Antiproliferative Effect of Thymoquinone on Human Colon Cancer Cells: Is It Dependent on Glycolytic Pathway?

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ABSTRACT

Purpose: In the present study, we aimed to investigate the anti-proliferative effect and metabolic activity of thymoquinone (TQ) on colon cancer cells (HCT-116).

Material and Methods: Cell viability was determined by MTT analysis. Cells were treated with different concentrations of TQ (40, 60, 80, 100, 150, and 200 µM) on HCT-116 cells and half-maximal inhibitory concentration (IC50) values were calculated by using the CompuSyn software program. In addition, glucose and lactate concentrations were measured from cell culture supernatants for RPMI medium, control and TQ (IC50 dose) groups. Statistical analyses were performed using GraphPad Prism 7.

Results: Thymoquinone was found to be antiproliferative particularly in 40-200 µM concentrations. The IC50 concentration of TQ was calculated as 68 µM. Glucose levels of supernatants were 478, 384±8.5 and 412±19.7 mg/dL in RPMI medium, control and TQ group, respectively. Lactate levels were found as 20±3.5 µM in the control group and 8±1.1 µM in TQ group.

Conclusion: The present study showed that TQ has an antiproliferative effect on HCT-116 in addition to its inhibitory effect on a glycolytic pathway.

Keywords: Colon cancer, Antiproliferative effect, Thymoquinone, Glycolytic Pathway
One of the most commonly diagnosed cancer types is colorectal cancer (CRC) and the deaths depending on CRC is increasing day by day (1). There has been a significant increase in the diagnosis of CRC in the West and other high-income countries over the past decade (1, 2). Increasing morbidity and mortality related to CRC led to developing new targeted treatment strategies. At this point cell culture systems, especially the cancer cell lines, have provided important research opportunities to illuminate the molecular basis of cancers. HCT-116 is one of the cell lines among the identified 70 colorectal cell lines (3).

The development of CRC may be due to bacterial causes (2) as well as genetic reasons such as chromosomal instability, DNA-repair defects, abnormal DNA methylation, mutational inactivation of tumour suppressor genes, activation of oncogene pathways, etc. (4). Surgical operations, radiotherapies and systemic therapies are used in colorectal treatments. Systemic therapies include the administration of fluoropyrimidines, alone or in combination with oxaliplatin (5). However, the effects of cytotoxicity, drug resistance or adverse reactions are the main encountered problems in systemic therapies. Less toxic and well-tolerated “natural products” are used in order to have better treatment outcomes and improve the life quality of the CRC patients. Alkaloids, polysaccharides, polyphenols, terpenoids and unsaturated fatty acids are among those natural compounds (6). Thymoquinone (TQ), as a phytocchemical, is found dominantly in Nigella sativa (black seed), possesses anti-oxidant, anti-inflammatory, anti-hepatotoxic and nephrotoxic, anti-diabetic, antimicrobial and antiproliferative activities (7). Studies have shown that TQ inhibits the division of the cancer cells at different stages of cell cycle and leads to apoptosis in cancer cell lines by activating the proapoptotic factors and suppressing the anti-apoptotic factors (8).

In normal cells, one molecule of glucose is converted to two molecules of pyruvic acid by glycolysis and in the presence of oxygen, lactate molecules are diverted to the citric acid cycle for further catabolic reactions. Electrons flow through the electron transport chain and in final step ATP is produced by ATP synthase. The process is called cellular respiration. However, cancer cells switch from cellular respiration to inadequate glycolytic pathways, although the ATP demand is extremely high in cancer cells (9). So, carbohydrate consumption and metabolism are changed in cancer cells.

In this in vitro study we will focus on the changes in glucose and lactate concentrations and the antiproliferative effect of TQ on HCT-116 cell line.

**MATERIAL AND METHOD**

**Materials**

The colorectal cancer cell line (HCT-116) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). RPMI medium, dimethyl sulfoxide (DMSO) and Thymoquinone were obtained from Merck. Glucose measurement kit (ref: 61 269) was obtained from bioMérieux (France), lactate kit (ref:1001330) was obtained from SPINREACT (Spain).

**Cell Culture**

HCT-116 cells were grown in RPMI medium containing 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin and 1% L-glutamine in an incubator adjusted at 37°C and 5% CO2. HCT-116 cells were seeded into each well so as to be 1x10⁴ and incubated for 24h. Following day, TQ was added from stock solution (DMSO concentration is less than 0.1% in stock solution) into the wells in different volumes in order to adjust the concentrations to 40, 60, 80, 100, 150 and 200 µM and left to incubation for 24h.

**Cell Viability**

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test was applied for cell viability. Briefly, after 24h incubation period with TQ, 10 µL MTT were added into the wells and incubated for 3 hours. Mixtures were removed from the wells and 100 µL DMSO added in order to dissolve formazan crystals occurred in the well. Purple coloured formazan crystals dissolved in DMSO were measured with a Varioskan Flash microplate reader (Skanit Software 2.4.5, Thermo Scientific) at a wavelength of 540 nm. The absorbance values were used to calculate the % cell viability. IC₅₀ value of TQ were calculated by CompuSyn software by using the fractional (Fa) values.

**Analysis of Glucose and Lactate Concentrations**

Glucose and lactate concentrations were analysed in order to reveal the changes on glycolytic pathway in control group and TQ-treated (only for IC₅₀ value of TQ) group. Briefly, HCT-116 were seeded into the cells and incubated for a night. Briefly, HCT-116 were seeded into the 6-wells microplate and incubated for a night. Following day, only RPMI medium were added into empty (without cells) wells and into control group and TQ were added into TQ-treated group wells and left for incubation throughout 24 hours. Samples were pipetted from wells into tubes after incubation and reagents were added as indicated in the manufacturer’s assay kit procedure and measured by spectrophotometer. Glucose concentrations were expressed as mg/dL and lactate concentrations as µM.
**Statistical Analysis**

The statistical analyses were carried out by GraphPad Prism 7. Since our data was normally distributed, to compare more than two independent samples with one another, one-way ANOVA test was applied. The results were given as mean (± standard deviation).

**RESULTS**

**The Antiproliferative Effect of Thymoquinone on HCT-116 Cells and IC50 Value**

There is gradually reduction in the % cell viability in TQ-treated groups compared to control group. According to the statistical analysis, the reduction of cell viability was non-significant when 40 μM concentration of TQ was compared with the control group. However, the range of concentrations of TQ from 60 to 200 μM showed significant decrease when compared to the control group (p<0.001) (Fig 1a). The IC50 value of TQ on HCT-116 cells was calculated as 68 μM over the Fa values by using the CompuSyn Software program (Fig 1b).

**Glucose Concentrations**

Glucose concentrations decreased in both control (p<0.001) and TQ (p<0.05) groups compared to RPMI medium group but according to the statistical analysis the drop was found extremely significant particularly in control group. Although, the lower levels were observed in TQ group compared the control group, the difference between them was found as non-significant (Table 1).

**Lactate Concentrations**

Lactate was not detected in RPMI Medium group. Lactate levels were significantly higher in TQ and control groups compared to RPMI Medium group, p<0.01. On the other hand, lactate levels were found to be significantly lower in TQ group when compared with control group (p<0.05) (Table 1).

**DISCUSSION**

The third most common cancer-related deaths are colorectal cancer in US. Approximately only 1 in 5 patients who are diagnosed with CRC can live more than 5 years (10). High mortality of CRC (3) has forced researchers to find effective and exact treatments.

Nowadays, natural products derived from plants, animals or microorganisms play an important role in cancer treatment (11) in addition to conventional treatment. TQ, a well-known antioxidant molecule, is one of the natural products found in black seed and it inhibits tumour growth and protects the cells against cancer development (12). Studies have shown that thymoquinone is effectively protective in different types of cancer and even in different stages of cancer (proliferation, metastasis and invasion) (13).

| Tablo 1. Glucose and lactate concentrations in RPMI medium, control and TQ groups |
|---------------------------------|-------|--------|---------|
|                                 | RPMI Medium | Control | TQ (68 µM) |
| Glucose Concentration (mg/dL) (mean±sd) | 478 | 384±8.5 *** | 412±19.7 ** |
| Lactate Concentration (µM) (mean±sd) | 0 | 20±3.5 ** | 8±1.1*** |

* Significant differences compared to RPMI Medium group; ** Significant differences compared to Control group; *** p<0.05; **** p<0.001.
In a study, it has been reported that TQ reduces the cell viability through increase the apoptotic pathways in a time- and dose-dependent manner in HCT-116 cells. It was demonstrated that 25 µM TQ killed approximately half of the living cells following to 24h incubation period (14). Gali-Muhtasib et al. (2004) demonstrated that IC50 value of TQ in HCT-116 cells is 35 µM in 24h incubation. Researchers attribute the antiproliferative effect of TQ to p53-dependent apoptosis (15). In our study, we found that TQ has antiproliferative effect on HCT-116 cells in different doses. The calculated IC50 value of TQ (68 µM) is showed similarity with the study that carried out by El-Far et al (2021)(16).

Glycolysis is unique pathway for catabolism of glucose in many types of cells. The catabolic pathway (glycolysis) starts with one molecule glucose and ends with two molecules pyruvic acid. The energy released is conserved as ATP and reducing agent NADH. In presence of oxygen, in another words in aerobic respiration, two molecule pyruvic acid are diverted to citric acid cycle for the further catabolic reactions and the ATP is produced at the end of the electron transport chain by the ATP synthase. In absence of oxygen, pyruvic acid is converted to lactic acid by lactate dehydrogenase and the NAD+ concentrations are kept stable in the cytosol (9). Of course, as expected, all these processes occur in normal cells. However, in cancer cells, the process switch from aerobic respiration to glycolysis although produced energy is lower at the end of the glycolysis. As the cancer cells grow rapidly, oxygen starts to be insufficient for the metabolism. Due to this, energy is predominantly provided by glycolysis in cancer cells. Adaptation to this hypoxic condition is achieved by hypoxia-inducible transcription factor (HIF). This factor increases the synthesis of glycolytic enzymes and glucose transporters (17). Lee et al. (2019) demonstrated that TQ has potential inhibitory effect on HIF-1α on renal cancer cell lines (Caki-1, Caki-2, A498). In addition, researchers showed that glucose levels increased while lactate levels decreased in TQ-treated group (18). In another study, similar results were expressed by Karim et al. (2022) in colorectal cancer cell lines (19). In the present study, lower glucose concentration is result of high consumption in control group. On the other hand, we can understand from the findings that lactate production is decreased in TQ group compared to control group. These changes in carbohydrate metabolism led us to reach in two results. First, as indicated in the studies, TQ increases cancer cell death by activating apoptosis. Thus, the reduction in lactate production and glucose consumption may be result of the antiproliferative effect of TQ. Briefly can be mentioned that the less amount of cancer cells the less production of lactate. Second, TQ may have inhibited the HIF and led to cancer cell death by decreasing the glucose catabolism because glycolysis is the main metabolic pathway to meet the energy demand of cancer cells.

CONCLUSION

In conclusion, we found that 68 µM TQ has antiproliferative effect and glucose metabolism is adversely affected in HCT-116 cells. In order to be more successful in cancer treatments, various of molecular mechanisms must be illuminated related to cancer forming and its treatment models by different studies. For further studies, we are thinking that it would be helpful to investigate some biomarkers in apoptotic pathways and focus on HIF.

DECLARATION

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Conflicts of Interest/Competing Interests
Authors has no conflict of interest to disclose.

Ethical Committee Approval
Ethical approval does not require for our study.

Availability of Data and Material
Available if it is requested.

Authors’ Contributions
EMA designed the study, carried out the experiments and analysed the results. MÖ carried out the experiments, analyzed the results and wrote the paper.

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