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EXPERIMENTAL STUDY

Investigation of the protective effect of thymoquinone of U87 cells ınduced by beta-amyloid

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ABSTRACT
BACKGROUND: Thymoquinone (TQ) is the active compound extracted from Nigella sativa which has been reported to possess various pharmacological attributes. This study shows that Thymoquinone (TQ) is an Alzheimer's disease (AD) model cell line on amyloid-beta (Aβ) -induced U87 (human astrocytoma cell line) in β-amyloid (Aβ)-induced in vitro Alzheimer's Disease (AD) model.
AIM: We aimed to investigate the effects on antioxidant and apoptotic pathways.
METHODS: In the study three groups were formed, the control group, the Aβ group, and the Aβ+TQ group obtained by adding TQ to the Aβ group. Firstly, the cytotoxic potential of TQ in U87 cells was investigated by the colorimetric MTT (3-4,5-dimethyl-thiazolyl-2,5 diphenyltetrazolium bromide) test. To determine the antioxidant status in the cell line treated with Thymoquinone, to examine the effects of superoxide dismutase (SOD) and catalase (CAT) activities, on apoptosis Caspase-3 protein levels were measured by ELISA method.
RESULTS: When compared to the control group, the SOD and CAT levels were significantly decreased in the U87 cell line exposed to Aβ; Caspase-3 levels were found to increase significantly. However, application of TQ to the Aβ-U87 cell line significantly increased SOD and CAT levels; it was found that it decreased the caspase-3 level.
CONCLUSION: In in vitro experiments, we determined that TQ has a protective effect by increasing antioxidant parameters in the amyloid beta-induced cell line *(Fig. 4, Ref. 41)*. Text in PDF *www.elis.sk*
KEY WORDS: thymoquinone, amyloid-beta, U87, SOD, CAT, caspase-3.

Introduction

Alzheimer's Disease (AD) is believed to be one of the most prevalent neurodegenerative diseases known and is characterized by a variety of symptoms, such as memory loss, decreased cognitive function, abnormal behavior, and psychiatric problems. Even though the exact cause of AD is not known, advanced age, hereditary factors, death of brain cells, impairment of neural conduction, and various toxic substances are thought to trigger the disease. Neuritic plaques and neurofibrillary tangles show pathological features of AD. AD is characterized by abnormal beta-amyloid (Aβ) accumulation in brain tissues, death of cholinergic neurons, tangles caused by hyperphosphorylation of microtubule-associated tau protein, dyshomeostasis of metals, such as copper, iron, zinc and aluminum, metal-induced oxidative stress, and various other factors. It is multifactorial with an unknown etiology, characterized by various pathological symptoms (1, 2, 3, 4).

There are studies reporting that factors like genetic mutations, synapse loss, aging, and the accumulation of biometals, such as iron, copper, and zinc, might be responsible for the pathophysiology of AD. However, the most widely accepted point of view is that oxidative stress and amyloid plaques in the extracellular area of the brain and Neurofibrils in neurons are most important. It is the classical theory based on the formation of tangles (5, 6, 7).

Aβ causes neurotoxic effects by triggering an increase in reactive oxygen/nitrogen products, resulting in loss of mitochondrial function, decreased ATP production, impaired intracellular calcium homeostasis, neuronal loss, and synapse, dendrite, and axon degeneration (8, 9).

The increased production of Aβ in the brain of Alzheimer's patients compared to a normal individuals leads to the formation of amyloid plaques in the brain tissue, which could be demonstrated histologically (10, 11, 12).

Currently, the most prominent targets for therapeutic intervention include the inhibition of amyloid precursor protein (APP) and Aβ production by blocking Aβ aggregation and the resulting inflammatory response and inhibiting Aβ-induced neurotoxicity. Age-related memory impairments have been depicted to be associated with decreased antioxidant mechanisms in the brain and plasma. The interaction of Aβ42 plaques with free radicals and the oxidative stress as a result of it may play a pivotal role in AD pathogenesis (13, 14).

The use of herbal medicines as an alternative approach is on an increasing trend due to the insufficiency of traditional medi-

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cines and the side effects of synthetic medicines. Thymoquinone (TQ) is the active compound extracted from Nigella sativa. Several pharmacological actions of TQ have been investigated, including anti-tumor, anti-histaminic, anti-inflammatory, and antioxidant effects and neuroprotective properties. Therefore, it could be a good candidate for the recovery of Alzheimer`s Disease (AD) pathology rather than current symptomatic relief (15–19).

It is thought that TQ could be utilized effectively for the treatment of Alzheimer's Disease and we anticipate that it could be evaluated among alternative treatment strategies in the field of medicine.

Materials and methods

Cell viability

Cell count was carried out for the U87 cell line and the viability of the cells was measured with trypan blue excretion test. By placing 10 µL of the cells + 10 µL of trypan blue on a slide, the viability was determined counting the cells that did not receive dye under the light microscope. The percentage of non-staining cells was calculated by counting 100 cells in the area.

Cell culture

Later, the cells were prepared with a medium at 104 cells per mL in the cell line. For each complex, 3 mL of cell suspension was added to 7 wells in a separate 12-well culture plate. All the plates were left to incubate for 24 hours in a CO2 incubator at 37 °C in an environment containing 5% CO2. Following the incubation, the cell concentrations we added to the 12-well plates, which comprised of U87 cell series, were added to the first well cell and medium, and the second well was determined by determining the solvent containing DMSO as a control. Active ingredients were added to the well at five different concentrations (200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM). The plates were again incubated in a CO2 incubator for 48 hours.

Working groups

Control group: 50 µl of saline was added to the medium of differentiated U87 cells and incubated for 48 hours. At the end of this period, Dimethyl Sulfoxide (DMSO) was added to the medium of the cells in a final concentration of 0.1 % and the cells were incubated for another 24 hours in the incubator.

Aβ group: Aβ1-42 was added to the medium of the differentiated U87 MG cells at a concentration of 5 µM and incubated for 48 hours. At the end of this period, DMSO was added to the medium of the cells in a final concentration of 0.1 % and the cells were incubated for another 24 hours in the incubator.

Aβ + TQ group: The differentiated U87 cells were added to the culture medium at a final concentration of 5 µM, 48 hours after Aβ1-42 application. 300 µmol/L Thymoquinone was added and the mixture was incubated for another 24 hours.

Cytotoxicity: At the end of the 48 hours, U87 cell line cells were transferred to 96 well plates with 100 µL of cell suspension per well and 10 µL of MTT solution was added to each well and incubated for 4 hours. The MTT solution was prepared by dissolving it in PBS as 5 mg/mL and transferring it to a bottle with sterile filtration. Afterwards, 100 µL of DMSO was added to each well and the well was kept in a CO_2 incubator at 37 °C for 10 minutes to dissolve the formazon crystals formed by MTT. Each well was read at 570 nm wavelength with a microplate reader and the cytotoxicity level was determined according to the absorbance value read. Their cytotoxicity levels were calculated employing the following formula.

1- (the absorbance of the test pad - the absorbance of the control pad/the absorbance of the test pad) x 100 the concentration with 50% cytotoxic effect relative to the control was accepted as the cytotoxic dose.

ELISA (Enzyme Linked Immunosorbent Assay) Test: To evaluate the antioxidant status in the cell line treated with TQ, SOD, CAT activities, and caspase-3 protein levels were measured based on ELISA method to examine its effect on the apoptosis process. Experimental protocols of ELISA kits vary for each kit.

Results

MTT test

The effect of thymoquinone-administered human brain cell line U87 (glioblastoma astrocytoma) on cell viability and proliferation was also investigated. Following the application of Aβ1-42, the number of viable cells decreased in all the groups except for the control group ($p < 0.05$). After the application of Timokino on U87 cells, there was a significant statistical increase in the number of cells in the Aβ + TQ group ($p < 0.05$) (Fig. 1). According to these results, it was determined that TQ increased cell survival in the *in vitro* AH model.

SOD activity

The application of amyloid-B to the cells significantly decreased the SOD (U/mg protein) enzyme level in all the groups except for the control group ($p < 0.05$). The SOD enzyme level increased significantly in the Aβ + TQ group after Timoquinone was applied on the U87 cell ($p < 0.05$) (Fig. 2). It has been determined that TQ triggers the antioxidant enzyme mechanism.

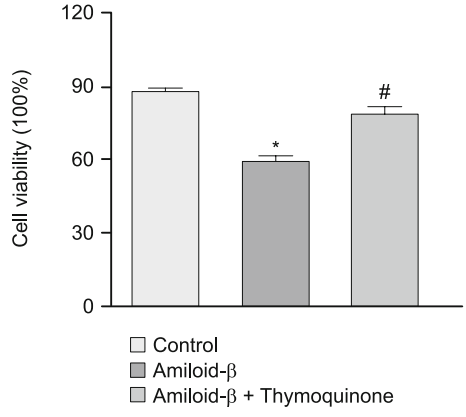

Fig. 1. Cell viability of the groups with TQ added versus Aβ1-42 application.

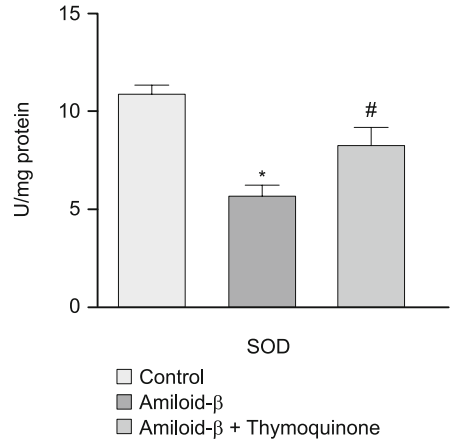

Fig. 2. SOD enzyme (U/mg) level values in the in vitro AH model cell lines.

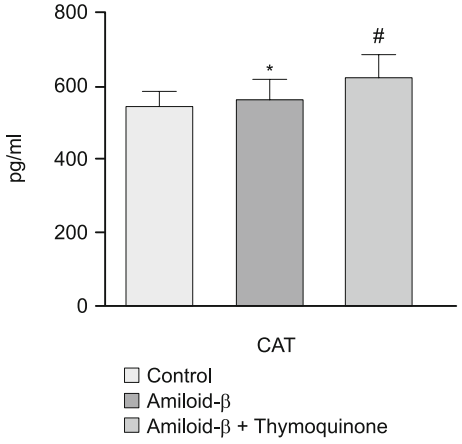

Fig. 3. CAT enzyme (pg/ml) level values in *in vitro* AH model cell lines.

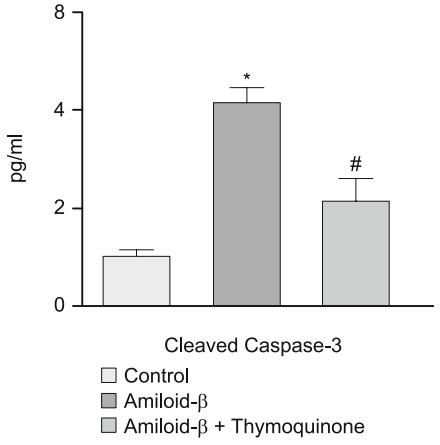

Fig. 4. Caspase-3 enzyme (pg/ml) level values in *in vitro* AH model cell lines.

CAT activity

Our findings indicated that CAT enzyme level (pg/ml) significantly decreased after Aβ1-42 administration in all the groups except for the control group ($p < 0.05$). We observed a significant statistical increase in CAT enzyme level in the Aβ + TQ group after Timoquinone was applied on the U87 cell ($p < 0.05$) (Fig. 3). Moreover, the results implied that TQ triggers the antioxidant enzyme mechanism.

Caspase-3 activity

The application of amyloid-β to the cells significantly increased the Caspase-3 enzyme activity (pg/ml protein) in all the groups except for the control group ($p < 0.05$). Caspase-3 enzyme activity was found to be statistically and significantly lower in the Aβ + TQ group after Timokinoon was applied on the U87 cell ($p < 0.05$) (Fig. 4).

Discussion

Reactive oxygen species (ROS) containing hydroxyl radicals (OH.), Superoxide anion, and hydrogen peroxide (H_2O_2) are considered to be of great importance in the formation of cancer and various diseases by acting on cell metabolism. Reactive oxygen species (ROS) are generated during aerobic cellular reactions and are effectively cleared by the cell's detoxification defense system, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in order to maintain redox homeostasis. However, once ROS production exceeds the cell detoxification capacity, the overproduced ROS can directly alter lipid, protein, or DNA and signal transduction pathways resulting in an irreversible oxidative modification (20, 21, 22).

The oxidative stress caused by the disruption of the balance between the production of reactive oxygen species (ROS)/reactive nitrogen species (RNS) and the antioxidant defense mechanism is another major factor implicated in the pathogenesis of Alzheimer's. Excessive accumulation of free radicals, the increase in protein oxidation, and lipid 71 peroxidation level due to the changes in the redox state in the brains of patients with Alzheimer's Disease appear as a common pathological features of AD. However, studies have shown that oxidative stress leads to neuronal cell dysfunction, Aβ accumulation, cell death, and cognitive dysfunction. This indicates that oxidative stress occurs in the early stages of AD. It is also known that Aβ causes mitochondrial damage by increasing ROS production in the brain. Therefore, antioxidant therapy is considered as a new therapeutic approach to the prevention and symptomatic treatment of AD23-27. In our study, we aimed to examine the effects of TQ on the human brain cell line U87 (glioblastoma astrocytoma), which is an Alzheimer's model. The MTT method was employed in order to examine the effects on cell proliferation and vitality and investigate the effects on the antioxidant defense system, superoxide dismutase (SOD), and catalase (CAT) activities. Caspase-3 protein levels were measured with ELISA method to examine the effects on the apoptosis process. In some studies, a difference has been reported between AD and controls in terms of SOD, yet it was not statistically significant. On the contrary, other studies have shown that the SOD level increased significantly.

According to the obtained data herein, amyloid-B administration significantly reduced SOD activity on human brain cell line U87 (glioblastoma astrocytoma) cells compared to the controls. We found that TQ application significantly augmented this decrease and implied an antioxidant effect. In the studies in the literature, different results were obtained with the change of SOD expression and activity in patients with Alzheimer's Disease. On top of the findings showing a significant decrease in SOD activity in the cerebral cortex and hippocampus, there are researches indicating no differences in SOD activity compared to controls. It has been determined that TQ triggers the antioxidant enzyme mechanism (28–32).

The results herein illustrated that CAT enzyme level (pg/ml) significantly decreased after Aβ1-42 administration in all the groups except for the control group ($p < 0.05$). Moreover, we observed a significant statistical increase in CAT enzyme level in the Aβ + TQ group after Timoquinone was applied on the U87 cell $p < 0.05$) (Fig. 2). It has been determined that TQ triggers the antioxidant enzyme mechanism.

In addition, the increase in the level of antioxidant enzyme CAT in the *in vitro* AD group compared to the control and its decrease with TQ application, along with the increase in the free radicals in AD, is indicative of an increase in enzymatic antioxidant activation in order to eliminate the toxic effects of these radicals. The oxidative stress induced by Aβ and the antioxidant defense mechanism associated with them are accepted as basic mechanisms in the etiology and pathogenesis of Alzheimer's. As a result of the disruption of the balance between antioxidant and oxidant systems, free radicals emerge. It protects the cell against oxidative stress by eliminating these radicals, which are SOD, GSH-Px, CAT; they are endogenous enzymes involved in the antioxidant defense mechanism(34-36).

Caspase-3, a protease, is thought to be an important driver caspase in apoptosis. The creation of active caspase-3 performs an irreversible step of the apoptotic pathway. Numerous studies have revealed high caspase-3 levels in AD. Caspase-3 was analyzed in the study groups, representing apoptotic pathways. We found that caspase-3 level increased significantly in U87 cell lines exposed to Aβ compared to that in the control group(36-41).

Conclusion

U87 supports the antioxidant defense system in the Alzheimer's cell line model, significantly increasing the levels of CAT and SOD while decreasing the level of caspase-3, the apoptotic pathway enzyme. TQ represented antioxidant activity and decreased neurodegeneration due to apoptosis. Therefore, the treatment with TQ could be suggested as a novel therapeutic approach to the prevention and symptomatic treatment of Alzheimer's.

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