DOI: 10.4149/BLL_2018_059

Bratisl Med J 2018; 119 (5)
312 – 316

EXPERIMENTAL STUDY

Antiproliferative and antiapoptotic effect of thymoquinone on cancer cells in vitro

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ABSTRACT
OBJECTIVES: *Nigella sativa* oil and thymoquinone were comparatively tested *in vitro* for their effects on human cancer cell lines (glioma,T98; prostate, LnCaP) as well as mouse embryonic fibroblast cell lines (3T3), and for the induction of apoptosis.
METHODS: Individual cell lines were treated with thymoquinone and *N. sativa* oil for 24 and 48 hr. Survival rate with MTT, apoptosis with flow cytometry and caspase-9 mRNA enzyme levels with RT-PCR were determined in vitro.
RESULTS: Application of respective concentrations of *N. sativa* oil (excluding 100 µg/mL for 48 hr) did not change the number of tested cell lines, however, treatment with thymoquinone reduced the number of all cells significantly. Thymoquinone also exerted its apoptosis inducing effect through the activation of caspase-9.
CONCLUSION: Differing with the type of cancer cells, thymoquinone posseses a strong contentration and time dependent survival reducing effect on cancer cells via apoptosis *(Fig. 6, Ref. 22)*. Text in PDF *www.elis.sk.*
KEY WORDS: thymoquinone, in vitro, apoptosis, antiproliferative, anticancer.

Introduction

The plague of this century, "cancer" is a group of complex pathologies and diseases characterized by "out of control" cell growth (1). During apoptosis, a variety of molecules with up-regulatory and down-regulatory properties have a dynamic interaction and can inhibit pro-apoptotic molecules or apoptotic factors. And cancer is caused by uncontrolled cell proliferation or failure of cells to pass through the apoptotic cell death (2–4). As a consequence, compounds which may trigger cancer cell apoptosis may lead to promising future drugs for the treatment of cancer.

Nigella sativa, well known also as black cumin seed of the Ranunculaceae is an annual plant, has a long history in traditional medicine with therapeutic effects including analgesia, antihypertensive, anti-eczema, diuretic, antimicrobial, gastrointestinal problems as well as in various cancer therapies (5).

Thymoquinone is one of the bioactive components of *Nigella sativa* seed oil and has a wide range of biological and pharmacological activities including anti-cancer, anti-tumoral, anti-oxidant and anti-proliferative properties. *In vitro* and *in vivo* pharmacological studies have been reported for their potential antidiabetic (6), neuroprotective (7), cardiovascular (8), gastroprotective, hepatoprotective (9) activities among others (10). Since there has baeen no encountered study examining the possible role of thymoquinone on glioma (T98), prostate cancer cells (LnCaP) and mouse embryonic fibroblast cell line (3T3), we tried to reveal its role on these cell lines in vitro.

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Materials and methods

General

Thymoquinone was obtained from commercial sources in high purity (> 98 %, Sigma-Aldrich) whereas *Nigella sativa* oil was obtained from local producer (> 10 % thymoquinone, AweCemre, Tokat, Turkey). Both samples were dissolved individually in dimethyl sulfoxide (DMSO, Sigma) and diluted further with Dulbecco's modified Eagle's medium (DMEM, Sigma) to obtain the required final concentrations for the assays listed below in detail.

Cell culture

T98, LnCaP and 3T3 cells were obtained from the American Type Culture Collection (ATCC, USA) and grown in complete medium containing DMEM supplemented with 10 % fetal calf serum (FCS, Sigma) and 1 % penicillin (10000 unit)-streptomycine (10mg/ml) solution (Sigma) in a humidified atmosphere of 95 % O_2 and 5 % CO_2 in air at 37 °C. After confluence achieved more than 95 %, the cells were detached with 0.25 % trypsin-EDTA (Sigma), centrifuged at 1200 rpm, 4 °C for 5 min and counted with

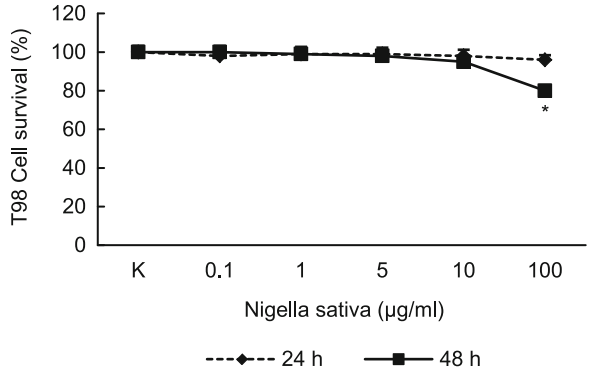

Fig. 1. Treatment of T98 cells with Nigella sativa oil for 24 and 48 hr.

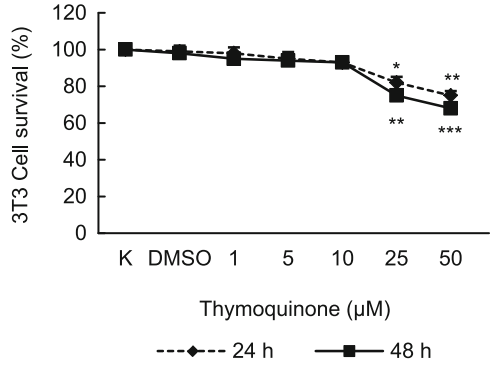

Fig. 2. Effects of Thymoquinone and DMSO (vehicle) on 3T3 cell survival during 24 or 48 h treatments. * p < 0.05, ** p < 0.01, *** p < 0.001.

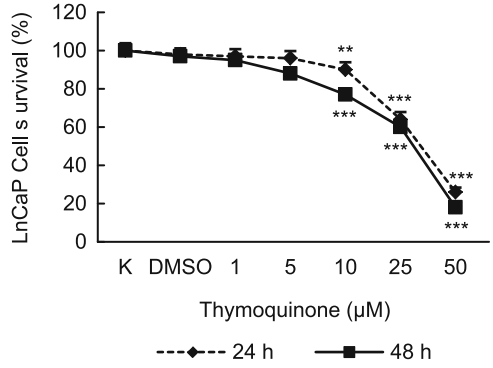

Fig. 3. The influence of Thymoquinone on LnCaP cells survival after 24 or 48 h.

a cell counter (CEDEX, Roche). The cells were then transferred to microplates for cell survival studies and to flasks for apoptosis and caspase measurements.

Test groups

Test groups were assigned as follows: control (complete medium only); dimethly sulfoxide (DMSO) (complete medium with a final concentration of 0.1 % DMSO, solvent); thymoquinone (treated with 1, 5, 10, 25 and 50 µM for 24 or 48 hr) and *N. sativa* oil (0.1, 1, 5, 10 and100 µg/mL for 24 and 48 hr).

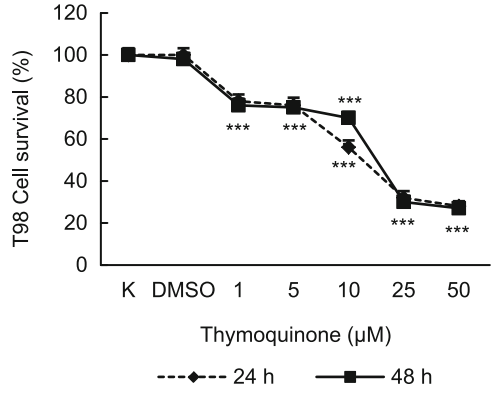

Fig. 4. The activity of 1, 5, 10, 25 and 50 µM thymoquinone on cell survival of T-98 cells by using MTT after 24 or 48 h.

Cell survival

The effects of thymoquinone and *Nigella sativa* oil on cell survival were determined by 3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazolium bromide (MTT, Sigma) colorimetric assay (11). MTT test was applied and cell survival was determined by measuring the formazan absorbance at 550 nm with a microplate reader (BioTek; Winooski,VT). Since the number of cells in each well is proportionate to the absorbance of the soluble formazan, the optical density read from the drug-treated wells was converted to the percentage of live cells versus control using the following formula:

Absorbance of treated cells in each well x 100 / the mean absorbance of control cells.

Flow cytometric analysis

To determine cell death type (necrosis or apoptosis) of cells, we used flow cytometric analysis with the 25 and 50 µM thymoquinone doses that diminish cell survival by more than fifty percent. Apoptotic cells were determined by staining with fluorescein isothiocyanate (FITC)-labeled Annexin V (Invitrogen) and subsequent flow cytometric analysis. Alive cells are negative for both PI and Annexin V, early apoptotic cells are PI negative but Annexin V positive while dead/late apoptotic cells are positive for both PI and Annexin V.

RNA extraction and determination of caspase-9 mRNA levels

The expression of caspase-9 mRNA was examined in control and thymoquinone treated cells. The mRNA levels of caspase-9 in relation to the housekeeping gene was determined by qT-PCR with TaqMan probes. RT-+PCR data were collected using the Roche lightcycler nano system.

Statistical analysis

All results are the mean of at least three independent assays and the p value less than 0.05 was considered to be significant for MTT results. Data were expressed as the mean percent fraction of control ± standart error of mean. Statistical significance ascertained by one way analysis of variance followed by Tukey's multiple comparison test. The apoptotic results were depicted as

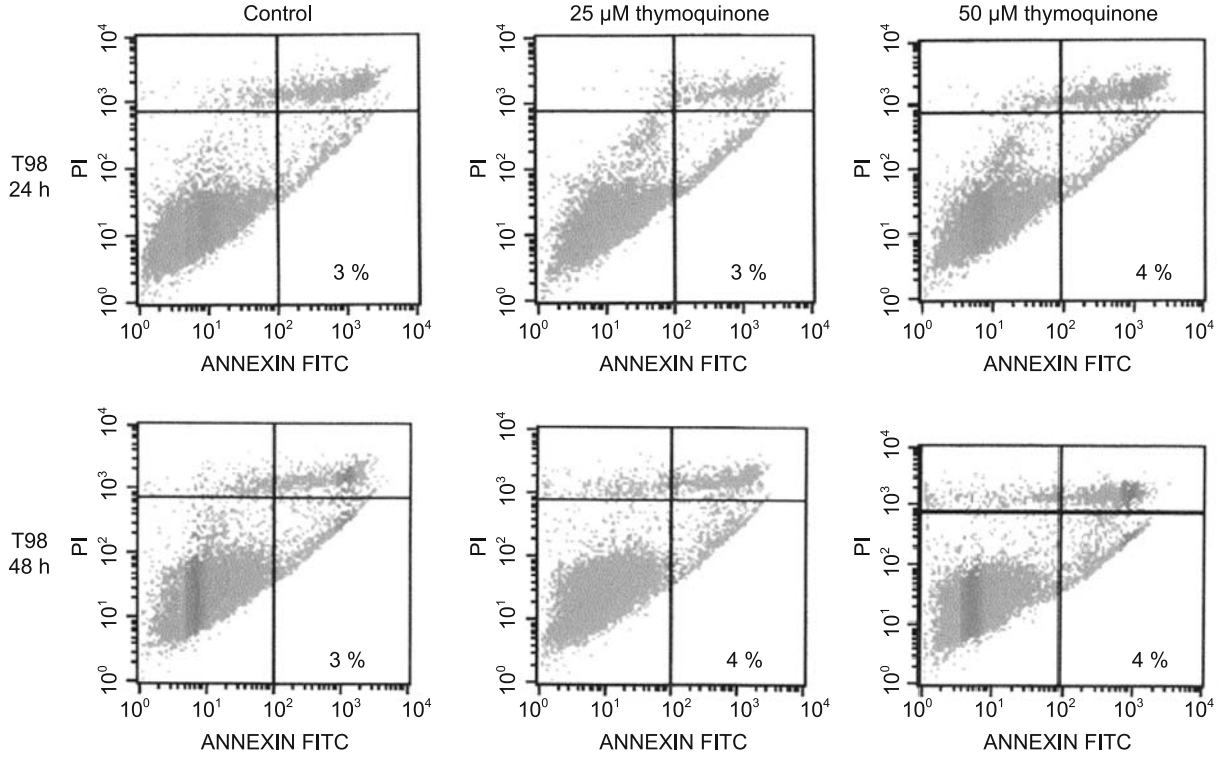

Fig. 5. T-98G cells, treated or non-treated with 25 or 50 µM Thymoquinone for 24 (A) or 48 (B) h, then stained with FITC Annexin V apoptosis assay kit with PI (Invitrogen). Lower left sections of all the Figure, AnnexinV/PI (−), living cells; lower right sections of all the Figure, AnnexinV (+)/PI (−), early apoptotic cells; upper left sections of all the figüre, Annexin (−)/PI (+), necrotic cells; upper right sections of all the Figure, Annexin (+)/PI (+), late stages of apoptosis and secondary necrosis. Results of only one independent experiment out of 3 is pointed.

percentage of cells. RT-PCR results were calculated by GraphPad software program. Transcript data were expressed relative to the control standart deviation.

Results

Cell viability assays

Treatment of the cells with DMSO did not cause any significant change in cell viability in pilot studies. Viability of T98, LnCaP and 3T3 cells was not changed after treatment with *Nigella sativa* oil for 24 hr. Culturing of cells with 100 µg/mL of *Nigella sativa oil* for 48 hr reduced the number of only T98 cells by 20 % (Fig. 1). However, treatment of cells with 1, 5, 10, 25 and 50 µM thymoquinone reduced the number of cells by 98, 95, 93, 82 ($p < 0.5$) and 75 % ($p < 0.01$) for 3T3 cells (Fig. 2); 97, 96, 90, 64 ($p < 0.01$) and 26 ($p < 0.001$) for LnCaP cells (Fig. 3) and 78, 76, 56, 32 and 28 ($p < 0.001$) for T98 cells (Fig. 4) for 24 hr, respectively. For 48 hr these rates were 95, 94, 93, 75 ($p < 0.01$) and 68 % ($p < 0.001$) for 3T3 cells (Fig. 2); 95, 88, 77, 60 and 18 ($p < 0.001$) % for LnCaP cells (Fig. 3) and 76, 75, 70, 30 and 27 ($p < 0.001$) % for T98 cells (Fig. 4) for 48 hr.

Flow cytometric analysis and gene expression

To further investigate the underlying mechanism of reduction of cell survival detected with MTT assay, we examined the apoptotic effect of the thymoquinone on cancer cells using flow cytometric analysis with 25 and 50 µM thymoquinone that decreases cell survival by more than 50 %. As seen in Figs 5 and 6, time and concentration dependent apoptosis of LnCap cells, not T98 cells, was detected. The analysis after 24 hr treatment with 25 and 50 µM thymoquinone demonstrated that 2 % and 23 % of the LnCaP cells underwent early apoptosis, whereas 3 and 76 % of the cancer cells underwent early apoptosis after 48 hr treatment. Caspase-9 mRNA level was increased in LnCaP cellline treated with 25 and 50 µMthymoquinone ($p>0.05$) whereas notchanged in T98 cell line.

Discussion

There are increasing number of scientific studies on the relationship between thymoquinone and cancer. *In vitro* and *in vivo* pharmacological studies have been reported for their potential antitumor, anticancer (12), antidiabetic (13), cardiovascular activity (8), gastroprotective, anti-inflammatory, hepatoprotective activity (9) and pulmonary activity (14).

Several other studies demonstrated that thymoquinone, one of the most active components in *Nigella sativa* seed, inhibits the proliferation of various cancer cell lines. Thymoquinone diminished cell survival and induced apoptosis of canine osteocarcinoma cells, human breast adenocarcinoma (MCF7) and human ovarian

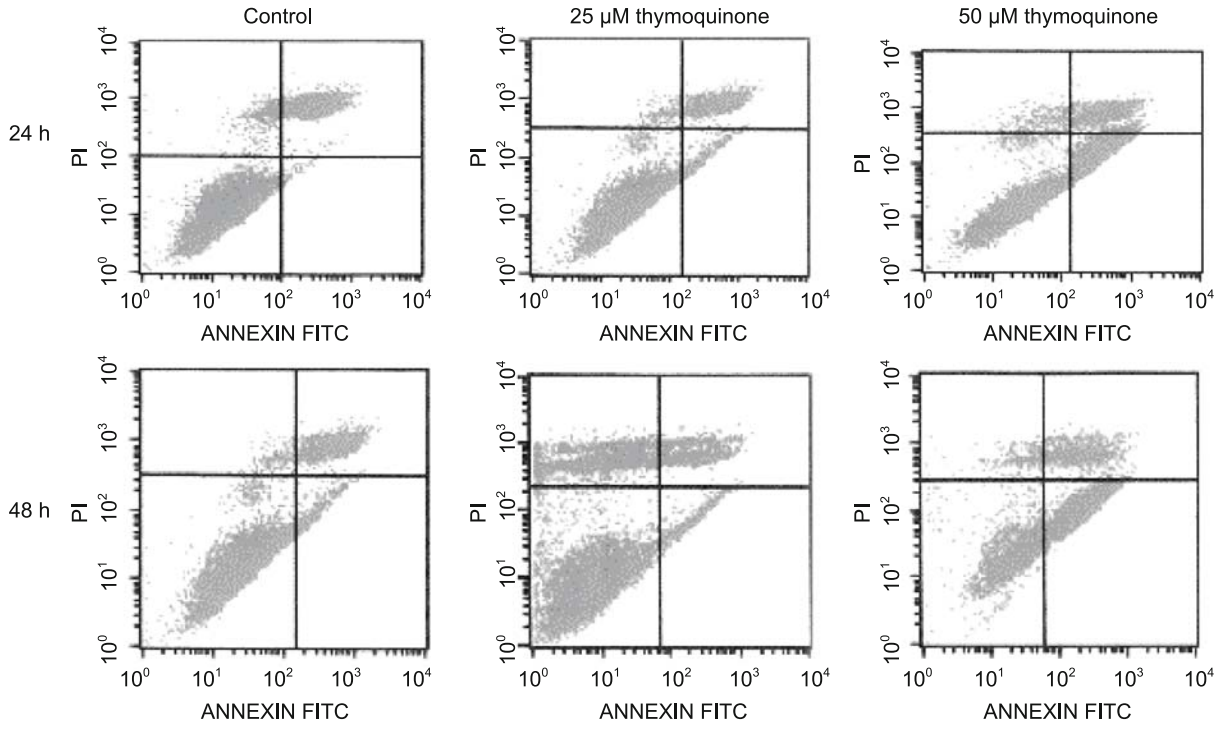

Fig. 6. The activity of 25 and 50 µM Thymoquinone on apoptosis of LnCaP cells by using Annexin V-FITC/PI after 24 or 48 h.

adenocarcinoma (BG-1) (15). Similarly, thymoquinone has been shown to inhibit cell proliferation in cultured cells derived from myeloblastic leukemia cells, fibrosarcoma cells, laryngeal neoplastic cells, pancreatic cells and human colon cancer cells (16, 17, 18, 19, 20). El-Mahdy et al. showed that thymoquinone induced apoptosis is p53-independent and occurs through the activation of caspase 3, 8 and 9 (21). In contrast, normal cells and primitive mouse keratinocytes are resistant to the apoptotic and antiproliferative effects of thymoquinone and their lack of significant changes in morphology and proliferation confirms the selectivity of this compound for cancer cells (15, 22).

According to our results, *N. sativa* oil did not affect the survival of cancer cells and 3T3 cells, however thymoquinone diminishes survival rate and induces apoptosis of only LnCaP cells in vitro and it possibly this effect through caspase-9. However, thymoquinone did not show the same effects on T98 and 3T3 cells. Our study shows thymoquinone has strong dose, time and cancer type dependent effects through decreasing cell survival and inducing apoptosis in prostate cancer cells. Although much work is needed, our data provides evidence of potential implications for the application of thymoquinone as a novel possible antiproliferative drug against prostate cancer.

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Received December 29, 2017.
Accepted February 5, 2018.