Original Article

Regulatory Effects of Thymoquinone on Dopamine Level in Neuronal Cells Exposed to Amphetamine: an in Vitro Study

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Abstract

Background: Amphetamine (AT) is a potent central nervous system stimulant that is capable of producing damaging effects to the central dopaminergic pathway; used both for medical purposes and also abused recreationally. One of the potential naturally occurring compounds is thymoquinone (TQ), an active compound of Nigella sativa; which is known for its cellular protective effects. The objectives of this study were to determine the IC50 values of AT and TQ on differentiated SH-SY5Y neuronal cells and to evaluate the changes of dopamine (DA) level in the cells exposed to AT after co-administering with TQ.

Materials and Methods: Differentiated SH-SY5Y cells were grown in a cell culture flask containing DMEM/F12 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. The IC50 value of TQ and AT in differentiated SH-SY5Y cells was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The DA level was determined by using the Enzyme-Linked Immunosorbent Assay (ELISA) kit.

Results: The IC50 values of AT and TQ were 1596 µM and 926 µM, respectively. Co-administration of 40 µM of AT and 30 µM of TQ demonstrated a significant increase in DA level at 48 hours of exposure when compared to the administration of AT group ($P \leq 0.05$).

Conclusion: These findings suggested that TQ has a role in maintaining the DA activity after long-term AT exposure.

Keywords: amphetamine, thymoquinone, differentiated SH-SY5Y cells, dopamine, lactate dehydrogenase activity

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Introduction

Amphetamine (AT) is a potent central nervous system stimulant that belongs to the psychoactive drugs group. It has been used as a treatment for several disorders such as attention deficit hyperactivity disorder (ADHD), narcolepsy, depression, and minor obesity (1). However, the illegal use of AT has gained popularity as a recreational drug among youths and teenagers due to its psychostimulant effects (2).

Administration of AT causes elevation of dopamine (DA) levels in the body. DA is a primary neurotransmitter involved in reward pathways for movement regulation, working memory, and euphoric effects (3,4). The elevation of DA level causes the DA systems to become dysfunctional and leads to DA depletion after a long-term AT exposure (5). The damaging effects of AT administration will be manifested as restless sleep, mood swings, and depression (6) as well as anxiety and fatigue (7). Overdose and uncontrollable consumption of AT will also lead to several medical disorders such as heart and kidney problems, increased risk of stroke, and neurotoxicity (6).

Antipsychotic drugs such as butyrophenone and benzodiazepine are reported as the options for pharmacological treatment of AT adverse effects (8). Unfortunately, antipsychotic drugs are associated with neurological side effects, including acute extrapyramidal syndromes (parkinsonism, dystonias), sedation, and hypotension (9). Apart from looking into a modern biological treatment, natural traditional remedies are also an alternative solution in reducing the effects of a drug used (10). Natural traditional medicines are used by a lot of people worldwide who depend primarily on these alternative medicines as their method to sustain their healthcare (11).

One of the sources of traditional medicines that has a high potential in treating several diseases is thymoquinone (TQ). TQ is a bioactive compound found in several plants including Nigella Sativa and Monarda fistulosa which provides multiple healthy medicinal properties (12,13,14,15). Previous studies discovered that TQ possessed antioxidant, anti-inflammatory, anti-cancer, as well as neuroprotective properties (16,17). The TQ was found to have a neuroprotective effect on primary dopaminergic neuronal cells against 1-methyl-4-phenylpyridinium (MPP+) and rotenone toxicities by preserving the tyrosine hydroxylase (TH) immunoreactive cells, most likely via its antioxidant properties (18).

Furthermore, TQ was found to affect neurotransmitters in counteracting the effect of arsenic (AS) induce toxicity. The TQ reduced the effects of AS by increasing the levels of DA, norepinephrine (NE), acetylcholine esterase (AChE), and decreased the levels of nitrite/nitrate (NO), serotonin (5-HT), lipid peroxidation (MDA), and tumor necrosis factor (TNF-α). The TQ acted as an antioxidant in this event (19).

Considering the benefits of TQ as described previously, this study was conducted to investigate the effects of TQ on the DA level in neuronal cells exposed to AT. For this purpose, the human neuroblastoma SH-SY5Y cell line was used as this cell line expresses neuronal markers such as DA and DA transporter (DAT). It also expresses different neuron-specific proteins as possessed by mature neuronal cells after being induced to undergo differentiation using retinoic acid (20,21,22). Hence, the differentiated human neuroblastoma SH-SY5Y cell line is a suitable in vitro model to be used to study the effects of AT on neuronal cells after AT exposure.

Methods

Experimental Design: This study was an in vitro experimental laboratory study. The purpose of this study was to investigate the effects of TQ on the DA level in differentiated SH-SY5Y cells exposed to AT. This study was done at the Chemical Laboratory and Cell Culture Laboratory, Faculty of Medicine, University of Sultan Zainal Abidin (UniSZA). This study was divided into 3 phases. In phase 1 of the study, differentiation of SH-SY5Y cells was carried out and the IC50 values of TQ and AT in differentiated SH-SY5Y cells were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Three concentrations of AT and TQ were determined for the second phase of the study. In this phase, the DA level for the selected concentrations of AT and TQ was determined using Enzyme-Linked Immunosorbent Assay (ELISA) kit. One optimum concentration of AT and TQ was later be determined from the result for further study in phase 3. In the phase 3 study, four experimental groups of differentiated SH-SY5Y cells were used which comprised the control group, AT group, TQ group, and co-administration of AT and TQ group. The concentration of DA level for each group was measured after incubating the cells with respective substances for 3, 12, 24, and 48 hours (Jamil et al., 2013). Finally, statistical analysis was carried out using Graph Pad Prism 6.

Reagents and Chemicals: The SH-SY5Y cell line was purchased from American Type Culture Collection (ATCC), Virginia, USA. Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), fetal bovine serum (FBS), phosphate-buffered saline (PBS), neurobasal medium, B27 supplement, trypsin EDTA, glutaMAX, and penicillin/streptomycin were obtained from Gibco (Invitrogen, USA). Dimethyl sulfoxides (DMSO) were purchased from Merck (Germany). Thymoquinone (>99% purity), trypan blue, MTT powder were purchased from Sigma-Aldrich (USA). Retinoic acid (RA) was purchased from Nacalai Tesque, Japan, and amphetamine were purchased from Arlesheim, Switzerland. The dopamine ELISA kit was obtained from Elabscience, Texas, USA.

Cell Culture Preparation and Differentiation of SH-SY5Y Cells: The SH-SY5Y cell line was grown in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin. The media was changed every 3 days and incubated at 37°C in a humidified atmosphere of 5% CO2. After the cells reached 70-80% confluency, the growth media was discarded from the flask and was replaced with neurobasal medium (containing B27 supplement and GlutaMAX) and 10 µM of RA to promote differentiation and neuronal phenotype for 5 days. The growth media was replaced every 48 hours. The differentiation of the cells was monitored microscopically for their morphological changes. Once the SH-SY5Y cells completed the differentiation, the cells exhibited long neurites with branched processes, reminiscent of dendrites or axons (23,24).

Determination of IC50 Value by MTT Assay: Determination of IC50 values of TQ and AT was evaluated using MTT assay as previously described by Mosmann, (1983) (25). Briefly, 2×105cells/ml 2×105 of cells in 100 µL growth media per well were seeded in a 96-well plate. Then, the well plate was incubated for 24 hours in 5% CO2 and the differentiation method was carried out as described previously. Following cell differentiation, serially diluted solutions of TQ (0, 95, 190, 381, 761, 1523, 3045, and 6090 µM) and AT (0, 116, 231, 462, 925, 1849, 3698, and 7396 µM) were prepared following the previous study from Al-Sheddi et al., (2014) (26). A 100 µL of each dilution was transferred to the cells in a 96-well plate and incubated for 72 hours. Subsequently, 20 µL of MTT (5 mg/mL) was added to the cells in the dark and incubated for 4 hours, covered in aluminum foil. After the incubation, all the mixture in each well was removed and 100 µL of DMSO was added to each well to dissolve the formazan crystals formed. The absorbance was immediately read by using the Tecan ELISA microplate reader (Infinite M200 Pro) at a wavelength of 490 nm as a measurement wavelength and using 630 nm as a reference wavelength. The potency of cell growth inhibition for the test agents was expressed as the half-maximal (50%) inhibitory concentration, IC50. Cell viability rate was calculated as the percentage of MTT absorption as follows (27):

50% of maximal Inhibition = [(mean of experimental absorbance/mean of control absorbance x 100%]

Cell Culture and Exposure to Amphetamine and Thymoquinone: Differentiated SH-SY5Y cell was used to measure the effect of TQ on the DA level. The SH-SY5Y cells were maintained in cell culture flask with DMEM/F12 medium containing 10% (v/v) FBS and 1% (v/v) pen/strep. The SH-SY5Y cells were grown as a monolayer in a humidified incubator supplemented with 5% CO2 at 37°C. Next, the cells were treated with AT and TQ to determine an optimum concentration for each substance. The measurement of DA level was carried out in phase 2 and phase 3 of the experiment as described previously. In phase 2 of the study, the result from the MTT assay was used to select the concentration below the IC50 value. Then, the range of safe concentration was determined to be used for the subsequent experiment. Firstly, the growth media was replaced with a fresh growth media containing the following concentrations of AT (10, 40, and 180 µM) and TQ (5, 30, and 150 µM). After that, DA level measurements for three concentrations of AT and TQ were conducted by incubating the differentiated SH-SY5Y cells for 3 hours (short-term effect) and 48 hours (long-term effect) (28). This step was done to demonstrate the DA level changes in the concentration of AT and TQ. One optimum concentration for each substance was determined from the result for further study in phase 3. Following that, phase 3 of the experiment was conducted with four experimental groups of differentiated SH-SY5Y cells comprising of the control group, AT group, TQ group, and co-administration of AT and TQ group. These groups were incubated for 3, 12, 24, and 48 hours

following Jamil et al., (2013) (29) with some modification before measuring the DA level from the supernatant.

Measurement of Dopamine Level: All groups in phase 2 and phase 3 of the experimental design were assayed for the DA concentration after incubation in differentiated SH-SY5Y cells by using the ELISA kit. (Elabscience, Houston, Texas). The procedure for this assay was applied according to the commercially available protocol from the DA ELISA kit user manual. Briefly, the growth media of the cells was removed and the cells were washed with a moderate amount of pre-cooled PBS. After that, the cells were dissociated by using trypsin, and the cell suspension was collected into a centrifuge tube containing growth media. The cell suspension was later centrifuged at 1000xg at 4°C for 5 minutes. The growth media was discarded and the cells were washed with pre-cooled PBS three times. Then, 1 mL of pre-cooled PBS was added to keep the cells in suspension and the cells in PBS were stored at -20°C. After two freeze-thaw cycles, the cell lysates were centrifuged at 1500xg at 4°C for 10 minutes. The supernatant of the cell lysates was added to the biotinylated detection antibody Ab working solution. This mixture was incubated in antibody-coated wells for 45 minutes at 37°C. Subsequently, the solution from each well was aspirated and 350 µL of buffer was added to each well. The microplate was patted to dry using clean absorbent paper. This wash step was repeated 3 times. Next, 100 µL Horseradish Peroxidase (HRP) conjugate was added to each well and incubated for 30 minutes at 37°C. The wells were then washed 5 times using the same washing steps as described previously. A 90 µL substrate reagent was added to the wells in the next step and the wells were incubated for 15 minutes at 37°C. The reactions were stopped by adding 50 µL stop solution and the results were determined using a microplate reader set at 450 nm.

Statistical Analysis: The determination of cell viability was calculated based on the measurement of wavelength at 490 nm and the reference wavelength of 630 nm using non-linear regression analysis. Results were presented as mean ± standard error mean (SEM). For grouped comparison in investigating the DA level, statistical analysis was determined using a one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test. P-value of less than 0.05 was considered as statistically significant. All the data were presented graphically in the form of graph and figure using a computer software program of Graph Pad Prism 6 (Graphpad Software Inc., San Diego, California, USA).

Results

The IC50 Values of Thymoquinone and Amphetamine in Differentiated SH-SY5Y Cells: Following the MTT assay, the dose-response curve was prepared by plotting the percentage of viable cells against the concentration of the compound. As shown in Figure 1(a) and (b), the IC50 values for TQ and AT were 926 µM and 1596 µM respectively. From this result, the range of concentrations below the IC50 values required for the subsequent experiment was identified. The AT concentrations of 10, 40, and 180 µM (1, 5, and 25 µg/mL) and TQ concentrations of 5, 30, and 150 µM (1, 5, and 25 µg/mL) were chosen as these concentrations showed cell viability of nearly 100% and less affected by the treatment (29).

Determination of Optimum Concentration of Thymoquinone on Dopamine level: for determination of the optimum concentration of amphetamine on dopamine level, the analysis was conducted to determine an optimum concentration of AT and TQ in differentiated SH-SY5Y cells. This analysis was carried out to observe and validate the optimum concentration of AT and TQ that cause an optimum increase in DA level in the cells. Differentiated SH-SY5Y cells were exposed to three different concentrations of AT (10, 40, and 180 µM) and TQ (5, 30, and 150 µM) for 3 hours and 48 hours. The result showed that at 3 hours of incubation, AT concentrations of 40 and 180 µM produced a significant increase in DA level compared to the control group ($P \leq 0.05$).

Whereas, at 48 hours of incubation all concentrations of AT (10, 40, and 180 µM) showed a significantly decreased level of DA ($P \leq 0.05$) ($P \leq 0.01$). Thus, the AT concentration of 40 µM was chosen for the subsequent analysis as the concentration significantly increased the DA level after the short-term effect (3 hours) and significantly caused DA level depletion after the long-term effect (48 hours)

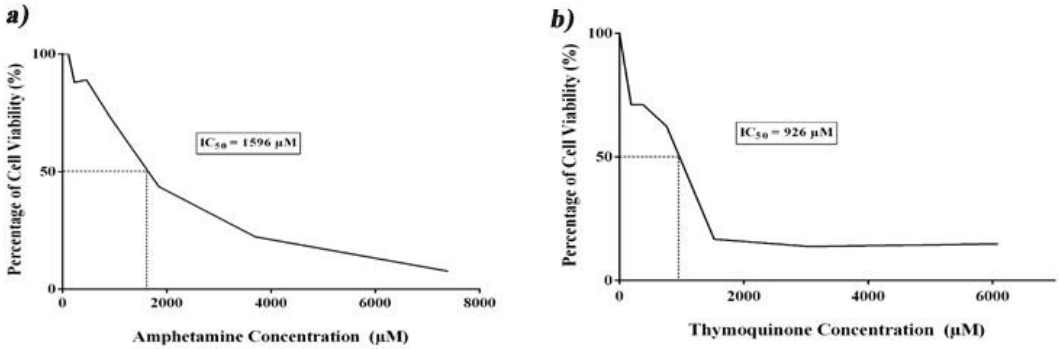

Figure 1. The IC_{50} values of AT (a) and TQ (b) in differentiated SH-SY5Y cells. The cells were exposed to series of AT (0-7400 µM) and TQ (0-6100 µM) concentrations and incubated for 72 hours. The IC_{50} values of TQ and AT were 926 µM and 1596 µM respectively. Data represent the mean ± SEM of three independent experiments.

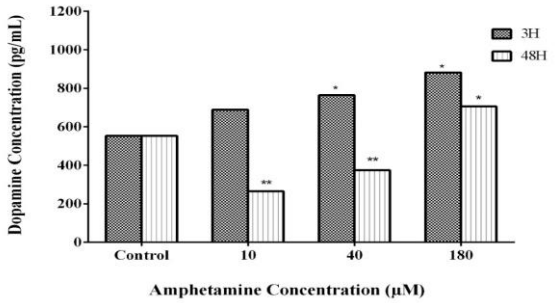

Figure 2. Dose-Response Effect of Amphetamine on Dopamine Level in Differentiated SH-SY5Y Cells at 3 and 48 hours of incubation. Statistical analysis was carried out using One-way ANOVA Followed by Dunnet's Multiple Comparisons Test. **P*≤0.05 vs Control Group, ***P*≤0.01 vs 3 Hours Group.

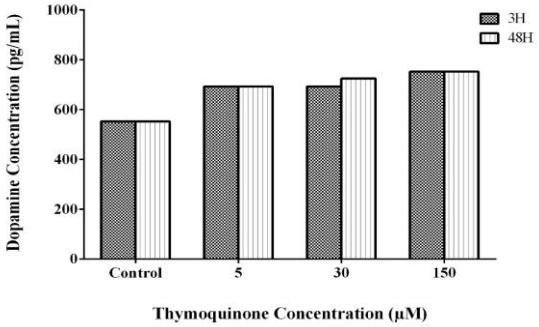

Figure 3. Dose-Response Effect of Thymoquinone on Dopamine Level in Differentiated SH-SY5Y Cells at 3 and 48 Hours of Incubation. Statistical Analysis was Carried out Using One-way ANOVA Followed by Dunnet's Multiple Comparisons Test.

compared to the control group (Oliveira et al.). The percentage of DA level changes (decrease) between 3 hours and 48 hours after exposure to 40 µM of AT is also high (51%) (Figure 2).

Determination of Optimum Concentration of Thymoquinone on Dopamine level: A further experiment was conducted to investigate the optimum concentration of TQ on the DA level. Exposure of cells to TQ for 3 hours exhibited an increased level of DA in all groups compared to the control group, although the difference in the level is not significant among the treated groups. Cell exposure to TQ for 48 hours showed no significant changes in the level of DA compared to groups exposed to TQ within 3 hours of exposure (Figure 3). This indicated that TQ does not produce damaging effects on the DA system. Since there is no significant difference in the level among the different concentrations of TQ, a concentration of 30

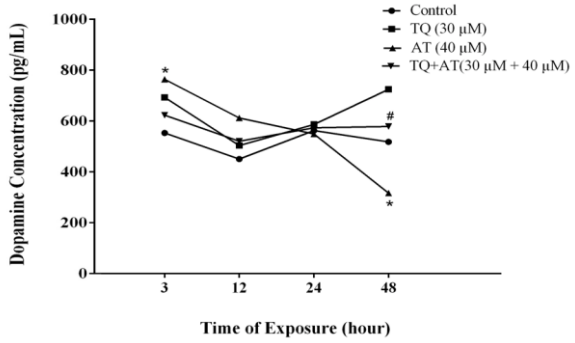

Figure 4. The Changes in Dopamine Level Against the Time of Exposure in the Experimental Groups. Differentiated SH-SY5Y Cells were Exposed with Amphetamine (40 µM), Thymoquinone (30 µM) and Co-administration of Thymoquinone (30 µM) and Amphetamine (40 µM) for 3, 12, 24, and 48 hours. One-Way Analysis of Variance (ANOVA) was Used to Determine the Significance between Groups Followed by Dunnet's Multiple Comparisons test. **P*≤0.01 vs Control Group, #*P*≤0.01 vs AT Group.

µM was chosen for subsequent analysis taking reference from a study by Babazadeh et al. (30).

The Effect of Dopamine Level after Co-Administration of Amphetamine and Thymoquinone: In this study, the effect of DA level following co-administration of TQ and AT was examined for 3, 12, 24, and 48 hours of incubation. To determine the effect of TQ in counteracting the depletion of dopamine levels produced by AT, differentiated SH-SY5Y cells were co-administrated with 40 µM of AT and 30 µM of TQ. Three other groups used in this study were the control group, AT group, and TQ group. For the control group, the pattern of the graph showed a slightly decreased DA level at 3 hours and 12 hours of incubation. Later, it showed a slightly increasing level at 24 hours followed by a slightly decreasing level at 48 hours of incubation.

Exposure to AT significantly increased DA level at 3 hours of incubation ($P \leq 0.05$) compared to the control group. Later, it showed significantly decreasing levels of DA with the lowest seen at 48 hours of incubation when compared to the control group ($P \leq 0.05$). The percentage of DA level decrease between 3 hours and 48 hours is 51%. The administration of TQ showed an increasing level at 3 hours of incubation. Later, it decreased at 12 hours of incubation. After 12 hours, it showed an increasing pattern until 48 hours of incubation. There is no significant difference in the DA level when compared to the control group. Co-administration of AT and TQ showed an increasing level at 3 hours of incubation and slightly decreased at 12 hours of incubation, but later, it showed a significant increase in DA level at 48 hours of incubation when compared to AT group ($P \leq 0.05$). The graph also showed that the pattern of DA level changes of this group (co-administration of AT and TQ) and the control group is similar (Figure 4).

Discussion

Amphetamine is a psychostimulant drug that affects the central nervous system by increasing alertness, energy, and euphoria as a result of an increase in DA concentration (31). Administration of AT caused an excessive accumulation of DA and produced hydroxyl and superoxide radicals that exert toxic effects on the cells (32). Illicit use of AT was reported to give a range of negative side effects on the body. Recently, several natural traditional remedies were reported to have therapeutic effects including thymoquinone. Previous studies demonstrated that TQ exerts a neuroprotective effect against MPP+ and rotenone toxicities on primary dopaminergic neuronal cells (18). TQ also has a role in suppressing oxidative stress and neuropathy in streptozotocin-induced diabetic rats by interacting with neurotransmitters as it preserved the norepinephrine and DA concentrations to control-like values (33).

In the present study, the effects of TQ on the changes of DA level in differentiated SH-SY5Y cells exposed to AT were examined. Initially, the IC50 values of TQ and AT were investigated in differentiated SH-SY5Y cells by using an MTT assay to determine the range of concentrations for both compounds which are going to be used in the subsequent analysis. The MTT assay is used to measure several viable cells following treatment by a certain compound and the procedure was originally established by Mosmann. The result showed that the IC50 values of TQ and AT were 926 µM and 1596 µM respectively. Therefore, the concentration of TQ and AT that were used for the subsequent experiment was chosen to be lower than these IC50 values so that enough viable cells were present for the experiment and the number of cell death due to the treated compounds were very minimal and could be ignored. Thus, a range of concentrations below the IC50 value of AT (10, 40, and 180 µM) and TQ (5, 30, and 150 µM) were used for further analysis. Further investigation on the optimum concentration of AT and TQ on DA level concentration was conducted in this experiment. From the result, AT at a concentration of 40 µM was chosen for the subsequent analysis as the concentration gives a significant increase in DA level after short-term exposure (3 hours) while the DA level was depleted after long-term exposure (48 hours) compared to the control group. The percentage of DA level changes (decrease) between 3 hours and 48 hours after exposure to 40 µM of AT is also high (51%). This finding corresponded with a result from previous studies that showed a depletion of DA level by about 80% after long-term exposure to AT in PC-12 dopaminergic neuronal cells (28).

Although the exact mechanism of AT inducing the damaging effects remains to be elucidated, several studies suggested that it involves several factors. A study conducted by Hotchkiss and Gibb (34) revealed that DA depletion after AT exposure corresponded to reduced activity of tyrosine hydroxylase. The DA depletion was also associated with a decrease in the number of DAT and vesicular monoamine transporter

2 (35). Furthermore, another study revealed that AT caused elevation in DA release that leads to DAT saturation and eventually resulted in DA reduction (36,37,38). The exposure of AT was also associated with high production of reactive oxygen (ROS) and nitrogen species causing a depletion at DA level (39).

Meanwhile, one optimum concentration of TQ on the DA level was determined to be used for the next experiment. Long-term TQ exposure at a concentration of 30 µM preventing a reduction in DA level after AT exposure when compared to the control group. This suggests that TQ can interact with the DA neurotransmitter system and protects the system from dysfunctioning. According to El-Shamy et al. (40), TQ potentially gives prominent effects to neurotransmitter changes after long-term exposure to nicotine as it restored almost all monoamine neurotransmitters including DA, 5-HT, and NE to a value near to the control group. Another study revealed that long-term exposure to TQ can protect primary dopaminergic cultured neurons as it significantly increased the number of neurons by 83 to 100% after exposure to MPP+ and rotenone (18). Hence, by considering a concentration of TQ that was also used in a previous study by Babazadeh et al., 30 µM of TQ concentration was chosen for further analysis of its effects. The effect of co-administration of AT and TQ on the DA level was investigated in this present study. In our knowledge, these findings provide the first evidence of the TQ effect on the changes in DA level after exposure to AT. The result demonstrated that 30 µM TQ potentially prevents depletion in the DA level after the administration of 40 µM AT. It restores the DA level to almost the value of the control group. Thus, TQ has a role to maintain DA activity after exposure to AT. This is due to the properties of TQ that have the capability as scavengers for free radicals or oxidative stress agents (41).

Oxidative stress is associated with depletion at the DA level (42). The purpose of this study is to investigate the TQ potential to counteract the effect of AT exposure. As mentioned by a study from Mansour et al., (43) TQ can act as a free radical scavenger including attenuating the ROS through its antioxidant properties. In the same way, TQ attenuated the ROS generation induced by beta-amyloid peptides in cultured hippocampal neurons (44). Supporting the explanation, a study from Badary et al. (45) reported that TQ also has a great capability to alleviate iron-dependent microsomal lipid peroxidation. The lipid peroxidation acts as an agent that promotes oxidative stress in an in vitro study. This event may be correlated with the ability of TQ to cross the physiological barrier and get access to cellular components, which then act as a radical scavenger (45,46). Besides that, Kassab and El-Hennamy demonstrated an antioxidant action of TQ against AS by reducing lipid peroxidase and MDA levels. Furthermore, it has been shown that pre-treatment with TQ effectively inhibited the degradation of antioxidant enzyme systems including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase in the red blood cells of rats (47). The TQ was reported to prevent inhibition of antioxidant enzymes, SOD, and CAT, as suggested by a study from Cobourne-Duval et al. (48), inactivated BV-2 murine microglial cell lines. They also found that an increase in antioxidant enzyme activity after TQ treatment in the PC-12 cell line exposed to amyloid β-peptide (49). Thus, the present result suggested that TQ protects against neurotransmitter depletion due to its antioxidant properties. The TQ enhances dopaminergic function by preventing the overproduction of free radicals or oxidative stress agents and attenuated degradation of antioxidant enzyme systems.

Conclusion

In summary, these findings concluded that TQ prevents long term DA depletion in neuronal cells after long term exposure to 40 µM of AT. The findings also provide information on the effects of TQ for the prevention of neuronal damage caused by AT exposure. Hence, further study is recommended to discover detailed mechanisms of neuroprotection of TQ following AT exposure.

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Conflicts of Interest

The authors declare that they have no conflict of interest based on this study.

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