Research Paper

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Nigella Sativa Extract Role on Mitochondria-Mediated Apoptosis Induction in Human Colorectal Cancer Cells *in Vitro*

Medical Science

KEYWORDS : thymoquinone, p53, Bcl-2, caspase-3.

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ABSTRACT

There has been a growing interest in naturally occurring compounds from traditional medicine with anti-cancer potential. Nigella sativa (black seed) is one of the most widely studied plants. This annual herb grows in countries bordering Mediterranean Sea and India. Thymoquinone (TQ) is an active ingredient isolated from Nigella sativa. The anticancer effect of TQ, via apoptosis induction resulting from mitochondrial dysfunction, was assessed in colorectal cancer (Caco-2) cells in vitro with an IC50 of 7µg/mL. Treatment of Caco-2 cells with TQ encouraged apoptosis and antiproliferative effect with cell death-transducing signals by a down-regulation of Bcl-2, up-regulation of p53 and caspases-3 expression were also observed in the treated cells. Additionally, a typical characteristics of apoptosis including DNA fragmentation which is caspase-3 dependent and cytological alterations was observed. Future study will may deal with further investigations of TQ possible usages as a new alternative or complementary chemotherapeutic agent for human cancer types specially colorectal cancer type.

INTRODUCTION

Nigella sativa, also known as black seed, is an annual herbaceous plant belonging to Ranunculacea family which grows in Mediterranean Sea countries and India[1]. It has been used as traditional herbal medicine for more than 2,000 years[2]. Apart from this complimentary medicinal use, it has been used in Asia, Middle East and Africa as a medicinal food to support health and fight diseases[3]. They are also used as a natural remedy for asthma, hypertension, diabetes, inflammation, bronchitis, headache, eczema, fever, dizziness and influenza[4]. The seeds are known to be carminative, emenagouge, galactogogue, and have been used in treating fever[5]. The biological activity of *N. sativa* seeds oil is attributed to their essential oil components as main compound is thymoquinone (30%–48%)[6]. Black seed oil has been widely commercialized and due to its amazing healing power, numerous research projects are being carried out from time to time to improve its quality. Thymoquinone is the most bioactive constituent of the volatile oil of this seed, which has been shown to possess anti-inflammatory, antioxidant and anti-carcinoma effects[7].

Over the past decade, there is continuous increase in colorectal carcinoma in the world as the most common malignant diseases. Colorectal cancer is the cause of more than 1/2 million deaths worldwide, and it was ranked as the third leading cause of cancer-related death after lung cancer and stomach cancer[8]. Epidemiological studies have shown strong evidence that diet and lifestyle play an important role in preventing cancer. In particular, an increased consumption of fruits and vegetables is associated with decreasing in cancer onset and mortality[9].

The major apoptotic pathways can be divided into caspase- and mitochondria-dependent pathways, according to caspase-3 activation which is generally considered to be a key hallmark of apoptosis[10]. Apoptosis is characterized by chromatin condensation and DNA fragmentation, and is mediated by the cysteine protease family called caspase-3[11].

Tumor suppressor protein p53 is a principal factor in regulation of growth arrest as well as apoptosis. It interacts with other proteins as a transcription factor[12]. Indeed, in response to various types of stress, p53 becomes activated as a consequence, cells can undergo marked phenotype changes, ranging from increased DNA repair to senescence and apoptosis[13].

Mitochondria are involved in a variety of key events, including release of caspase-3 activators, changes in electron transport, loss of mitochondrial membrane potential, and participation of both pro- and anti-apoptotic Bcl-2 protein[14]. Alterations in mitochondrial structure and function have been shown to play a crucial role in caspase-3-dependent apoptosis and Bcl-2 expression[15]. Bcl-2 is the founding member of family of genes that either prevents or promotes cellular apoptosis. Bcl-2 itself is an antiapoptotic gene that prevents initiation steps of apoptosis and programmed cell death[16]. Therefore, TQ being generally considered as safe, consumption of natural compounds is currently a major interest in health care. In the current study, we evaluated the potential of TQ on human colorectal cancer cells using Caco-2 cells as an *in vitro* model.

MATERIALS AND METHODS

Chemicals: TQ, MTT salt [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide], dimethylsulfoxide (DMSO), commercial methanol, ethanol and acetone, Tris-HCl, edetic acid, Triton-X100, RNase A, proteinase K, NaCl, 2-propanol, phosphate-buffered saline (PBS), ethidium bromide, agarose gel, Peroxidase, trypsin, Hematoxylin and eosin (Hx&E) stain, rabbit polyclonal antibodies against cleaved caspase-3, primary monoclonal antibody against Bcl-2 and against p53, AB reagent, biotinylated immunoglobulin secondary antibody and Tween 20 were purchased from Sigma-Aldrich, Egypt.

Cell line and cell culture: Caco-2 cell line, was obtained from American Type Culture Collection (ATCC, USA). They were subcultured as mono-layer according to the instructions provided by ATCC in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated (56°C, 30min) fetal bovine serum, 2mmol/L L-glutamine, 100U/mL Penicillin-Streptomycin and 100U/mL Amphotericin B at 37°C in a humidified atmosphere of 5% CO2. Cells were used when monolayer reached 80% confluence in all experiments. Cell propagation media was purchased from Invitrogen (Carlsbad, CA).

Methods:1.Cell Viability Assay: *In vitro* evaluation of antiproliferation effect: growth inhibition was evaluated by MTT assay. MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was reduced by mitochondrial dehydrogenases to water blue insoluble formazans[17]. Viable cell number/well is directly proportional to formazans production. 8.25×103 cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12h), replaced with fresh medium containing TQ at concentrations: 3µM/L, 5µM/L, 7µM/L and 9µM/L for 72h at 37°C in an incubator with 5%CO_2. After incubation, TQ modified medium was replaced by 100µL of MTT (0.5mg/mL) medium for incubation (3h at 37°C and

5%CO_2). MTT medium was then replaced with 100µL of DMSO and left for 10min on a platform shaker to solubilize converted formazan. The absorbance values were determined at 570nm test wavelength and 630nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as a positive control cells and all values were correlated with this set of data. The experiment was performed in triplicates. Inhibition Percentage=[1–(net Absorbance of treated well/net Absorbance of control well)]x100%, then was plotted against TQ concentrations.

2.Determination of DNA fragmentation by DNA laddering assay: cells were seeded in 60-mm petri dishes at density 4x105 cells/plate (treated cells by IC50 concentration of TQ or positive control cells). Adherent and floating cells were collected by centrifugation at 1000×g/5min. Cell pellet was suspended in cell lysis buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 10mmol/L pH8.0, Triton-X100 0.5%) and kept at 4ºC/10min then, lysate was centrifuged at 25.000×g/20min. Supernatant was incubated with RNase A 40µg/L/1h (37ºC), incubated with proteinase K 40µg/L/1h (37ºC), mixed with NaCl 0.5mol/L and 50% 2-propanol overnight (-20ºC), then centrifuged at 25.000×g/15min. After drying, DNA was dissolved in buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 1mmol/L pH 8.0) and separated by 2%agarose gel electrophoresis at 100V for 50min. DNA was visualized under ultraviolet light after staining with ethidium bromide[18].

3.Cytological changes investigation: detached and trypsinized cells (IC50 concentration of TQ treated cells and positive control cells) were collected and centrifuged at 2000 rpm for 5min. Cell pellet was re-suspended with 100µL of PBS (pH7.3). 10µL of the suspension were smeared on a glass slide, allowed to air-dry, fixed with cool methanol for 5min before proceeding by Hx&E stain and examined under light microscope[19].

4.Immunocytochemical investigations: by detection of Bcl-2, p53 and Caspase-3 by immunocytochemistry staining kits. The procedure was done according to the manufacturer's instructions, simplified as follows: 1-2 drops of Peroxidase was applied to cells (IC50 concentration of TQ treated cells and positive control cells) on the slide (10min), followed by blocking solution (10min). Cells were fixed in ethanol:acetone (9:1) for 30min at -20°C and then rinsed again with cold PBS at room temperature. Cells were incubated overnight with rabbit polyclonal antibodies against cleaved caspase-3 at 4°C, then AB reagent and substrate-chromogen mixture (30min). Between each step, the slide was washed with washing buffer (PBS) with 0.1% Tween 20). Cells were incubated overnight with primary monoclonal antibody against Bcl-2 and against p53 at dilution of 1:75 at 4°C, then in Tris buffer and biotinylated immunoglobulin secondary antibody was used[20]. The slides were then mounted and examined under light microscope.

5.Statistical analysis: results were presented as mean±standard deviations (SD). Analysis of variance (ANOVA) for two variables (Two Way-ANOVA) was used together with student t-test. Significant analysis of variance results were subjected to post hoc. Statistical significance was set at $P<0.05$ and high significance was set at $P\leq 0.01$[21].

RESULTS

1.Cell viability assay: *In vitro* evaluation of antiproliferation effect.

Cytotoxic effect of TQ concentrations (3µM, 5µM, 7µM and 9µM)/72h on Caco-2 cells was determined by MTT assay (Figure 1). Cells number started to reduce immediately after treatment with TQ concentrations in a dose dependent manner. All concentrations were found to be high significantly different ($P\leq 0.01$) in respect to their antiproliferative and apoptotic effects when compared with positive control cells. Cell inhibition percentage was gradually increased with TQ concentration increasing and 95% of cell inhibition was observed in treated cells with 9µM/72h. Cell proliferation reduced about 25% and 35% when cells were treated with 3µM and 5µM for 72h, respectively. Cells proliferation decreased to 55% as treated with concentration 7µM/72h.

2.Determination of DNA fragmentation by DNA laddering assay.

DNA degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis. Nuclear DNA isolated from Coca-2 cancer cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 72h when treated with TQ concentration at 7µM whereas untreated cells did not show typical ladder (Figure 2). Results indicated that TQ induced DNA fragmentation which was caused by apoptosis.

3.Cytological changes investigation.

Positive control cells group had round nuclei, distinct small nucleoli and homogeneous chromatin with accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by TQ concentration: 7µM/72h, apoptotic cells were identified by a series morphological changes as experimental proof of underlying processes alterations appeared as: bleb plasma membrane, cellular shrinkage, chromatin condensation granules, vacuolated cytoplasm, degrading nucleus and apoptotic bodies formation were observed (Figure 3b, 3c and 3d).

4.Immunocytochemical investigation.

After Coca-2 cells treatment by TQ concentration at 7µM/72h, the reaction of caspase-3 protein was considered positive (over expression of caspase-3 protein) when over 50% of treated tumor cells had a clear brown cytoplasm staining, with slight degrading in the intensity in the same field (Figures 3f). Specially those fields that had necrotic or apoptotic nucleus as sign for TQ treatment effect, but fields of positive control cells have negative reaction of caspase-3 (cytoplasm did not show the brownish reaction stain) (Figure 3e). On the other hand regarding to the positive control Coca-2 cells, Bcl-2 protein reaction was considered positive (over expression of Bcl-2 protein) when over 55% of cells had nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane brown staining, with slight intensity degrading in the same field (Figures 3g). After Coca-2 cells treatment by TQ concentration at 7µM/72h, those fields that had necrotic or apoptotic nucleus as sign for TQ apoptotic effect with Bcl-2 negative reaction (faint to non-brown stain) (Figure 3h). Also, when applying p53 stain, p53 protein reaction in the positive control Coca-2 cells, was showed negative reaction (no brown stain) (Figure 3j). Treated Coca-2 cells, those fields had necrotic or apoptotic nucleus for TQ effect showed p53 positive reaction (over expression of p53 protein) when over 55% of cells had nuclear brown staining, with slight intensity degrading in the same field (Figures 3i).

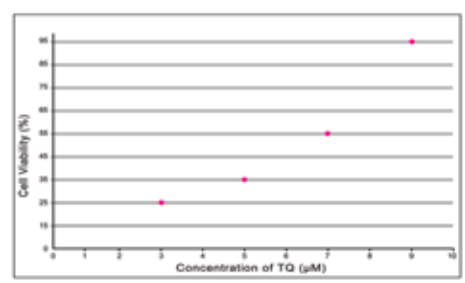

Figure 1: Effect of TQ with different concentrations on the cells viability of Caco-2 cells. The experiment was performed in triplicates and values means were calculated [mean±SD, n (for each concentration)=4].

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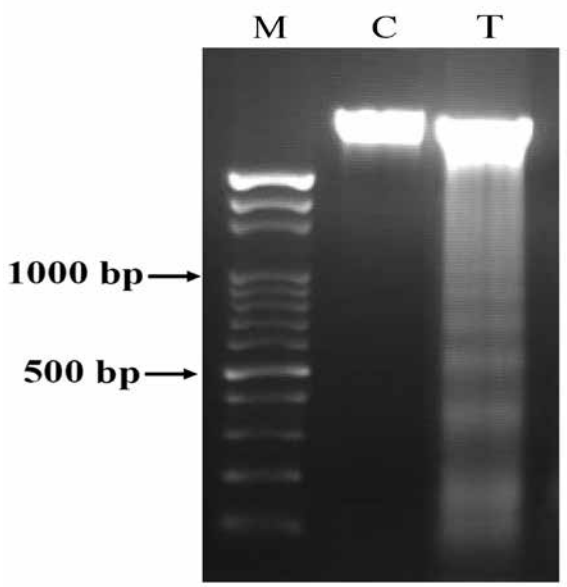

Figure 2: DNA fragmentation by DNA laddering assay of extracted DNA from TQ treated cells and positive control cells. DNA laddering, typical for apoptotic cells, which were visible in treated Coca-2 cells (T), and there was no any apoptotic features in the positive untreated cells (C) where M indicating to marker.

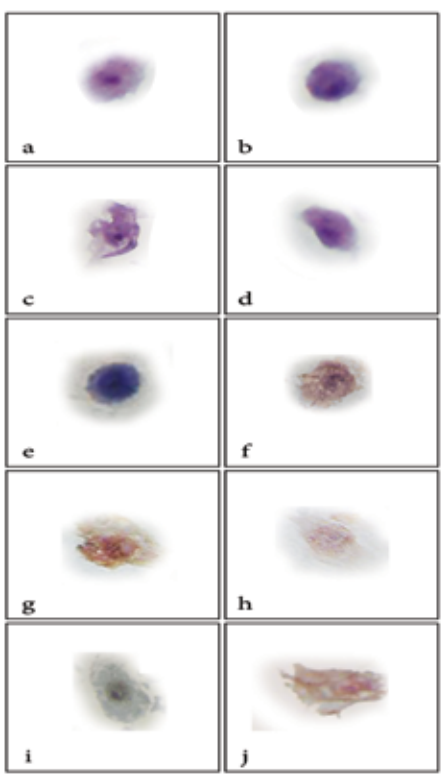

Figure 3: Cells in different stages of apoptosis in treated cells are easily distinguishable. Cell with normal morphology (a). Complete apoptotic cell (b). Nuclear condensation is evident in cells (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (c). Degradation of nucleus, vacuolated cytoplasm with apoptotic bodies (d). Immunocytochemistry of caspase-3 protein. Control positive cell showing cytoplasm negative reaction for caspase-3 protein (e). Treated cell showing cytoplasm positive reaction for caspase-3 protein (f). Control positive cell showing Bcl-2 protein nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane showing brownish positive reaction (g). Treated cell showing negative reaction of apoptotic cell apoptosis (h). Treated cell showing p53 protein nuclear positive reaction (i). Control positive cell showing nuclear negative reaction (j).

DISSCUTION

TQ and its effects on different cancer cell lines have been widely studied; these effects include inhibition of cancer cell viability. In almost all tested pancreatic cancer cell lines, the inhibition was up to 70%[22]. Anti-proliferative and pro-apoptotic activities of TQ in both NSCLC and SCLC cell line[23]was investigated. Breast cancer cell lines and liver cell lines have also been studied, including in some animal models[24]. One of the main problems of the present chemotherapy in treating tumour patients is the toxicity of the drugs used. Most of the existing anticancer drugs, unfortunately, also attack normal proliferating cells[25]. The aim of this study is to verify if its action of TQ on other human colon cancer cell lines such as Caco-2 cells. Our results showed that TQ strongly inhibited proliferation of Caco-2 cells and blocked the cell cycle as previously described[26].

Programmed cell death or apoptosis plays an important role in normal development and is impaired in many types of cancer. Apoptosis occurs under a variety of physiological and pathological conditions that control the development and homeostasis of multicellular organisms[27]. P53 has been shown to be involved in the induction of apoptosis, cell-cycle arrest and differentiation responses that prevent further proliferation of stressed or damaged cells and so protect from the outgrowth of cells harboring malignant alterations. P53 role in the repair of DNA damage has also been described and the ability of p53 to induce reversible cell-cycle arrest may contribute to the ability of cells to repair and recover from damage before reentering a normal proliferative state[28]. It is of great importance to understand the mechanisms of apoptosis in cancer cells, as apoptosis is believed to be one of the major consequences of anticancer drug treatment against malignancies[29].

Caco-2 cells which were treated with TQ exhibited increased levels of p53 expression at concentration of 7μM/72h, which suggested that p53 involved in TQ-induced Caco-2 cell death. Bcl-2 family of proteins serves as critical regulators of pathways involved in apoptosis. The main protagonists are suggested to be anti-apoptotic and known as Bcl-2[30]. Caco-2 cells which was treated with TQ exhibited reduced levels of Bcl-2 expression. These results suggested that the mitochondrial pathway was involved in TQ-induced Caco-2 cell death.

Apoptosis, as programmed cell death, is a highly organized cell death process characterized by an early obvious condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of nucleases, enzymatic cleavage of DNA into oligonucleosomal fragments and segmentation of the cells into membrane-bound apoptotic bodies. DNA fragmentation, a hallmark of apoptosis, is regulated by a specific nuclease called caspase-activated DNase and its inhibitor[31]. Apoptosis has specific signals instructing the cells with specific morphological change as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation and DNA fragmentation that are considered as landmarks of the apoptotic process[32]. That was agreed with the results of recent study after treatment by TQ. TQ decreased the viable percentage of cell number (dose dependent effect) and induced apoptosis of Caco-2 cells. Therefore, we may presume that as primary mechanism involved in TQ growth-inhibitory effects as it considered main apoptotic signals. In response to apoptogenic stimuli, the mitochondrial protein apoptosis-inducing factor (AIF) translocates through the outer mitochondrial membrane to the cytosol and to the nucleus, resulting in the induction of nuclear chromatin condensation and

large DNA fragmentation in a caspase-independent manner. It is well known that a family of cysteinyl proteases, caspases, is involved in the apoptotic cell death. Caspase-3, one of the active executioners, promotes apoptosis by cleaving cellular substrates such as ICAD[33]. That was agreed in the recent study by caspase-3 expression inhibition. We found that TQ induced caspase-3 activity in Caco-2 cancer cell line.

CONCLUSION

In summary, we demonstrated that TQ caused an inhibition of cell growth with apoptosis induction by DNA fragmentation and p53 activation in human colon (Caco-2) cancer cell line. Moreover, a large part of our study essentially focused on the mitochondrial pathway and we investigated that TQ's action was caspase-3 dependent according to its inhibitory effect on Bcl-2 expression. There was also noticeable cytological alterations. These new findings suggest that TQ-induced effects may have novel therapeutic applications for the treatment of different cancer type as previously described for breast cancer. Future *in vitro* and *in vivo* study will may deal with further investigations of the possible usages of TQ as a new alternative chemotherapeutic agent.

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