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Potential Effect of Thymoquinone on HNO-97 Cell Line

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Aim: The aim of the current study was to assess the effect of thymoquinone (TQ) on tongue squamous cell carcinoma cell line (HNO-97).
Materials and Methods: Tongue squamous carcinoma cell line (HNO-97) was obtained, and maintained in cultured flasks and were divided into; Group I (Control): Untreated (HNO-97) cells and three treated groups: HNO-97-treated cells with three different concentrations of TQ. All cells were incubated for 24 h. The viability of HNO-97 cells treated with TQ was determined 24 h post treatment. Determination of the effect of TQ on HNO-97 cell line was done by Methyl Thiazol Tetrazolium (MTT) cytotoxicity assay, flow cytometry cell cycle assay, and ELISA test for detection of TNF-α.
Results: MTT assay showed that TQ cytotoxicity was dose dependent. The mean percentage of viability of the treated cells decreased in a dose dependent manner. Moreover, TQ treatment induced reduction in the percentage of cells in the G1 to S phases of the cell cycle and increased the percentage of cancer cells in the G2/M phase. Furthermore, TQ treatment significantly decreased TNF-α level in a dose dependent manner.
Conclusion: The effect of TQ decreased the viability of tongue squamous cell carcinoma cell line in a dose dependent manner.

Key words: Carcinoma, Squamous, Thymoquinone, Tongue.

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Introduction

Oropharyngeal cancer is one of the most prevalent malignancies that occur in the head and neck region with rising incidence worldwide.[1] It is responsible for approximately 2.8% of new cancer cases and 1.9% of cancer-related deaths in 2023.[2]

Oral cancer is considered a major worldwide public health issue due to its growing prevalence. Its disease burden has changed substantially during the last three decades.[3] According to the National Cancer Institute survey, the incidence rates of oral cancer are significantly higher in males than in females. Its incidence increases with age, with a more rapid increase after age 50, especially in adults aged 65 and older.[4, 5] Smoking, drinking alcohol, and infection with human papilloma viruses are the main risk factors of oral cancer.[6]

Squamous cell carcinoma is the most common type of malignant tumors found in head and neck regions, accounting for 90% of all diagnosed cases, including the oral cavity and lip.[7, 8] Its incidence has increased especially in young people.[9, 10]

Oral SCC is treated with multimodal approaches including surgery, radiation therapy, systemic chemotherapy, targeted therapy, hormonal therapy, and immunotherapy.[11] Recently, there are interesting treatment strategies that use phytochemicals in order to reduce its side effects and normal cell toxicity.[12-14]

Thymoquinone (TQ) showed an increased range of pharmacological properties including antimicrobial, analgesic, anti-inflammatory, antihypertensive, anticarcinogenic, histamine release inhibitor, antioxidant, hypoglycemic, and hepatoprotective properties.[12] Moreover, TQ has been reported as one of those phytochemicals that showed promising results in cancer management.[12] It demonstrates antiproliferative effects on variable cancer cell lines.[12, 15]

Prior work has shown that TQ treatment in human oral cancer activates the apoptosis and autophagy pathway, resulting in cell death.[16-19] Therefore, the objective of this research was to examine the different effects of TQ on HNO-97 cells.

Materials and methods

The research ethics committee of the Faculty of Dentistry, Minia University approved the protocol (approval number: 66/2022).

Samples Preparation

(HNO-97) cells were obtained from the Cell Culture Department at Nawah Scientific in Cairo, Egypt. Cells were imported and authenticated by Cytion. Cells were confirmed as human by immunofluorescence and maintained in cultured flasks.

Samples Grouping

HNO-97 cells were divided into; Control untreated group: (HNO-97) cells. Treated groups: (HNO-97) cells treated with three concentrations of TQ (7.25µM/ml to 23.05µM/ml). All cells were incubated for 24 h.

Cell Culture Protocol

HNO-97 cells were rinsed with 0.25% trypsin to remove any serum-containing trypsin inhibitor. Then cells were observed under an inverted microscope until they get detached from the surface within 5 to 15 min. The growth medium was added, and cells were then aspirated. The cell suspension was centrifuged for five to ten minutes, then the pellet was suspended in fresh growth medium. Aliquots of the cell suspension were transferred to new plates and incubated for 24 h at 37°C.

Drug Preparation

Thymoquinone, obtained from the Cell Culture Department, Nawah Scientific and was dissolved in normal saline.

Treatment of HNO-97 Cells with TQ

The prepared cell line was treated in a 96-well culture plate with serially diluted test materials starting from 100µM. Plates were incubated with daily observation using the inverted microscope for the detection of cytological changes. Maintenance medium as a negative control was also observed. Cells were incubated at 37°C for 24 h.

Determination of the Effect of TQ on HNO-97 Cell Line

The viability of TQ- treated HNO-97 cells was determined 24 h post treatment.

MTT Cytotoxicity Assay

For MTT assay, HNO-97 cells were grown in 96- well micro-titer plates and treated with different TQ concentrations for 24 h. The cells were then incubated with 10 µL of MTT (0.5mg/ml stock) solution at 37°C for 24 h. The formed purple Formosan crystals were dissolved in MTT solubilization solution. The plates were quantified by measuring the absorbance at 570 nm using a spectrophotometer (Dynatech MR5000). The experiment was performed in triplicate.

The obtained data were analyzed using Master Plex Reader Fit program to determine the pre IC50, IC50, and post IC50 of TQ for 24 h.

Flow Cytometry Cell Cycle Analysis

Following treatment with different concentrations of TQ for 24 h, cells were collected by trypsinization. They were then washed thoroughly with a cold saline solution twice. Next, the cells were placed in a solution of 60% ice-cold ethanol, kept at 4°C for 1 h.

HNO-97 cells were first fixed and washed. Then they were re-suspended in 1 mL of phosphate buffer saline containing 10 µg/mL propidium iodide and 50 µg/mL RNAase A, and then incubated for 20 min in dark at 37°C. Then the cells were analyzed for DNA using FL2 (λex/em/ACEA NovocyteTM flowcytometer). Cell cycle distribution was calculated using NovoExpressTM software.

Detection of TNF-α by ELISA

The micro-ELISA plate was coated with an antibody for rat TNF-α, followed by a biotinylated detection antibody. The optical density of TNF-α conjugated with the antibody was measured at 450 nm.

Statistical Analysis

Experimental data were collected, tabulated, and statistically analyzed by IBM SPSS software version 16.0.

Results

MTT Cytotoxicity Assay Results

The cytotoxic effect of TQ on HNO-97 tongue carcinoma cells were assessed for 24 h. The obtained data revealed that the cytotoxicity of TQ was dose dependent. The treated cells showed decreased mean viability percentage compared to control cells as the drug concentrations increased from 7.25µM/ml to 23.05µM/ml in TQ **(Table 1).**

Table (1): The mean viability percentage of TQ treated HNO-97 cells with low concentration (7.25µM/ml), medium concentration (12.52µM/ml), and high concentration (23.05µM/ml) for 24 h incubation.

Sample	HNO-97 cells treated with TQ for 24h incubation					
Conc. (µM)	7.25 Pre IC_{50}	8.57	12.52 IC_{50}	15.78	19.23 Post IC_{50}	23.05
Viability %	75.25%	57.74%	**50%**	40.92%	25.63%	31.18%

Flow Cytometry Cell Cycle Results

G1 Phase

On comparing TQ treatment on HNO-97 cells in three different doses (IC 25,

50, and 75), a gradual decrease in the number of cells entering the cell cycle was observed in a dose-dependent manner, when compared to the control group. The lowest levels of cells were observed in the lowest concentration of TQ and the highest levels were observed in the control group.

S Phase

Regarding the S phase, the TQ-treated group in the 3 doses IC 25, 50 and 75, respectively, showed a gradual decrease in the number of cells in a dose-dependent manner. The least number of cells were detected at the highest dose IC75. On comparing with their control group, the highest number of cells was detected in the control group while the least amount was observed in the highest dose of TQ (IC75).

G2 Phase

Regarding the G2 phase, the TQ-treated group in the 3 doses IC 25, 50 and 75, respectively, showed a gradual increase in the number of cells in a dose dependent manner. The highest number of cells was detected at the highest dose IC75.

Comparing TQ treatment with their control group, the highest number of cells was detected in the control group while the least amount was observed in the highest dose of TQ treatment.

Apoptotic Phase

A gradual increase in the number of apoptotic cells in the TQ treated groups was revealed in a dose dependent manner. The control group showed very low % of apoptosis, while the highest dose of TQ treatment showed the highest % of apoptosis **(Fig. 1)**.

Detection of TNF-α by ELISA

Expression of TNF-α showed a significant decrease with TQ treatment, where TNF-α expression decreased in a dose dependent manner, while the untreated group (control) showed the highest expression **(Table 2)**.

The control group showed an average expression of TNF-α of 83.4 pg/ml, while TQ treatment group showed a significant downward expression of the marker from an average 74 to 65 pg/ml as we increase the dose from IC25 to IC75.

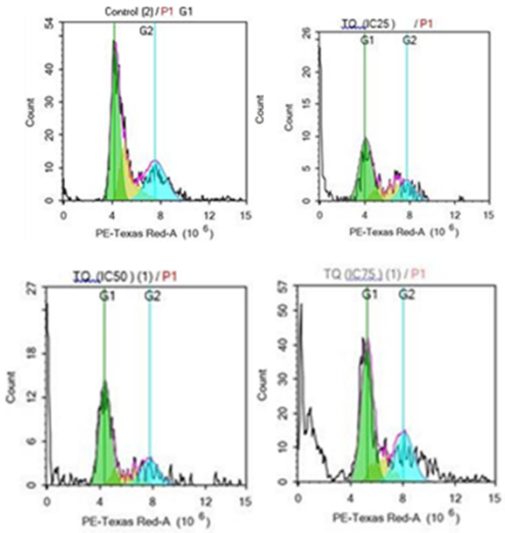

Figure (1): Cell cycle distribution histograms of untreated (control group) and TQ-treated HNO-97 cell line.

Table (2): Average expression values of TNF-α of the control group and TQ treated cells

Customer code	Nawah code	Tested parameter	Test method	Test result	Unit
Control	SO14506-01	Human TNF-α	ELISA	83.40	pg/ml
TQ IC25	SO14506-03	Human TNF-α	ELISA	74.27	pg/ml
TQ IC50	SO14506-04	Human TNF-α	ELISA	68.86	pg/ml
TQ IC75	SO14506-05	Human TNF-α	ELISA	65.42	pg/ml

Cell Cycle Analysis

As regards G1, there was a significant difference between G1 percentages in the two groups (control and TQ with different concentrations), ($P < 0.001$, Effect size = 0.984). Pair-wise comparisons revealed a statistically significant difference between groups; a statistically significantly lower percentage was observed with TQ (IC50) followed by TQ (IC75); all showed

statistically significantly lower values.

As regards S, there was a statistically significant difference between S percentages between the groups (P <0.001, Effect size = 0.990). Pair-wise between groups revealed that control group showed the significantly highest percentage. Lower mean values were observed with TQ (IC75).

As regards G2, there was a significant difference between G2 percentages between the 2 groups (P <0.001, Effect size = 0.958). Pair-wise among groups revealed that the TQ (IC75), showed the highest percentage with a statistically significant difference from TQ (IC25). The lowest mean percentage was observed with the control group.

Finally for Sub-G1, there was a significant difference between Sub-G1 percentages in different groups (P <0.001, Effect size = 0.983). Pair-wise between groups revealed that TQ (IC75) showed the statistically significantly highest percentage. A significantly lower mean value was observed with TQ (IC25). The control group showed the statistically significantly lowest mean percentage **(Table 3).**

Table (3): Descriptive statistics and comparison of the cell cycle percentages across different groups

	G1		S		G2		Sub-G1	
Group	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	44.7 ^{A}	1.3	33.7 ^{A}	4.9	19 ^{B}	3.5	2.6 ^{G}	0.5
TQ (IC25)	40.1 ^{A}	3.6	10.8 ^{B}	2.1	22.5 ^{A}	1.5	20.6 ^{F}	3.2
TQ (IC50)	38 ^{B}	3.9	10.5 ^{B}	2.2	20.5 ^{AB}	3.4	31 ^{E}	4.2
TQ (IC75)	27 ^{D}	2.5	7.1 ^{C}	1.9	16.8 ^{C}	2.4	49.1 ^{C}	3.5
P	<0.001*		<0.001*		<0.001*		<0.001*	
Effect size (Eta squared	0.984		0.990		0.958		0.983	

** P ≤ 0.05, Different superscript within a column means significant difference between groups*

TNF-α Expression

There was a statistically significant difference between TNF-α expression in the four groups (*P* <0.001, Effect size = 0.867). Pair-wise comparisons between groups revealed that control group showed the statistically significantly highest mean TNF-α expression. TQ treatment showed statistically significantly lower value in a dose dependent manner, where the highest dose of TQ showed the lowest expression and lowest dose showed the higher expression as follows **(Table 4).**

Table (4): Descriptive statistics and comparison of the TNF-α expression (pg/ml) across different groups

Group	TNF- α		P	Effect size (Eta squared)
	Mean	SD		
Control	83.4 ^{A}	3.2	<0.001*	0.867
TQ(IC25)	69.5 ^{B}	3.2		
TQ(IC50)	44.5 ^{C}	14.7		
TQ(IC75)	40.6 ^{C}	1.7		

*** P ≤ 0.05, Different superscripts indicate significant difference between groups**

Discussion

SCC, the preponderant malignant tumor affecting the head and neck region, which encompasses the oral cavity and lip, constitutes approximately 90% of all diagnosed cases of malignancies.[7, 8]

The frequency of oral squamous cell carcinoma (OSCC) has witnessed an upward trend in numerous nations, particularly among the younger demographic.[9, 10] Consequently, OSCC was the primary focus and chosen lesion in the present investigation.

Regarding the management of OSCC, the conventional treatments have been linked to significant morbidity as a result of the engagement of crucial anatomical structures, adverse effects, and therapeutic resistance. Consequently, there is a pressing necessity for innovative alternative therapies for such a medical condition. Thankfully, multiple avenues of evidence have proposed the potential utilization of dietary constituents derived from fruits and vegetables, as they have exhibited noteworthy efficacy against oral cancer over the past few decades.

These bioactive dietary phytochemicals, which occur naturally, are not toxic and do not have significant adverse effects. As a result, they are considered as potential options for treating cancer. Taking into consideration the significant benefit of utilizing these substances in cancer treatment, this study aimed to explore a novel approach that is both natural and less invasive, which has gained popularity in recent years.[11, 20]

Nigella sativa (NS) has been described as the "miracle herb of the century" and is also known as black cumin, black seed.[18] The most pharmacologically active compounds isolated from NS are TQ, dithymoquinone, thymol, and thymohydroquinone.[18]

Thymoquinone, the most efficacious constituent of NS, has been the subject of extensive studies and has been determined to possess a broad spectrum of pharmacological attributes, encompassing antimicrobial (specifically antibacterial, antifungal, anthelmintic, and antiviral) properties; anti-inflammatory effects; analgesic properties; inhibition of histamine release; antihypertensive capabilities; hypoglycemic effects; anticarcinogenic potential; antioxidant activity; and hepatoprotective qualities.[12, 15]

In this investigation, several methodologies were employed, encompassing the MTT assay, flow cytometry, and ELISA. With regards to the MTT assay, it was executed due to its status as one of the dependable techniques for evaluating cellular viability. Furthermore, it is renowned for its discerning detection of cellular proliferation, as it quantifies the rate of cellular growth through the utilization of a linear correlation between cellular activity and absorbance.[21, 22]

On the contrary, flow cytometry was the preferred methodology for the identification of apoptosis as well as the quantification of cells undergoing apoptosis. In relation to ELISA, it stands as one of the most convenient and dependable techniques for the identification and analysis of diverse markers.[23]

In the present study, the therapy involving TQ in different concentrations manifested the most potent cytotoxic impact, accompanied by a minimal proportion of viable and actively dividing cells. Likewise, it exhibited the greatest percentage of cells undergoing programmed cell death and the least amount of TNF-α concentration expression.

This positive outcome was consistent with findings of Zheng,[24] Ballot,[16] and Lei[25], who demonstrated that TQ had a therapeutic effect on breast cancer cell line in vitro, colorectal cancer cells, and in gastric cells, respectively, both in vitro and in vivo.

This work reveals that TQ has an anticancer effect by inhibiting the cell cycle and increasing apoptosis in a dose-dependent manner. These findings are consistent with those of Xu,[26] and Chu,[17] who reported similar results in human cholangiocarcinomas, human squamous cell carcinoma, and breast cancer cells, respectively.

Similar to this, additional phytochemicals including graviola extract and cucurbitacin E had a potent cytotoxic effect on OSCC and HNSCC cell lines that were concentration dependent.[13, 27]

The current study's striking reduction in the percentage of cells in the G1 to S phases of the cell cycle suggests that the DNA content of cells entering the cycle has changed, which can result in a deregulated cell cycle and inhibit the growth and proliferation of cancer cells in all treatment groups in a dose-dependent manner with varying significance degrees.

Conversely, all treatment groups had a higher percentage of cancer cells in the G2/M phase, suggesting that the highest dose

of TQ caused irreversible DNA damage, which resulted in cell cycle arrest and an accumulation of cells in this phase of the cell cycle before apoptosis.

In the same context, TQ was shown to significantly induce cell cycle arrest at the G2/M phase against human CCA cell lines, resulting in the induction of death.[26] They declared that the fundamental mechanism was linked to higher pro-apoptotic protein expression and lower anti-apoptotic protein expression.

In the current investigation, compared to the control group, the expression of TNF-α was dramatically downregulated in the highest dose of TQ therapy. This suggested that apoptosis was actively occurring.

These findings corroborated those of Adinew[28] who demonstrated that TQ induced apoptosis in breast cancer cells and downregulated TNF-α in TNF-induced breast cancer cells. Additionally, Arjumand[29] demonstrated that TQ reduced TNF-α, IL-1, and NF-κB expression levels in rheumatoid arthritis.

Thymoquinone had an anticancer impact with increasing dose, this study clarifies the prospect of employing TQ as a supplemental treatment for traditional cytotoxic medicines.

Conclusions

Thymoquinone decreased the viability of HNO-97 cells in a dose dependent manner. Moreover, TQ avoided cell cycle progression of HNO-97 cells at the G2/M checkpoint in a dose dependent manner. Furthermore, TQ decreased the expression of TNF-α in a dose dependent manner.

Recommendations

Further in vivo and clinical studies are recommended to investigate the therapeutic efficacy of TQ. In addition, the study of possible synergistic effects of different extracts that provide chemo preventive benefits should be addressed in the future.

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