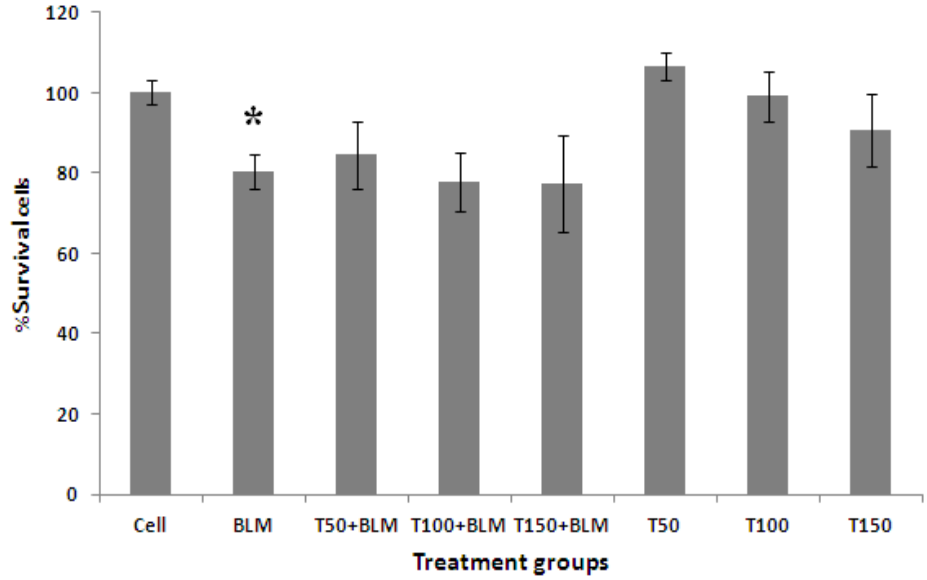

Figure 2 Anti-proliferative effects of thymol (T) (50, 100 and 150 µM) with bleomycin (BLM) (10 µg/ml) on ovarian cancer cell (SKOV-3). $P < 0.05$ comparison control with BLM

toxicity and pathogenesis of pulmonary fibrosis induced by BLM (5). Several studies reported that antioxidants and anti-inflammatory agents have protective roles in mitigation of BLM toxicity in normal tissues. N-acetylcystein amide, ellagic acid, corilagin, and andrographolide attenuated production oxidative stress and pro-inflammatory process in lung toxicity caused by BLM, and had protective effects (4, 27-30). Oxidative stress and inflammations are contributed in DNA damages and caused chromosome instability and mutagenesis induced by BLM in the cells (31-33). The role pro-inflammatory process and oxidative stress are different in the normal and cancer cells. However anti-inflammatory agents and antioxidants protect normal cells against serious effect of oxidative stress, there is an interaction between tumor cells and pro-inflammatory mediators. Cytokines and pro-inflammatory conditions promote tumor cell proliferation, growth, and invasion. Pro-inflammatory mediators and ROS play critical roles in tumor cell growth. Based these mechanisms, anti-inflammatory agents and antioxidants block inflammation pathways and oxidative stress to may reduce the growth of cancer cells (34-36). Thymol as a naturally monotherpene compound has numerous biological activities on cells mainly through antioxidant and anti-inflammatory effects.

Thymol prevented oxidative stress-induced damage to liver cells through suppression of ROS and MDA levels and increase of GSH level (37). Thymol protected oxidative stress-myocardial infarction in rats by its anti-lipid peroxidation and antioxidant properties and near normalization of reduced GSH, vitamin C, and vitamin E levels (38). Thymol ameliorated airway inflammation in ovalbumin-induced mouse asthma, through inhibiting NF-κB activation (39). Thymol exerted anti-inflammatory property in lipopolysaccharide-stimulated mouse mammary epithelial cells by interfering the activation of nuclear factor kappa **(**NF-κB) and mitogen-activated protein kinases (MAPK) signaling pathways (40). These results showed thymol has anti-inflammatory effects through suppression of several biomarkers involved in inflammation. It is suggested that the protective effect of thymol against genotoxicity induced by BLM is probably related to its antioxidant and anti-inflammation.

Conclusion

The present study shows that thymol could reduce the genotoxic effect of bleomycin on human lymphocyte. It was also shown that neither enhanced cell death nor cell protective effect was observed using thymol pre-treatment of SKOV-3 cancer cells. On the basis of these results, further investigations and as well as *in vivo* experiments must be performed to suggest

the possible clinical applications of these combinations in patients.

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Conflict of interest statement
The authors declared no potential conflict of interest with respect to the authorship, and/or publication of this study.

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