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Novel combination of thymoquinone and resveratrol enhances anticancer effect on hepatocellular carcinoma cell line

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ABSTRACT

Hepatocellular carcinoma remains one of the most dominant malignancies worldwide. Neutraceuticals have become under focus in anticancer treatment. Resveratrol is one of the major components of *Polygonum Cuspidatum* and is known as chemo-preventive agent. Thymoquinone is one of the most potent constituents in *Nigella Sativa* and has many medicinal effects. The aim of the present study is to investigate the combined effect of thymoquinone and resveratrol on treatment of hepatocellular carcinoma cells (HepG2). We evaluated the effect of thymoquinone and resveratrol separately and in combination on HepG2. Cell viability, caspase-3 activity, glutathione and malondialdehyde content were determined. The IC50 values of thymoquinone and resveratrol were (46 μM and 64.5 μM) respectively, where each showed potent anti-tumor activity on HepG2. The cell viability was 47.2% and 49.9% respectively. Comparing to the control group, treatment with thymoquinone and resveratrol increased caspase-3 enzyme by 77% and 98.5% respectively, while content of glutathione decreased by 22.8% and 35.6% while malondialdehyde content decreased by 18% and 29.6% correspondingly. The combination (thymoquinone + resveratrol) affected the cell viability leading to further decrease by 9.9% and 12.6%. The content of caspase-3 increased by 89% and 67.5% while the glutathione content had further decrease by 25.6% and 12.8%. Malondialdehyde content decreased by 32.3% and 20.7% all are comparing to thymoquinone and resveratrol separate treatment.

Thymoquinone and resveratrol combination showed significant cell inhibition and increase in caspase-3 indicating cell apoptosis. Both drugs raised reactive oxygen species leading to decrease of glutathione and minor effect on lipid peroxidation, all these results give a new promising combination with enhanced anticancer effect.

1. Introduction

Hepatocellular carcinoma (HCC) the primary malignancy of the liver is considered as the second most common cause of cancer-related death [1]. Many therapeutic agents have been involved in HCC treatment such as sorafenib, brivanib, oxaliplatin and many other chemotherapeutic agents [2]. Chemotherapy remains unsatisfactory. As many side effects decrease the patient compliance and quality of life. To improve the outcome of the therapy and increase the patient's quality of life, many natural compounds are under the spotlight. (see Table 1)

In addition to chemotherapeutic drugs, natural remedies have been used to support the treatment of cancer. *Nigella Sativa*, that is also called Habbah Al-Baraka in Egypt dates back to the Egyptian Pharaoh Tutankhamun and is commonly used in the Middle East countries as a folklore medicine for the treatment of various diseases [3]. Thymoquinone (TQ) which is one of the main constituent in *Nigella Sativa* has been shown to exert anticancer effect [4]. The molecular pathways of TQ as anti-cancer agent include anti-proliferation, apoptosis induction cell cycle arrest and anti-angiogenesis [5]. Moreover, TQ exhibits anticancer activity through the activation of caspases and generation of reactive oxygen species [4,6]. Previous studies showed that TQ induced

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Table 1
Summary for the results of the four experimental groups: control (0.1%DMSO), TQ (46.03 μM) Resveratrol (64.54 μM) and as combination after determining the cell viability, caspase-3, GSH and MDA contents. The table is pointing to a synergistic action for the combination between TQ + resveratrol.

Test	Control (0.1%DMSO)	TQ (46.03 μM)	Resveratrol (64.54 μM)	TQ + resveratrol
MTT (% of Cell Viability)	97.69 ± 0.5467%	52.83 ± 1.635%[a]	49.91 ± 2.552 %[a]	37.27 ± 0.9473 %[a,b,c]
Caspase-3(n mol/10^6)	14.73 ± 0.36	26.06 ± 1.72[a]	29.23 ± 2.466[a]	39.17 ± 2.436[a,b,c]
GSH (n mol/10^6)	136.52 ± 3.90	105.29 ± 2.30[a]	95.09 ± 3.91[a]	76.33 ± 2.77[a,b,c]
MDA (n mol/10^6)	10.88 ± 0.08	8.9 ± 0.17[a]	9.88 ± 0.26[a]	6.977 ± 0.25[a,b,c]

Data is reported as mean ± SEM for four independent experiments for each parameter.
Date at $p < 0.05$ showed.
a: Significant difference from Control (0.1% DMSO).
b: Significant difference from TQ (46.03 μM) group.
c: Significant difference from Resveratrol (64.54 μM) group.

apoptosis and inhibited proliferation in pancreatic ductal adenocarcinoma cells [7]. Many other studies reported that TQ exhibited inhibitory effects on cell proliferation of many cancer cell lines, including colon, ovarian, lung, and myeloblastic leukemias [8].

Many other neutraceuticals have beneficial effect in treatment of several types of cancer, such as resveratrol which is a polyphenol compound belonging to the class of the stilbenes present in many vegetables and fruits including grapes [9]. Resveratrol possesses an apoptosis-dependent anticancer activity and minimal toxicity to normal cells at certain doses [10]. Since many tumors show sensitivity to resveratrol including lung carcinoma, acute myeloid leukemia, promyelocytic leukemia, multiple myeloma, prostate cancer, oral epidermoid carcinoma, and pancreatic cancer great attention has been given to resveratrol [11].

Resveratrol has various anti-cancer cellular mechanisms including inhibition of angiogenesis and metastasis [12,13]. Additionally resveratrol modulates many cell cycle signaling factors resulting in cell cycle arrest or apoptosis and it also increases caspases which are apoptosis associated enzymes [14]. Caspase-3 is one of the executioner caspases that are believed to be responsible for the actual damage of the cell [15]. Many agents, which induce apoptosis, are either oxidants or stimulators for cellular oxidative metabolism [16]. TQ and resveratrol have oxidative stress effect within cancer cells that makes them potential candidates for treatment of cancer [4,17]. ROS production is one of apoptosis predisposing factor within the cell leading to glutathione (GSH) reduction and the loss of cellular redox balance [18].

A need for a safer and more effective therapy is required either by using single or combined drugs, by offering a great potential in treating HCC and counteracting the side effects of chemotherapeutic agents. Here we investigate the effect of TQ and resveratrol either separately or in combination as anticancer agents on HCC cell line (HepG2).

2. Material and methods

2.1. Chemicals

TQ (2-methyl-5-propan-2-ylcyclohexa-2, 5-diene-1, 4-dione) drug, Resveratrol (3,4,5-trihydroxy-trans-stilbene) drug and reduced glutathione (GSH) kit all were purchased from Sigma-Aldrich St.louis, Mo, USA. Caspase-3 immune assay kit was purchased from Quantikine ELISA, USA. Fetal bovine serum (FBS), RPMI-1640 medium (culture media) and streptomycin/penicillin were purchased from Lonza Co., Almaadi, Egypt.

2.2. Cell line and culture condition

The HCC cell line (**HepG2**) was purchased from VACSERA **(Dokki, Giza, Egypt)**. Cells were cultured in PRMI media with 10% fetal bovine serum FBS and 100 U/mL Penicillin in 5% CO_2 at 37 °C.

2.3. Design of the work

Cells were classified as follow; control cell lines with 0.1%DMSO, TQ treated cells with 46 μM concentration, Resveratrol treated cells with 64.5 μM concentration and (TQ + resveratrol) in combination treated cells.

2.4. Cytotoxicity assay

Cells were plated on 96-well plates at a density of 5000 cells/well. They were plated in triplicate for each concentration. Different concentrations of TQ and resveratrol were prepared by serial dilution. All serial dilutions were transferred to the cells in the 96-well plate, each plate included untreated cells as control with 0.1% DMSO and was incubated for 24 h. After incubation the viability of the cells was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) 20 μl were added to cells and incubated for 2–3 h at 37 °C. After incubation, all of the media were removed and 100 μl of DMSO were added to the cells for solubilization. The absorbance was measured at a wavelength of 570 nm using a micro plate reader. The percentage of cell viability was calculated as survival fraction = T/C × 100 (T mean absorbance of test /C mean absorbance of control).

Data analysis was carried out using prism software program (Graph pad Software incorporated, version 5).

2.5. Caspase-3 immuno-assay

In-vitro determination of the proteolytic activity of the enzymes in lysates of HepG2 cells were performed. TQ and resveratrol as single therapy and then as combination (TQ + resveratrol) induced apoptosis in cells at different time intervals (12, 24 and 48 h) using the Quantikine ELISA Kit. Briefly, cells were collected, washed with cold PBS (1800 rpm in 10 min) and subsequently re-suspended in protein lysis buffer and incubated for 10 min on ice. Then, centrifugation was done at 1500 rpm for 1 min, and the supernatant (protein) was collected. Protein was transferred to 96-well plates in triplicates, then 50 μl of reaction buffer and 5 μl of caspase were added to each well, then incubated for 1–2 h at 37 °C. The plates were read using the micro plate reader at a wavelength of 405 nm.

2.6. Glutathione content determination

Determination of non-protein sulfhydryl compounds (NPSH) in HepG2 cell lysate (indication to reduced glutathione) was performed to each micro centrifuge tube. The tubes were gently shaken intermittently for 10–15 min. This was followed by centrifugation at 2000 rpm for 5 min at room temperature. Then