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Original Article

In Vitro Challenge using Thymoquinone on Hepatocellular Carcinoma (HepG2) Cell Line

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Abstract

Black seed (*Nigella sativa*) is considered as a biological response modifier. Thymoquinone (TQ) is the bioactive and the most abundant constituent of the volatile oil of this seed which has been shown to possess anti-inflammatory, antioxidant and anti-neoplastic effects. In this study, the effect of TQ on HepG2 cell line was investigated in an attempt to identify its potential mechanism of action. Cell viability and proliferation were assessed in presence of different concentrations of TQ, which revealed a remarkable inhibition of HepG2 cells by TQ in a dose dependant manner. TQ ability to induced apoptosis was determined by Flowcytometry and colorimetric measurement of Caspases 3 and 9. The apoptotic effect of TQ was much more dramatic after 12 h treatment and the activity of Caspases 3 and 9 was increased. Also, Flowcytometric analysis of cell cycle revealed an early G_1/S arrest of cells, which is characteristic of apoptosis. It could be concluded that Thymoquinone is a promising anti-cancer agent for hepatocellular carcinoma.

Keywords: Black seed; Thymoquinone; Apoptosis; Cell cycle; Flowcytometry.

Introduction

Cancer has become an important issue in medicine as it is a major cause of death in both the developed and developing countries and it is now well thought-out as second to myocardial infarction (1). A great majority of human cancers (about 80-90%) are attributable to environmental factors (2). However, it is not an easy task to eliminate carcinogenic factors from the environment. While modern surgery has significantly reduced the cancer mortality, the use of additional treatments such as radiotherapy and chemotherapy has resulted in no more than 5% reduction in the number of deaths (2). Therefore, there is an ongoing search for better controlling and preventive methods in order to reduce cancer mortality and related side effects. Many investigations are now being carried out to discover naturally occurring compounds that can suppress or prevent the process of carcinogensis (3, 4).

Cancer treatment is a paradox: on one hand delivering powerful toxicity to a tumor, but on the other hand spreading toxicity to the rest of the body. The side effects of such treatments may cause death even before the cancer does. Biological response modifiers (BRMs)

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are compounds that have a unique effect on physiological functions and can diminish the side effects of cancer treatments while increasing their efficiency (5).

Herbal therapies are commonly used for prevention and treatment of cancer despite the little understanding of their molecular and cellular basis of action. Black seed (*Nigella sativa*) grows in India and countries bordering the Mediterranean sea, and has been used in Middle East and Africa to promote health and fight diseases (6). Black seed is considered as a BRM because studies have shown that the extracts from the seeds are toxic to cancer cells, and in mice, prevents blood cell toxicity caused by anti-cancer drug cisplatin (7).

Thymoquinone (TQ), the bioactive and the most abundant constituent of the volatile oil of black seed, has been shown to possess an anti-inflammatory and antioxidant effects (7-9). Also, TQ exerts an anti-neoplastic effect and is thought to be a promising dietary chemopreventive agent. Despite the promising antineoplastic activities of TQ, the molecular mechanism of its pharmacologic effects is poorly understood (10, 11).

Cell death can follow distinct pathways: apoptosis or necrosis. Necrosis appears to be the result of acute cellular dysfunction in response to severe stress condition or exposure to toxic agents (12). Apoptosis is a physiological form of cell death that occurs during the development of multicellular organisms or the process of the immune response. In addition, the apoptotic process can be activated in response to stress conditions, toxic chemicals, physical agents, etc (13). Several protease families are implicated in apoptosis, the most prominent being caspases, which are cystein containing aspartic acid specific proteases having similar site specific proteolytic activity (14, 15).

This study was designed to investigate the possible beneficial effects of thymoquinone on hepatocellular carcinoma cell line (HepG2) in an attempt to identify its potential mechanism of action. This study was started on may 2006 as a cooperation between National Cancer Institute and National Research Center, Cairo, Egypt.

Experimental

Reagents and drugs

A purified preparation of TQ (>99 % pure) was purchased from Fisher Scientific GmbH (Germany). Propidium iodide (PI) was purchased from Molecular Probes (Eugene, OR, USA). Dimethyl sulphoxide (DMSO) was purchased from Merck (Darmstadt, Germany). RNase and Trypan blue were obtained from Sigma Chemical Co. Tumor cell line, HepG2 cell line, was obtained frozen in liquid nitrogen (-180 °C) from American Type Collection. The tumor cell line was maintained in National Cancer Institute, Cairo, Egypt, by serial sub-culturing in 75 cm^2 cell culture flasks (Fisher Scientific, Pittsburgh, PA, USA) using 10 ml of RPMI-1640 (supplemented with 1% (2 mM) glutamic acid, 10% unheated fetal bovine serum (FBS), 100 µ/ml penicillin and 100 µg/ml streptomycin) obtained from Gibco-BRL (Gaithersburg, MD, USA). All cells were grown at 37°C under an atmosphere of 5% CO_2.

Cytotoxic test

The SRB colorimetric assay was optimized based on the Papazisis et al method (16). Cells in exponential growth phase were washed, trypsinized and resuspended in RPMI medium. For experiments, cells were seeded on 6 well plates at a density of 105 cells per well and left to grow. Cells were treated with defined concentrations of TQ. Cell proliferation and cytotoxicity were determined using the cell Titer 96TM non-radioactive cell proliferation assay. Cells were placed on microtitre plate wells and incubated for 24 h under a humidified atmosphere, during which a partial monolayer was formed. The cells were then exposed to different concentrations of TQ in methanol (400, 200, 100, 50 and 25 µM/ml) prepared by serial two-fold dilutions of the test drugs in triplicate using a constant volume of 20 µl and maintaining the total well volume of 200 µl. The methanol concentration in treated and control wells did not exceed 0.1%. The cells were incubated at the same pervious concentrations for a period of 48 h. The changes of the cell cultures were examined using an inverted microscope (Olympus IX2 model), and the cellular viability

was determined by light microscope using trypan blue dye exclusion technique at 12 and 24 h time intervals (17, 18). 50 µl of cold (50% TCA) was added to the 200 µl culture medium in each well to produce a final TCA concentration of 10%. Microplates were left for 30 min at 4 °C and subsequently washed 5 times with deionized water. Microplates were then left to dry at room temperature for at least 24 h. Then, of a 100 µl 0.4% (w/v) solution of sulforhodamine B (SRB-Sigma) in 1% acetic acid was added to each well and left at room temperature for 20 min. SRB was removed and the plate washed with 1% acetic acid for 5 times, before air drying. Bound SRB was solubilized with 200 µl of 10 mM unbuffered Tris-base solution (Sigma) and the plate was left on a plate shaker for at least 10 min. The absorbance was read in a 96 well plate reader at 492 nm subtracting the background measurement at 620 nm.

Apoptosis assay

Apoptosis was detected by MoFlow Flowcytometer, Dako Cytomation using phosphatidyl serine detection TM Kit. Cells growing on 6 cm culture dishes were collected by trypsinization after treatment with TQ for 6 and 12 h and, washed with PBS. The calcium buffer was diluted 20 times in deionized water and stored at 4 °C.The cells were washed and the concentration was readjusted to 1.5×10^6 cells/ml in calcium buffer. 10 µl of Annexin V FITC was added to 100 µl of cell suspension and then incubated for 20 min in ice in the dark. The cells were washed with calcium buffer and 10 µl of propidium iodide was added. The cells were then kept at 4°C until ready to be analysed by flowcytometry. Viable cells were not stained; apoptotic cells excluded PI and expressed phosphatidyl stain by green colour while necrotic cells were permeable to PI which associates with nuclear DNA, and were visible by red fluorescence (19).

The caspase 3 and 9 colorimetric protease assay1

ApoTarget TM kit was used for the in vitro determination of proteolytic activity of the enzymes in lysates of mammalian cells (BioSource International, Inc., USA).

Apoptosis was induced in cells by TQ at different concentrations. Cells were counted and pellets of 3-5 million cells per sample were prepared. The cells were resuspended in 50 µl of chilled cell lysis buffer and then cells are incubated on ice for 10 min. The samples were centrifuged and the protein concentrations were determined by Bradford method (Figure 1) in cytosol extract. Each cytosol was diluted to a concentration of 50 to 200 µg protein per 50 µl. The cell lysis buffer, i.e. reaction buffer (containing DDT), was added to each sample. 5 µl of the 4 mM DEVD-pNA substrate (200 µM final concentration) was added and the samples were incubated at 37°C for 2 h in the dark. Then, the absorbance at 405 nm was recorded (20).

Cell cycle analysis by flowcytometery

The cells were seeded in 6-well microplates at a density of 5×10^5 cells per well. They were incubated and allowed to grow to 40-50% confluence after which they were treated with TQ and incubated for a further period of 6-12 h. The cells were then harvested by trypsin release, washed twice with PBS (pH 7.4), and permeabilized with 70% ethanol. They were washed twice with ice-cold PBS, treated with 1% RNase and incubated for 10 min at room temperature followed by addition of propidium iodide (100 µg/ml final concentration). Distribution of cells in G_1, S and G_2 phases with different DNA contents was then determined using a MoFlow Flowcytometer, Dako Cytomation (21).

Results

Growth and proliferation inhibition

Hepatocellular carcinoma cells (HepG2) were treated with graded concentrations (25-400 µM) of TQ for 12-24 h. The viability and cell proliferation tests were performed. The data indicated that treatment of cells with concentrations lower than 200 µM for 12-24 h resulted in significant inhibition of cell viability, as compared with control (Figure 1). TQ was found to remarkably inhibit the proliferation of HepG2 cells in a dose dependent manner with an IC_{50} (concentration of 50% inhibition) of 350 µM (Figure 2).

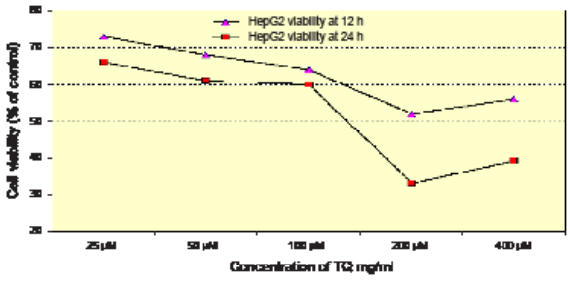

Figure 1. Dose response curve of the effect of TQ on HepG2 viability at 12 h and 24 h of exposure determined by Trypan Blue exclusion test.

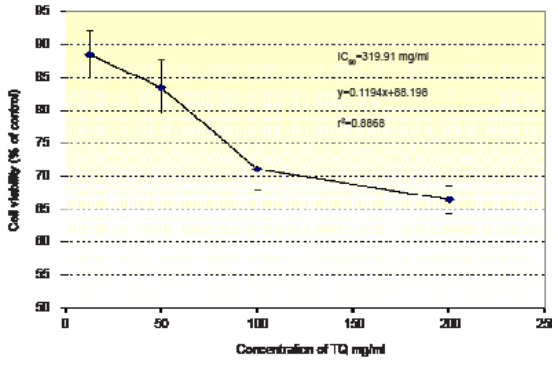

Figure 2. SRB cytotoxicity assay for determination of the mortality of HepG2 cell line under different concentrations of TQ.

TQ induced apoptosis

Treatment of HepG2 cells with TQ resulted in no necrotic cells after 6 and 12 h. However, the apoptotic effect by TQ was much more dramatic as more than 57% of the cells became apoptotic after 12 h of treatment, compared with control (Figure 3, 4).

The apoptotic effect of TQ was also examined by measuring the activities of caspase 3 and 9 enzymes. The enzyme activities were determined according to the different protein contents (50-250 µg/sample) of cells treated with graded dose of TQ (100, 200, 400 µM). The results showed an increase in the activity of both enzymes with an increase in the dose of TQ (Figure 5, 6).

Cell cycle analysis

To search for the mechanism of the anti-proliferative effects of TQ, it was important to determine whether the growth inhibitory effect of TQ is associated with any specific change in cell cycle progression. HepG2 cells were treated with TQ for 6 or 12 h and harvested for

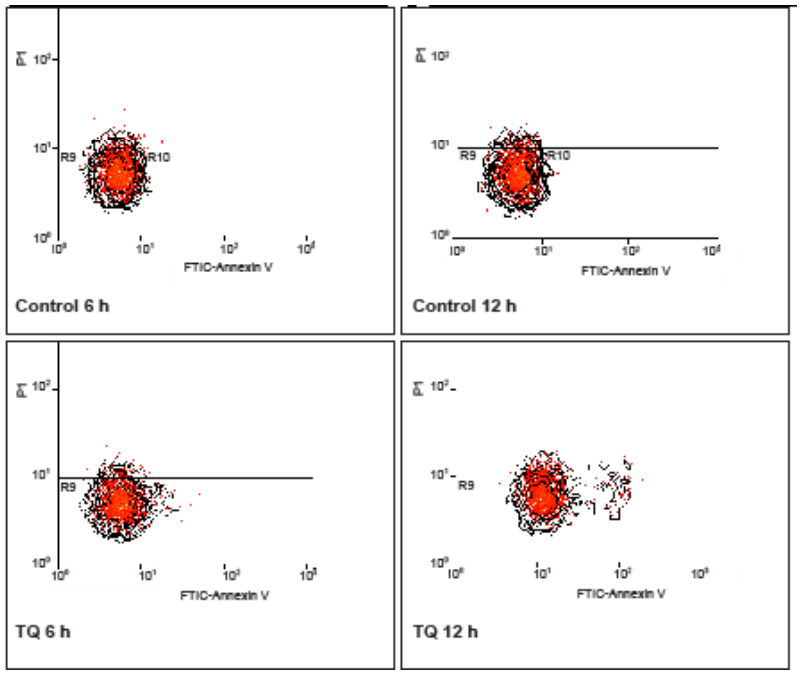

Figure 3. Flowcytometric detection of apoptosis in HepG2 cell line under the effect of TQ (at IC_{50}).

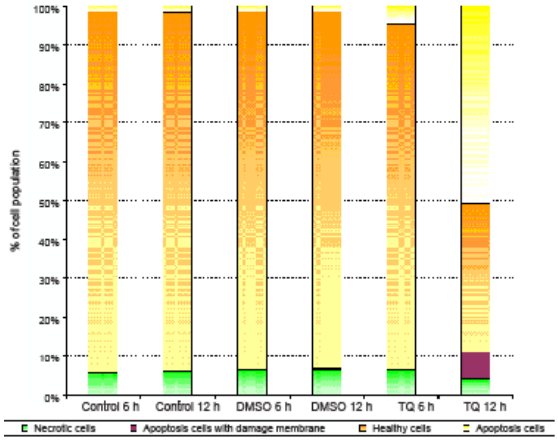

Figure 4. Apoptosis/Necrosis analysis of HepG2 cell line upon exposure to TQ (at IC_{50}).

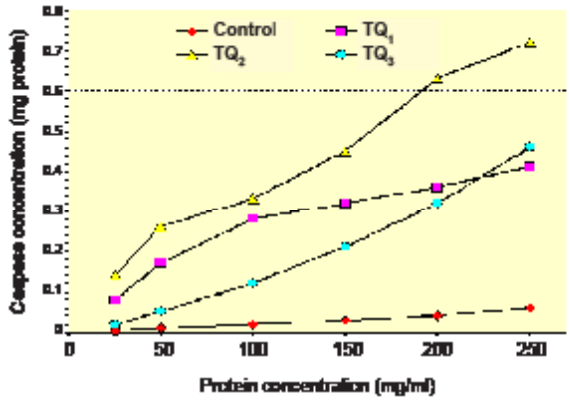

Figure 5. Caspase 3 concentration (Mg protein) in Hep G2 cell line upon exposure to different concentrations of TQ.

flowcytometeric analysis of cell cycle and DNA content determination by PI staining. Treatment with 350 µM of TQ caused a concentration-dependent decrease the number of HepG2 cells in S-phase, thus providing evidence of G_1 arrest (Figure 7, 8).

Discussion

Hepatocellular carcinoma (HCC) represents the fifth most common malignancy the main cause of mortality in patients with chronic liver diseases. HCC is a tumor characterized by high local invasiveness and high metastatic potential. There is no doubt that certain herbal products contain chemically defined components that can protect the liver from oxidative injury, promote virus elimination, block fibrogenesis, and inhibit tumor growth. (22). In the present study, we investigated the effect of thymoquinone on HepG2 cell line and attempted to identify its mechanism of action.

Although the thymoquinone has proved significant in vitro and in vivo antineoplastic activity against different cell lines, the mechanism and molecular pathways of TQ action have not been fully examined (5, 10). However, assumptions indicated that the anti-tumor effect of TQ may be mediated by one or more of the following mechanisms: Antioxidant activity, immunomodulatary action and cytotoxicity (23).

The results of this study revealed that TQ inhibited the viability and proliferation of tumor cells by a mechanism that involves cytotoxicity. In vivo studies previously revealed that TQ protects rat liver against induced hepatocarcinogenesis and showed that the protective effect of TQ is due to its antioxidant activity. Also, it has been revealed that TQ has the ability to kill several types of tumors without significant cytotoxicity to normal cells indicating that this compound may be a potentially effective chemotherapeutic agent (24).

Apoptosis is an active, energy dependent mechanism in which cells participate in their own destruction whereas necrosis is a passive process of cellular metabolic collapse followed by cellular disintegration (25). The predominant form of cell death is likely the apoptosis since evidence of apoptotic cell death was seen initially. However, at longer times of incubation and at a higher concentration, necrotic cell death was observed. Therefore, TQ causes both forms of death, i.e. apoptosis and necrosis; the incidence of these forms of death is dose and time dependant. Our results are consistent with the findings of other studies which prove that TQ produces both apoptotic/necrotic effects in different cancer cell lines (14, 21, 26).

Caspases are proenzymes that contain an active site of cystein nucleophile (27) which is prone to oxidation or thiol alkylation (5, 22). It is therefore not surprising that the

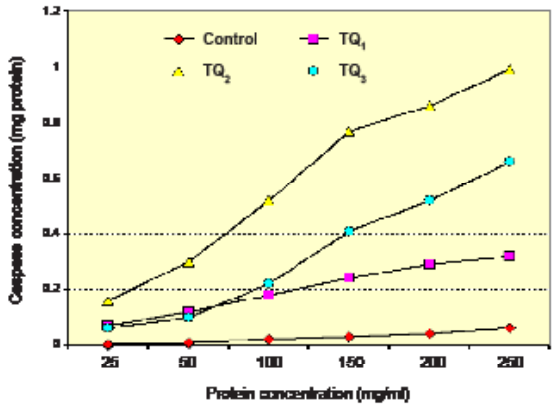

Figure 6. Caspase 9 concentration (mg protein) in HepG2 cell line upon exposure to different concentrations of TQ.

activity of caspases is optimal unders reducing environments. Any dying cell could be detrimental to caspase and render them inactive (28-30). Inhibition of caspase activity in cells that would otherwise undergo apoptosis has recently been shown to drive cells into necrosis (31, 32). The results of the present study have shown that TQ is an initiator of apoptosis through increasing the activity of caspase protease enzymes (Figure 3, 4). The results also suggest the ability of TQ to increase the activity of both Caspases 3 and 9 in a dose dependant manner.

Cell cycle checkpoints and apoptosis play key roles in developmental biology and represent a new set of potential targets for chemotherapeutic agents. Quinones represent a class of drugs that induce both effects (31, 33). Quinones induce free radical mediated DNA strand breaks, and also alkylate and cross link DNA (34, 35). In this study, flowcytometric analysis points to possibility of an early G_1/S arrest at 350 µM concentration of TQ (Figure 6). A reduction in number of S-phase cells and an increase in the G_1 peak are feature characteristics of the early stage of G_1/S phase arrest. Other mechanisms of toxicity include interference with cell cycle with upregulation of p21 protein by mutant p53, an inhibitor of cyclin dependent kinases and an important negative regulator involved in cell cycle control (36, 37).

Although the result of our study revealed an increase in cytotoxic effect of TQ in vitro, the previous in vivo studies reported that TQ was non toxic and also protective against quinine (doxorubicin) induced cardiotoxicity and nepherotoxicity without compromising its anti-tumor activity (38, 39).

In conclusion, TQ has shown a cytotoxic effect on HepG2 cells, a triggering effects on caspase activation and apoptotic cell death and cell cycle arresting activity. These results suggest that TQ can be a promising anti-cancer therapeutic agent for hepatocellular carcinoma while preventing sustaining chemotherapy induced damages on non-tumor tissues.

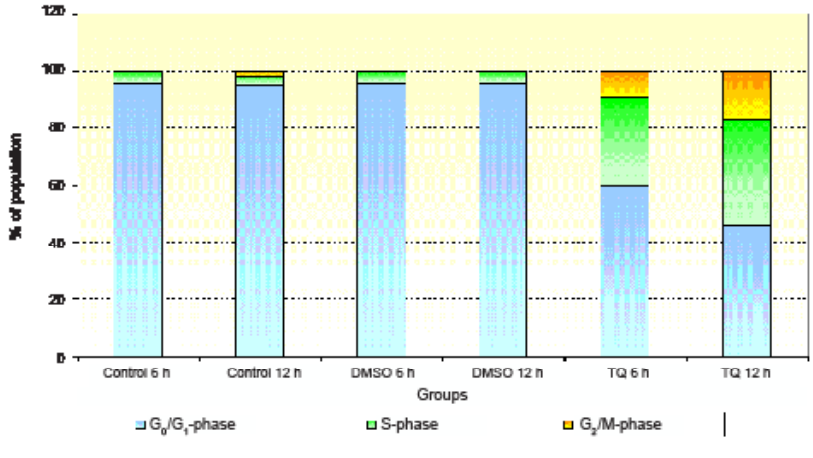

Figure 7. Cell cycle analysis of HepG2 cell line upon exposure to TQ (at IC_{50}).

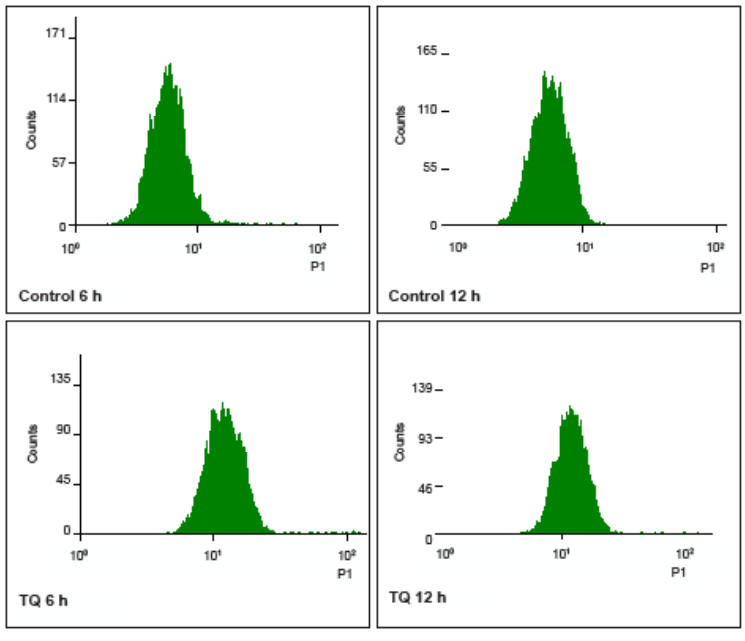

Figure 8. Cell cycle analysis of HepG2 cell line upon exposure to TQ(at IC_{50}) by flowcytometry.

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