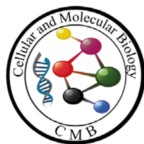

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Original Research

In Vitro evaluation of thymoquinone on apoptosis and oxidative DNA damage in high glucose condition

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Abstract: The study was planned to investigate the effects of thymoquinone (TQ), which is a compound in *N. sativa*, on caspase dependent apoptosis and oxidative DNA damage in high glucose treated PC12 cells. PC12 cells were treated with high glucose (G1-150 mM, G2-250 mM, G3-350 mM), TQ (20 µM), and their combinations. Oxidative DNA damage (8-OHdG (8-Oxo-2'-deoxyguanosine)), and apoptosis (caspase 3, caspase 8, caspase 9 enzymes and M30 protein) parameters were analyzed with ELISA. The 8-OHdG levels decreased in all combination groups compared to the control ($p \leq 0.001$). There was no statistically significant difference between caspase 3 and 9. Caspase 8 in TQ, G3, TQG1, TQG2 groups were higher than the control ($p \leq 0.002$). Low M30 levels were observed in TQG1 group ($p \leq 0.002$). In conclusion, it was observed that in PC12 cell line treated with the high glucose concentrations, TQ administration had a statistically significant effect on oxidative DNA damage and some apoptotic parameters (caspase 8 and M30 protein).

Key words: Caspases; DNA damage; High glucose; *In vitro*; Thymoquinone.

Introduction

Although insulin and similar drugs are used in diabetes treatment, there is an increasing interest in the search for new natural methods. In several parts of the world, various plants are used for the treatment of diabetes in traditional approaches. Certain traditional plant treatments have been considered in scientific circles and the World Health Organization (WHO) supports the studies conducted in this field (1-3).

In cells which are not required by an organism, intracellular signaling systems are activated and apoptosis process is initiated. The change in the equilibrium for or against apoptosis might explain the pathogenesis of several important diseases. Caspases are found in the cytoplasm as zymogens (inactive messengers) and called cysteine proteases since they contain cysteine in their activity centers. Apoptotic signal cascade is initiated as a result of apoptotic signals that occur due to reduction of growth factors and in hypoxia, DNA damage, oxidative stress, and ischemic damage (4,5).

Hyperglycemia leads to oxidative stress. This indicates that oxidative stress may play an important role in the pathogenesis of chronic complications in diabetes. Reduction in antioxidant levels leads to oxidative DNA damage due to the removal of DNA repair mechanisms or an increase in ROS (reactive oxigen species) formation (6,7).

For an extended period of time, studies on antineoplastic, anti-inflammatory and antioxidant effects of thymoquinone (TQ) have been conducted. It was determined that TQ processed hypoglycemic and anti-diabetic properties as well (8-12).

The beneficial effects of TQ on hyperglycemic conditions are known. The present study was planned to investigate whether the beneficial effects of TQ, which is commonly used in various forms and for different reasons, on caspase induced apoptosis and oxidative DNA in PC12 cell line treated with various high glucose concentrations.

Materials and Methods

Cell culture

PC12 neuroendocrine tumor cells, originated from medulla region of rat adrenal glands, were used in this study. PC12 cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Capricorn Scientific, Germany) that contained 5% FBS (Capricorn Scientific, Germany), 10% horse serum, 1% L-Glutamine, 1% penicillin/streptomycin (Capricorn Scientific, Germany) and 0.0125% gentamycin in flasks (or plates) (SPL Life Sciences) coated with polylysine under standard culture conditions.

Cytotoxicity (MTT cell viability) test

Cytotoxicity effects of compounds on cells were determined with MTT assay. Cells were plated in 96-well plates at $8x10^3$ cells/well density, and incubated overnight for attachment to the surface of the plate. Cells

were treated different concentrations of molecules after adhesion process. A % DMSO amount was added to wells as a negative control for TQ groups. Cell viability test was conducted with MTT assay after 72 hours of incubation. 5 mg/ml MTT stock solution was prepared in a sterile PBS and sieved through a 0.22µm filter. After 3 h incubation with MTT solution, lysing solution was added into each well for solubilization of formazan crystals. The IC_{50} concentration (the half of maximal inhibitory concentration) was determined with GraphPad Prism 5 software.

Biochemical analyses

The dose value of Glucose, TQ and their combination were determined according to MMT assay results. For biochemical analysis, cells were cultured in 25 cm^2 flasks with $1x10^6$ cell density and incubated overnight for the adhesion of these cells to the flask surface. After incubation, The determined concentration of Glucose and TQ and their combination were administered to cells. Biochemical analyses were conducted after 72h incubation. The supernatants in the culture flasks were transferred into sterile tubes, centrifuged at 1200 rpm for 5 min, and supernatants were discarded. The collected cells and supernatants were prepared with freeze-thaw method. Biochemical analyses were performed according to manufacturer's protocol for Caspase 3, Caspase 8, Caspase 9, and M30 on obtained cell samples. To measure the cellular oxidative DNA damage, 8-OHDG levels were determined with DNA Damage ELISA kit (EKS-350, Enzo Life Science).

Statistical Analysis

Descriptive statistics were expressed in median, mean, standard deviation, minimum and maximum values for the studied parameters. Kruskal-Wallis test was used to determine whether there were differences between the groups based on these parameters. Dunnet multiple comparison test was used to identify different groups. In the calculations, statistical significance level was accepted as 5% and SPSS (v. 22.0) statistical software was used for the calculations

Results

Cytotoxicity assay with MTT

Based on MTT assay results, it was determined that IC_{50} values for IC_{50} values for Glucose was 250 mM (Figure 1) and TQ was 34.2 µM (Figure 2). In biochemical analyzes, IC_{25} concentrations of TQ was used in single and combination groups in order to determine the protective effect of TQ against the cytotoxic effect that may be caused by cellular glucose content. Thus, for this purpose, Glucose G1 (150 mM=IC_{25}), G2 (250 mM=IC_{50}) and G3 (350 mM=IC_{75}), TQ (20 µM= IC_{25}) were selected as single concentrations and TQ and Glu-

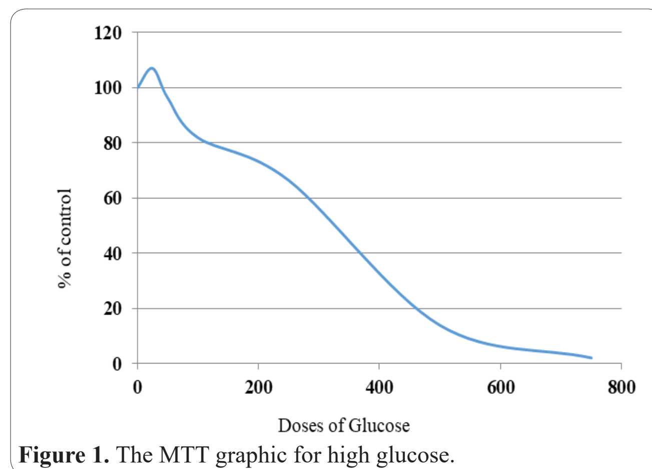

Figure 1. The MTT graphic for high glucose.

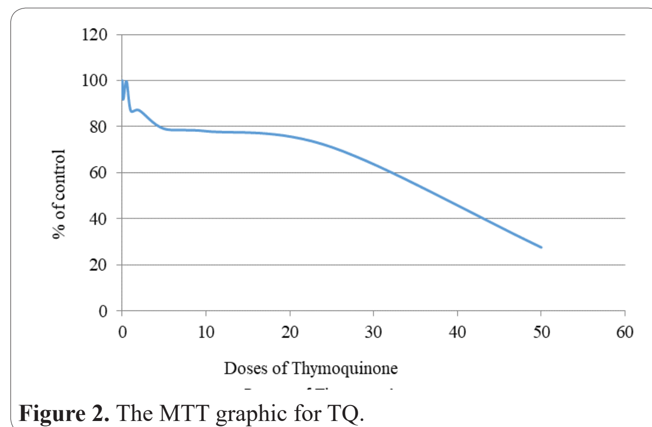

Figure 2. The MTT graphic for TQ.

Table 1. The TQ, Glucose, Combination Group designations.

GROUPS	APPLICATION
Control	N/A
TQ	20 µM TQ
Glucose1	150 mM glucose
Glucose 2	250 mM glucose
Glucose 3	350 mM glucose
TQG1	20 µM TQ - 150 mM glucose
TQG2	20 µM TQ - 250 mM glucose
TQG3	20 µM TQ - 350 mM glucose

Table 2. DNA damage and apoptotic parameters.

GROUP	8-OHdG (nm/ml)	Caspase 3 activities (ng/ml)	Caspase 8 activities (ng/ml)	Caspase 9 activities (ng/ml)	M30 concentrations (ng/ml)
Control	1.439± 0.107[ab]	1.551±0.265	1.147±0.056[b]	1.679±0.078	1.718±0.072[a]
TQ	1.469±0.097[a]	1.622±0.055	1.581±0.138[a]	1.580±0.087	1.503±0.177[ab]
Glucose1	1.372±0.156[ab]	1.506±0.123	1.334±0.312[ab]	1.674±0.023	1.698±0.027[a]
Glucose 2	1.402±0.037[ab]	1.586±0.118	1.144±0.155[b]	1.609±0.124	1.466±0.192[ab]
Glucose 3	1.159±0.054[b]	1.743±0.082	1.532±0.102[a]	1.605±0.086	1.631±0.303[ab]
TQG1	1.168±0.036[b]	1.618±0.078	1.651±0.045[a]	1.659±0.061	1.196±0.086[c]
TQG2	1.049±0.092[b]	1.622±0.044	1.466±0.047[ab]	1.648±0.109	1.385±0.297[bc]
TQG3	1.111±0.068[b]	1.510±0.272	1.368±0.092[ab]	1.629±0.089	1.427±0.105[bc]

The difference between group averages indicated with different letters is significant.

cose combination groups are presented in Table I.

Biochemical results

The data obtained with biochemical parameter analysis conducted on PC12 cell culture samples were analyzed statistically. The values determined as a result of statistical analyses performed on these concentrations are summarized in Table 2.

In the statistical analysis of 8-OHdG concentrations, G3 and G3 + TQ groups were decreased according to control, TQ and G1 and G2 groups ($p \leq 0.001$).

There were no significant differences between the groups based on caspase 3 and 9 activities. Caspase 8 enzyme activity analysis demonstrated that the levels in TQ, G3, TQG1, TQG2, groups were significantly higher than control group ($p \leq 0.002$). G2 was significantly lower than other groups. TQ had an augmenting effect on G2 caspase-8 activity. No significant differences were caused by the administration of TQ to glucose groups on G1 and G3. The analysis of M30 protein concentrations identified that the levels in TQ were significantly lower that of the control group ($p \leq 0.002$). There was no difference between the glucose groups.

Discussion

Nigella sativa, which is commonly used in phytotherapy, has been used in the treatment of several diseases such as colds, headache, rheumatism and several others in both seed and oil form for thousands of years. Thymoquinone, known as the active ingredient of *Nigella sativa*, was shown to have anti-diabetic, anti-oxidant, antihistaminic, anti-inflammatory, anti-microbial, antitumor and immunomodulatory properties (2,3,13,14).

Several studies were investigated to determine the role of TQ in the treatment and prevention of diabetes and diabetic complications (10,11). TQ has a significant regulatory effect on blood glucose, insulin, free radical, plasma cytokines and lipid metabolism and levels (15). It was reported that TQ treatment normalize hepatic enzyme activities, glucose and HbA (1C) levels and prevent anti-hyperglycemic effects in experimental diabetes (12).

Reactive oxygen species (ROS) that were demonstrated to increase under hyperglycemic conditions in various studies have cytotoxic effects. To prevent and stabilize ROS-induced oxidative stress and lipid peroxidation, it is necessary to administer exogenous substances to supplement the existing antioxidant potential of the organism. The application of TQ and *N. sativa*, which are significant antioxidants, has a major impact on cell viability and sustenance of healthy cellular functions (16).

In a study on the use of TQ against hypoinsulin and hyperglycemia in diabetic rats, it was reported that TQ had antioxidant, anti-inflammatory and antidiabetic effects based on the concentration of administration (17,18).

TQ was reported to have beneficial effects on prevention of DNA damage induced by various factors. Studies were conducted on the effects of TQ on apoptotic pathways in addition to its several other effects. It was reported that TQ had apoptotic effects on HL-60 cells (19,20).

In the present study, the statistical analysis conducted on oxidative DNA damage biomarker (8-OHdG) demonstrated that there were no statistical changes between the TQ and glucose treated groups. Furthermore, the reduction in 8-OHDG levels in the groups treated with glucose and TQ combinations, where oxidative DNA damage increased, was noted and considered to reflect the protective effect of TQ.

TQ, an antioxidant and anti-apoptotic substance, affects apoptosis in normal cells (21). Previous studies demonstrated that TQ had both activation and inhibition effects on apoptotic pathways in cell lines depending on the cell type, concentration and time (22,23). TQ also acts by suppressing the expression of proteins responsible for cell proliferation and vitality (24,25).

In the present study, concentrations of M30 protein, an apoptotic biomarker, were analyzed for the assessment of apoptosis and it was found that the concentration was lower in TQG1 and TQG2 groups compared to the control ($p \leq 0.002$). It was observed that administration of TQ led to an increase especially in the low concentration glucose group.

In the present study, analysis of caspase enzymes demonstrated that the differences between the groups based on caspase 3 and 9 activities were not statistically significant. Based on caspase 8 enzyme activities, TQ, G3, and TQG1, TQG2, and combination group levels were significantly higher than control, and, TQ supplementation increased the activities in G1 and G2 concentrations and as a result triggered apoptosis. It was considered that this might have led to the possible strengthening of apoptosis that could possibly occur due to the current glucose concentrations by TQ.

In explaining cellular death, ROS and apoptosis mechanisms are important. Treatment of Caov-3 cells with TQ induces plasma membrane permeability and reduces the mitochondrial membrane potential (26). In PC12 cells exposed to hypoxia, caspase 3 was significantly activated and Vcl-2 was over exposed and necrosis and apoptosis were observed (27). Apoptosis is induced by the increase in oxidative stress production, and TQ administration increases this factor. TQ is also active in the mitochondrial ROS production (28, 29).

Several antioxidants decrease oxidative stress-induced cellular damage by increasing endogenous defense capacity (30). TQ plays an important part in this process. However, there are also studies showing that TQ administration does not always result in the strengthening of the antioxidant system. In a study on cytogenetic and genotoxic effects of TQ administration in primary rat hepatocyte cultures, GSH depletion and liver damage were demonstrated. Although this does not fit in the current knowledge on antioxidants, it was reported that high TQ concentrations consume antioxidant enzymes, leading to DNA damage, which is probably caused by increased ROS production and oxidative stress (31).

Furthermore, it was reported that TQ might also exhibit a protective effect by inhibiting oxidative stress. Studies also demonstrated that TQ inhibited iron-dependent microsomal lipid peroxidation by accumulating superoxide anions due to its antioxidant properties (10,32).

It was determined that TQ decreased DNA fragmentation exhibited by Comet assay in PC12 cells with

serum/glucose deprivation (SGD) induced apoptosis and thus, demonstrating anticancer and anti-apoptotic effects. In PC12 cells with H_2O_2 induced experimental apoptosis and DNA damage, it was reported that caspase 3 activities were increased and following the inhibition of caspase 3 activity, apoptosis was inhibited as well. In the inhibition of PC12 cell death, it was reported that Bcl-2 was overexpressed, the number of apoptotic cells and Caspase 3 and 9 activities decreased significantly (33).

In conclusion, it was determined that oxidative DNA damage (8-OHDG) increased in TQ and glucose-treated groups compared to the control in the present study. It decreased with TQ application, and this data was considered significant in demonstrating the protective effect of TQ. There was no statistically significant difference between the groups based on caspase 3 and 9 enzyme activities. TQ+glucose groups exhibited induced apoptosis via caspase 8 and the probably the apoptosis was induced based on the present glucose concentrations. Based on the Caspase 8 and M30 protein findings, it could be argued that the administration of TQ had a beneficial effect on the glucose groups, especially at low concentrations.

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Interest conflict
The author declares no conflict of interest, financial or otherwise.

Author's contribution
A.F.G, S.D., V.Y., S.C. and M.T. contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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