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Attenuation of Aβ-induced neurotoxicity by thymoquinone via inhibition of mitochondrial dysfunction and oxidative stress

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Abstract Beta-amyloid (Aβ) peptides are considered to play a major role in the pathogenesis of Alzheimer's disease (AD) and compounds that can prevent pathways of Aβ-induced neurotoxicity may be potential therapeutic agents for treatment of AD. This study examined the hypothesis that thymoquinone (TQ) would reduce oxidative stress and mitochondrial dysfunction in differentiated pheochromocytoma (PC 12) cells exposed to Aβ fragment 25–35 (A$\beta_{25–35}$). To test this hypothesis, Aβ was used to induce an in vitro model of AD in differentiated PC 12 cell line of rat. After 24 h of exposure with A$\beta_{25–35}$, a significant reduction in cell viability and mitochondrial membrane potential (MMP) was observed. In addition, a significant elevation in the TBARS content and nitric oxide (NO) and activity of acetylcholine esterase (AChE) was observed which was restored significantly by TQ pretreatment. Furthermore, TQ also ameliorated glutathione and its dependent enzymes (glutathione peroxidase, glutathione reductase) which were depleted by A$\beta_{25–35}$ in PC 12 cells. These results were supported by the immunocytochemical finding that has shown protection of cells by TQ from noxious effects of A$\beta_{25–35}$. These results indicate that TQ holds potential for neuroprotection and may be a promising approach for the treatment of neurodegenerative disorders including AD.

Keywords Alzheimer's disease · PC 12 cells · A$\beta_{25–35}$ · Oxidative stress · Thymoquinone

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder of aged brain associated with progressive dementia and cognitive dysfunction [1]. It is characterized by the presence of senile plaques in several regions of the

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brain. The major protein component of the plaques is the amyloid β-peptide (Aβ), which is a 39–43 amino acid peptide [2, 3]. It has been established that A$\beta_{25–35}$ exhibits toxicity to neurons either in vitro or in vivo [4–6]. Although the exact mechanism of Aβ-induced neurotoxicity remains obscure, several studies have evoked that oxidative stress may play a key role in Aβ-mediated neuronal toxicity [7, 8]. One probable way to prevent the cellular injuries induced by oxidative stress is to augment or potentiate endogenous oxidative defense capacity through dietary or pharmacological intake of antioxidants. Other than oxidative stress, mitochondrial dysfunction is also one of the central pathways leading to apoptosis in AD [9]. Mitochondria are essential organelles in cells that not only generate adenosine triphosphate (ATP) but also execute critical functions such as calcium buffering, reactive oxygen species (ROS) generation, and apoptotic signaling [9]. Nitric oxide (NO) and its derivatives, also belonging to the group of ROS, are known to inhibit the mitochondrial respiration [10].

Herbal therapies are commonly used for the prevention and treatment of central nervous system (CNS) disorders despite the little understanding of their molecular and cellular basis of action. Thymoquinone (TQ) (2-isopropyl-5-methyl-1,2-benzoquinone), the bioactive and the most abundant constituent of the volatile oil of black seed, has been shown to possess an anti-inflammatory and antioxidant effects [11–13]. Moreover, TQ also has chemopreventive, anticarcinogenic, and antimutagenic activity [14, 15]. TQ mediates its inhibitory effect on NO production [16]. Despite the promising antioxidant activity of TQ, the molecular mechanism of its action is poorly understood.

In this study, we used A$\beta_{25–35}$ a neurotoxin commonly used to generate experimental models of AD, investigated the effect of TQ on oxidative stress and explored the protection of TQ in vitro in A$\beta_{25–35}$-induced PC 12 cells.

Materials and methods

Cell culture

The PC 12 cell line was obtained from National Facility for Animal Tissue and Cell Culture (NCCS), Pune, India and since then has been maintained in our laboratory. Cells were cultivated in F-12 Hams supplemented with 2.5 % fetal bovine serum (FBS), 15 % horse serum (HS), 0.2 % sodium bicarbonate and 1.5 % (100× solution) of antibiotics and antimycotic. Medium was replaced twice a week. A 100 µl of cell suspension was stained with trypan blue (0.2 %) and viable cells were counted using a hemocytometer. All treatments were performed with 80 % confluent cells. Before experiments, the cells were precultured for 8 days with 50 ng/ml nerve growth factor (NGF).

Treatment

Cells were cultured in flasks or plates. After 8 days of differentiation, the medium was replaced and fresh medium containing TQ was added for 24 h. Thereafter, the fresh medium containing A$\beta_{25–35}$ was added for 24 h replacing medium containing TQ.

Cytotoxicity and cell viability

The protective effect of TQ on PC 12 cell line was determined by the MTT dye-uptake method as described earlier [17]. In brief, cells (1×10^4/well) were seeded in poly-L-lysine pre-coated 96-well tissue culture plates and allowed to adhere for 24 h in CO_2 incubator at 37 °C. Cells were differentiated for the indicated time period. Thereafter, the medium was replaced with the medium containing TQ (0.78–400 µM) for a period up to 24 h. Tetrazolium bromide salt (5 mg/ml of stock in PBS) 10 µl/well was added in 100 µl of cell suspension and plate was incubated for 4 h. At the end of incubation period, the reaction mixture was carefully taken out and 200 µl of DMSO was added to each well by pipetting up and down several times until the content gets homogenized. The plates were kept on rocker shaker for 10 min at room temperature and then read at 550 nm using microplate reader (Biorad, USA).

Neutral red uptake (NRU)

PC 12 cells were seeded in 96-well culture plate at the same density as for MTT and the cell viability was also assessed by NRU assay as described by [18]. The medium was replaced with the medium containing TQ (0.78–400 µM) for a period of 24 h. Briefly, 0.4 % aqueous stock solution of NRU dye was prepared and an aliquot was added to the complete medium to make the final concentration of 50 µg/ml. The neutral red 200 µl was added in the wells medium and incubation for 3 h at 37 °C. The viable cells stained with the dye. Thereafter, the dye medium was taken out and cells were washed rapidly with 0.5 % formaldehyde. Thereafter 200 µl of 1 % acetic acid and 50 % ethanol was added, followed by an incubation of 20 min at 37 °C. The plates were read at 540 nm using multiplate reader (BioRad Model 680). Results were expressed as percentage absorbance.

LDH assay

Cells were seeded in 96-well plate at a density of 2×10^4 cells/well in culture medium. After NGF treatment, medium was replaced and cells were exposed to varying concentrations of TQ (0.63, 1.25, 2.5, 5, and 10 µM). Cells were incubated for 24 h and then exposed to

10 μM $A\beta_{25-35}$ for 24 h. The LDH activity was measured using in vitro toxicology assay kit (Biovision) in accordance with the manufacturer's instructions.

Preparation of cell lysate

For each enzymatic and non enzymatic assay 1×10^6 cells were seeded in 25-cm^2 flasks and differentiated with NGF. Differentiated cells were exposed to TQ at concentrations of 1, 2, and 4 μM for 24 h. After 24 h, the medium was replaced by fresh medium containing 10 μM $A\beta_{25-35}$ and incubated for 24 h. Thereafter, the cells of each flask were scrapped and centrifuged at $5{,}000\times g$ for 5 min at 4 °C. The supernatant was discarded and pellet was washed twice with 1 M PBS. 500 μl of chilled lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, and 1 % Triton X-100) containing 10 μl/ml protease inhibitor was added to each tube. After 30 min of incubation, the cells were sonicated in SONICS Vibra Cells Sonicator.

TBARS content

The TBARS assay was done according to the method of [19], as modified by us [20]. The cell lysate 0.25 ml was incubated in 13 × 100 mm test tube at 37 ± 1 °C in a metabolic shaker (120 strokes/min) for 1 h. Similarly, 0.25 ml of the same cell lysate was pipetted in a centrifuge tube and incubated at 0 °C. After 1 h of incubation, 0.25 ml of 5 % chilled TCA was added followed by 0.5 ml of 0.67 % TBA in each test tube and proper mixing was done after each addition. The mixture was centrifuged at $4{,}000\times g$ for 10 min. Thereafter, supernatant was transferred to another test tube and placed in boiling water bath for 10 min. The test tube was cooled and the absorbance of the color was read at 535 nm. The rate was expressed as nmol of TBARS formed/h mg^{-1} protein using molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Reduced glutathione (GSH)

GSH content was determined by the method of [21] with slight modification. Cell lysate was mixed with 4.0 % sulfosalicylic acid in a 1:1 ratio (v/v). The samples were incubated at 4 °C for 1 h, and later centrifuged at $1{,}500\times g$ for 15 min at 4 °C. The assay mixture contained 0.1 ml of supernatant, 1.0 mM DTNB, and 0.1 M PB (pH 7.4) in a total volume of 1.0 ml. The yellow color developed was read immediately at 412 nm in a spectrophotometer (UV-1601, Shimadzu, Japan). The GSH content was calculated as nmol of GSH/mg protein using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

Glutathione peroxidase (GPx)

GPx activity was estimated according to the procedure described previously [22]. The reaction mixture consisted of phosphate buffer (0.05 M, pH 7.0), EDTA (1 mM), sodium azide (1 mM), glutathione reductase (GR, 1 EU/ml), glutathione (1 mM), NADPH (0.2 mM), hydrogen peroxide (0.25 mM), and 0.05 ml of cell lysate in the final volume of 1 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was expressed as nmol of NADPH oxidized/min mg^{-1} protein using molar extinction coefficient 6.22×10^3 M^{-1} cm^{-1}.

Glutathione reductase (GR)

GR activity was assayed by the method of [23] as modified by [22]. The assay mixture consisted of phosphate buffer (0.1 M, pH 7.6), NADPH (0.1 mM), EDTA (0.5 mM), oxidized glutathione (1 mM), and 0.05 ml of cell lysate in total volume of 1 ml. The enzyme activity was quantified at room temperature by measuring the disappearance of NADPH at 340 nm and expressed as nmol of NADPH oxidized/min mg^{-1} protein using molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Acetylcholinesterase (AChE) activity

AChE activity was determined by a modified method [24]. Briefly, 2.6 ml of Tris–HCl buffer (0.1 M, pH 8.0), 20 μl cell lysate and 0.1 ml of buffered Ellman's reagent (DTNB 10 mM, $NaHCO_3$ 15 mM) were mixed and allowed to preincubate for 5 min at room temperature. The reaction was started by adding 40 μl of 0.075 M acetylthiocholine iodide and optical density was measured at 412 nm within 3 min. AChE activity was expressed as nmol thiocholine formed/min mg^{-1} protein using a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}.

Assay for Na^+/K^+-ATPase

Na^+/K^+-ATPase activity was measured as described previously [25]. The Na^+/K^+-ATPase activity was determined in two reaction media, A and B. The reaction mixture A consisted of 0.2 M KCl, 1.0 M NaCl, 0.1 M $MgCl_2$, 0.2 M Tris–HCl buffer (pH 7.4), and 0.1 ml of cell lysate in a total volume of 2.0 ml. The reaction mixture B consisted of 0.1 M $MgCl_2$, 10 mM ouabain, 1.0 M NaCl, 0.2 M Tris–HCl buffer (pH 7.4), and 0.1 ml of cell lysate in a total volume of 2.0 ml. The enzyme reaction was started by adding 0.2 ml of 25.0 mM ATP at 37 °C and terminated after 15 min by adding 1.0 ml chilled 10 % TCA. The mixture was centrifuged and supernatant (0.5 ml) was used

for the estimation of inorganic phosphorous according to method of [26].

Measurement of intracellular ROS

ROS formation was detected using a nonfluorescent compound, $DCFH_2$-DA. Once inside the cell, the de-esterified product becomes the fluorescent compound, $DCFH_2$ on oxidation by ROS. The fluorescent signal given by $DCFH_2$ is proportional to ROS production [27]. After the exposure with TQ and $A\beta_{25-35}$ for the mentioned time period, the cells (2×10^6/ml) were added to 800 µl PBS and incubated with 10 µM $DCFH_2$-DA at 37 °C for 20 min, in an atmosphere of 95 % air and 5 % CO_2. After loading with $DCFH_2$-DA, the cells were washed with the same buffer and fluorescence was measured at 365 nm excitation and 430 nm emission.

Mitochondrial membrane potential (MMP) assessment

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazo lylcarbocyanine iodide) was used following the method of [28]. Briefly, cells were seeded in 24-well plate at a density of 1×10^6 cells/well and the treatment was given as mentioned above. The harvested cells were incubated in 0.5 ml JC-1 (10 µM) for 8 min at room temperature in dark. After centrifugation for 5 min at $500\times g$ and washing twice by PBS (pH 7.4) to remove unincorporated dye, pellets were resuspended in 2 ml PBS (pH 7.4). Red fluorescence (excitation 570 nm, emission 610 nm) and green fluorescence (excitation 490 nm, emission 535 nm) were measured using an spectrofluorimeter (Spectrometer LS50B, Perkin Elmer). Results were expressed as the ratio of green to red fluorescence.

Nitric oxide determination

Nitric oxide was determined by measuring the nitrite content in culture medium [29]. Briefly, the cells were seeded in 96-well plate at density of 2×10^4 cells/well and incubated overnight. Thereafter, media was discarded and cells were exposed to medium containing TQ with three doses and thereafter beta amyloid treatment was given. After 24 h, media from each well was transferred to fresh tube and centrifuge at $500\times g$ for 5 min at 4 °C. The supernatant 100 µl was transferred to fresh 96-well plate and mixed with an equal volume of Griess reagent (0.04 g/ml PBS, pH 7.4). After incubation at room temperature for 10 min, the absorbance of resultant color was measured at 540 nm using a microplate reader (Bio-Rad, USA). Concentration of nitrite in medium of treated and untreated cells was calculated using a standard curve of sodium nitrite and expressed as % of control.

Immunocytochemical assay

Immunocytochemical analysis was performed to determine the expression of iNOS in treated cells. Cells were seeded in PLL coated 24-well plate at a density of 2×10^4 cells/well. Cells were differentiated with NGF for 8 days and subsequently exposed to TQ (4 µM) for 24 h. Thereafter, cells were exposed to $A\beta_{25-35}$ (10 µM) for another 24 h. Media was carefully removed and cells were washed twice with PBS (pH 7.4) and fixed with 4 % paraformaldehyde for 30 min. Again cells were washed thrice with PBS and incubated with 0.5 % H_2O_2 in methanol for 1 h followed by incubation in 0.02 % Triton X-100 in 0.1 % BSA for 15 min to block the non specific binding sites. Finally, cells were washed and incubated with anti iNOS antibody (1:500) overnight at 4 °C and then immediately incubated with secondary antibody (1:500) for 2 h. Further cells were washed thrice with PBS and visualized by 3,3′-di-aminobenzidine tetrahydrochloride (DAB) under microscope.

Determination of protein

Protein was determined by Lowry et al. [30] using bovine serum albumin (BSA) as a standard.

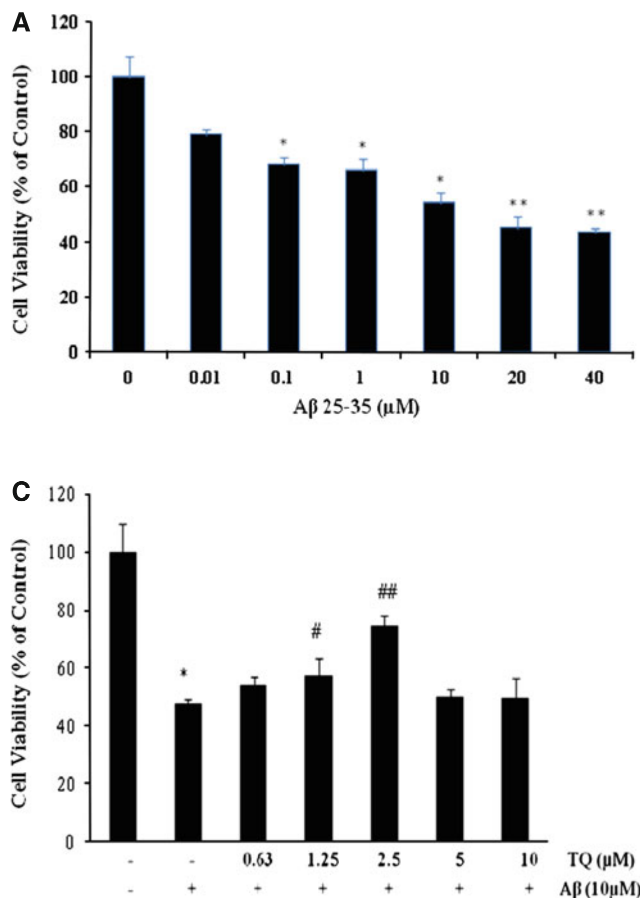
Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey's Kramer test. The $p \leq 0.05$ was considered statistically significant.

Results

Cell viability of PC 12 cells with different concentrations of $A\beta_{25-35}$ and TQ

The cytotoxicity of $A\beta$ was evaluated based on its effect on cell growth (MTT assay). Figure 1a deals with PC 12 cells exposed with $A\beta_{25-35}$ at a series of concentrations (0, 0.01, 0.1, 1, 10, 20, and 40 µM) for 24 h. Figure 1a, $A\beta_{25-35}$-induced marked decrease in cell viability in a dose-dependent manner and 10 µM $A\beta_{25-35}$ significantly reduced cell viability to 54.12 ± 3.81 % ($p < 0.05$). The possible cytotoxicity of TQ was evaluated based on its effect on cell growth by both MTT and NRU assays (Fig. 1b). Differentiated PC 12 cells were exposed to varying concentrations of TQ (0–400 µM) for 24 h. Figure 1b shows that IC 50 for TQ by MTT was 5.15 µM while IC 50 by NRU was 5.9 µM. Thus for further experiments, higher cytotoxic doses were not used.



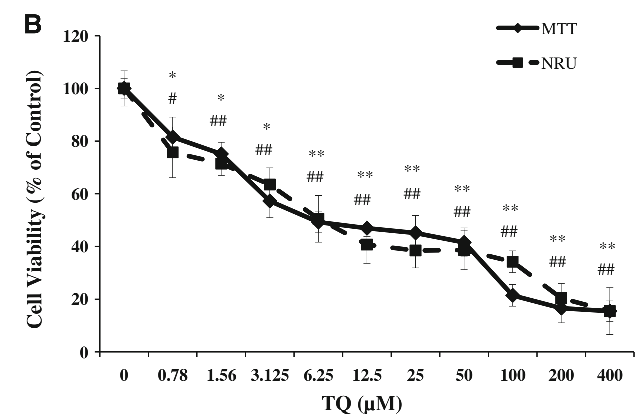


Fig. 1 **a** Effect of $A\beta_{25\text{–}35}$ on differentiated PC 12 cells. The cell viability (% of control) was significantly and dose dependently decreased in $A\beta_{25\text{–}35}$-treated groups (*$p < 0.05$ vs. control, **$p < 0.01$ vs. control). **b** Effect of TQ on differentiated PC 12 cells. The cell viability (% of control) was significantly decreased with increasing concentration of TQ (*$p < 0.01$ vs. control, **$p < 0.001$ vs. control (MTT), $^{\#}p < 0.01$ vs. control, $^{\#\#}p < 0.001$ vs. control (NRU)). Data are presented as mean ± SEM of three independent experiments, each in triplicate. **c** TQ diminished $A\beta_{25\text{–}35}$-induced cell death. Data are presented as mean ± SEM of three independent experiments, each in triplicate. The cell viability (% of control) was significantly decreased by $A\beta_{25\text{–}35}$ treatment (*$p < 0.01$ $A\beta_{25\text{–}35}$ vs. control). TQ pretreatment significantly increased the cell viability ($^{\#}p < 0.01$ TQ [1.25 μM] vs. $A\beta_{25\text{–}35}$ and $^{\#\#}p < 0.001$ TQ [2.5 μM] vs. $A\beta_{25\text{–}35}$)

Thymoquinone ameliorates $A\beta_{25\text{–}35}$ induced loss of cell viability

The cell viability was assessed by MTT reduction. Incubation of PC 12 cells with $A\beta_{25\text{–}35}$ (10 μM) for 24 h decreased the cell viability to 54.12 % as compared to control. Pretreatment of the cells with TQ substantially reduced the cell death caused by $A\beta_{25\text{–}35}$ in a concentration-dependent manner (Fig. 1c). Significant protection was achieved at 1.25 and 2.5 μM. With the concentration of 1.25 and 2.5 μM, the viability of cells was 57.3 ($p < 0.01$) and 74.3 % ($p < 0.001$), respectively.

Effect of thymoquinone on activity of lactate dehydrogenase

$A\beta_{25\text{–}35}$ (10 μM) significantly increased the activity of LDH ($p < 0.01$) which was restored by 2.5 μM TQ

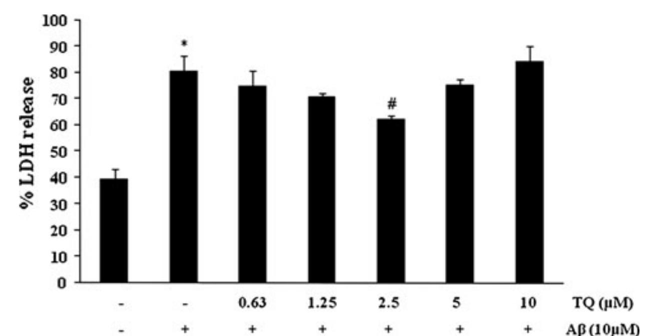


Fig. 2 TQ prevented the $A\beta_{25\text{–}35}$-induced increase activity of LDH. Data are presented as mean ± SEM of three independent experiments, each in triplicate. LDH activity was significantly increased by $A\beta_{25\text{–}35}$ treatment as compared to control (*$p < 0.01$ $A\beta_{25\text{–}35}$ vs. control). TQ pretreatment significantly decreased the LDH activity as compared to $A\beta_{25\text{–}35}$-treated group ($^{\#}p < 0.05$ TQ [2.5 μM] vs. $A\beta_{25\text{–}35}$)

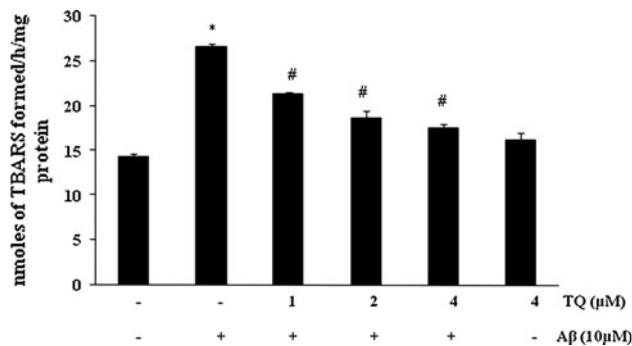


Fig. 3 Effect of TQ pretreatment on TBARS content in PC 12 cells exposed to $A\beta_{25-35}$. Data are presented as mean ± SEM of three independent experiments, each in triplicate. TBARS content was significantly increased in $A\beta_{25-35}$ group as compared to control group (*$p < 0.001$ $A\beta_{25-35}$ vs. control). TQ pretreatment significantly decreased the TBARS content as compared to $A\beta_{25-35}$-treated group ($^{\#}p < 0.001$ TQ [1, 2, and 4 μM] vs. $A\beta_{25-35}$)

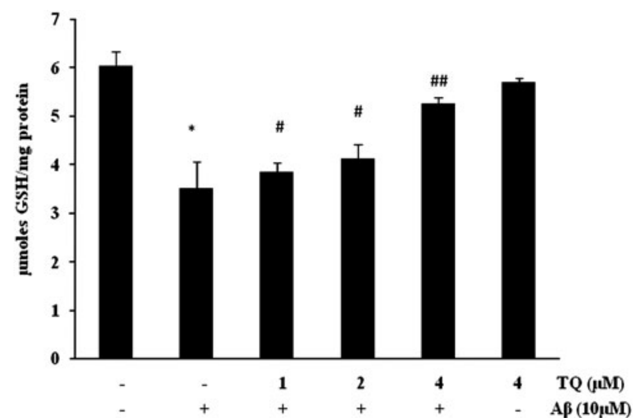


Fig. 4 Effect of TQ pretreatment on GSH content in PC 12 cells exposed to $A\beta_{25-35}$. Data are presented as mean ± SEM of three independent experiments, each in triplicate. GSH content was significantly decreased in $A\beta_{25-35}$ group as compared to control group (*$p < 0.01$ $A\beta_{25-35}$ vs. control). TQ pretreatment significantly increased the content of GSH as compared to $A\beta_{25-35}$-treated group ($^{\#}p < 0.05$ TQ [2 μM] vs. $A\beta_{25-35}$, $^{\#\#}p < 0.01$ TQ [4 μM] vs. $A\beta_{25-35}$)

($p < 0.05$) (Fig. 2). Therefore, three non toxic doses of TQ were selected for further studies viz. 1, 2, and 4 μM.

Effect of thymoquinone on TBARS level

The effect of TQ on TBARS content was measured to demonstrate the oxidative damage on lipid peroxidation in $A\beta_{25-35}$-treated PC 12 cells. A significant increased content of TBARS ($p < 0.001$) was observed in $A\beta_{25-35}$-treated PC 12 cells when compared to control group. Cells of TQ pretreatment group exhibited significant attenuation ($p < 0.001$) in comparison to $A\beta_{25-35}$ group (Fig. 3).

Effect of thymoquinone on GSH level

The GSH level was significantly ($p < 0.01$) decreased in $A\beta_{25-35}$-treated cells as compared to control cells. Pretreatment with TQ has protected GSH level significantly ($p < 0.05$ and $p < 0.01$) and dose dependently. TQ 4 μM alone has exhibited no significant changes on GSH level as compared to the control group (Fig. 4).

Effect of thymoquinone on the activities of antioxidant enzymes in PC 12 cells

The activities of antioxidant enzymes (GPx and GR) were decreased significantly in $A\beta_{25-35}$-treated cells as compared to control cells ($p < 0.05$). On the other hand, the activities were protected significantly by TQ pretreatment in PC 12 cells when compared to $A\beta_{25-35}$-treated cells ($p < 0.01$). The activity of these enzymes in TQ pretreated cells was not attenuated significantly, as compared to its control (Table 1).

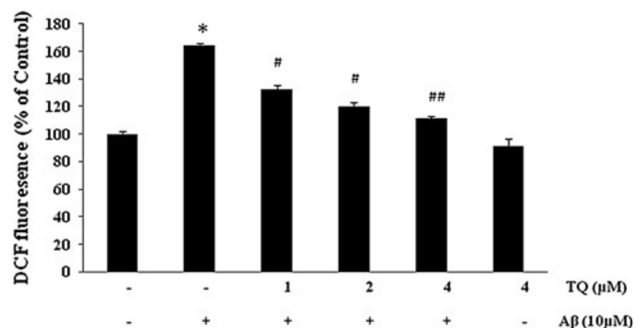


Fig. 5 Effect of TQ pretreatment on intracellular ROS in PC 12 cells exposed to $A\beta_{25-35}$. Data are presented as mean ± SEM of three independent experiments, each in triplicate. DCF fluorescence was significantly increased in $A\beta_{25-35}$ group as compared to control group (*$p < 0.01$ $A\beta_{25-35}$ vs. control). TQ pretreatment significantly decreased DCF fluorescence as compared to $A\beta_{25-35}$-treated group ($^{\#}p < 0.01$ TQ [1 and 2 μM] vs. $A\beta_{25-35}$, $^{\#\#}p < 0.001$ TQ [4 μM] vs. $A\beta_{25-35}$)

Protection of intracellular oxidative stress by thymoquinone in $A\beta_{25-35}$-treated PC 12 cells

To clarify the possible antioxidant effect of TQ, the accumulation of ROS was evaluated. As illustrated in Fig. 5, there was a significant increase in the intracellular ROS in cells treated with $A\beta_{25-35}$ ($p < 0.01$) compared to control taken as 100. TQ pretreatment has decreased the ROS level significantly and dose dependently ($p < 0.01$, 0.01, and 0.001, respectively) when compared to $A\beta_{25-35}$-treated cells.

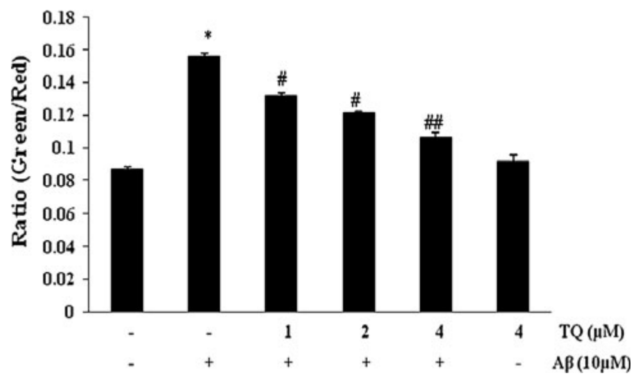


Fig. 6 Effect of TQ pretreatment on MMP in PC 12 cells exposed to $A\beta_{25-35}$. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. The ratio of green to red fluorescence was significantly increased in $A\beta_{25-35}$ group as compared to control group ($*p < 0.01$ $A\beta_{25-35}$ vs. control). TQ pretreatment significantly decreased the ratio as compared to $A\beta_{25-35}$-treated group ($^{\#}p < 0.05$ TQ [1 and 2 μM] vs. $A\beta_{25-35}$, $^{\#\#}p < 0.01$ TQ [4 μM] vs. $A\beta_{25-35}$)

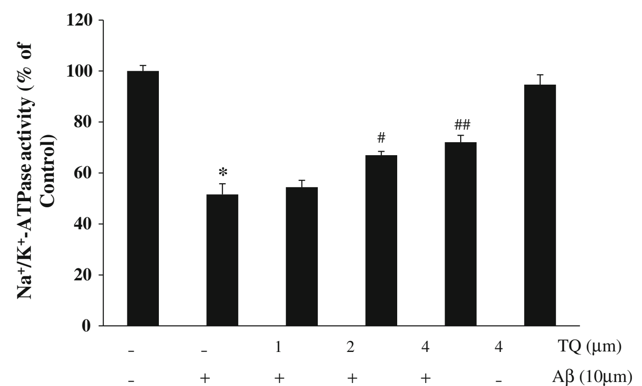


Fig. 7 Effect of TQ pretreatment on the activity of Na^+/K^+-ATPase in PC 12 cells exposed to $A\beta_{25-35}$. Data are presented as mean ± SEM of three independent experiments, each in triplicate. Na^+/K^+-ATPase activity was significantly decreased in $A\beta_{25-35}$ group as compared to control group ($*p < 0.01$ $A\beta_{25-35}$ vs. control). TQ pretreatment significantly increased Na^+/K^+-ATPase activity as compared to $A\beta_{25-35}$-treated group ($^{\#}p < 0.01$ TQ [2 μM] vs. $A\beta_{25-35}$, $^{\#\#}p < 0.001$ TQ [4 μM] vs. $A\beta_{25-35}$)

Effect of thymoquinone on mitochondrial membrane potential (ψm)

Disruption of MMP has been used to study the mitochondrial health. In this study, JC-1 dye was used as the marker of membrane disruption. The green/red ratio was found to be increase significantly in $A\beta_{25-35}$-treated cells ($p < 0.01$) as compared to control group suggesting decreased membrane potential. TQ pretreatment augmented the membrane potential significantly ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) and in a dose-dependent manner as compared to $A\beta_{25-35}$-treated cells (Fig. 6).

Effect of thymoquinone on Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was decreased ($p < 0.01$) by ~50 % in cultured PC 12 cells exposed for 24 h to 10 μM $A\beta_{25-35}$ (Fig. 7). In contrast, cells pretreated with TQ showed significant protection ($p < 0.01$ and $p < 0.001$) as compared to $A\beta_{25-35}$ group.

Effect of thymoquinone on acetylcholine esterase (AChE) activity

Table 1 shows significant increase ($p < 0.05$) of AChE in $A\beta_{25-35}$-treated cells as compared to control group. TQ at

Table 1 Effect of TQ on activities of antioxidant enzymes [GPx and GR] and acetylcholine esterase (AChE) in $A\beta_{(25-35)}$-treated differentiated PC 12 cells

Groups	GPx (nmol NADPH oxidized/min mg^{-1} protein)	GR (nmol NADPH oxidized/min mg^{-1} protein)	AChE activity (μmol thiocholine formed/min mg^{-1} protein
Control	741.02 ± 36.21	648.94 ± 44.14	2.78 ± 0.42
Aβ (25–35) (10 μM)	372.04 ± 12.41*	364.82 ± 45.62*	5.86 ± 0.99*
	(−49.79 %)	(−43.78 %)	(−52.60 %)
TQ (1 μM) + Aβ (25–35)	434.09 ± 31.57	408.10 ± 73.74	4.07 ± 0.21
	(16.68 %)	(11.86 %)	(30.49 %)
TQ (2 μM) + Aβ (25–35)	597.15 ± 97.21#	437.44 ± 45.89	3.62 ± 0.53#
	(36.14 %)	(19.91 %)	(38.16 %)
TQ (4 μM) + Aβ (25–35)	649.03 ± 29.61#	548.22 ± 35.87#	3.32 ± 0.42#
	(74.45 %)	(50.27 %)	(43.36 %)
TQ (4 μM)	720.79 ± 58.99	683.69 ± 45.95	3.19 ± 0.77
	(−3.39 %)	(−7.9 %)	(−11.4 %)

Protective effect of TQ on glutathione peroxidase (GPx), glutathione reductase (GR), and AChE in PC 12 cells exposed to $A\beta_{25-35}$. Data are presented as mean ± SEM of three independent experiments, each in triplicate. $A\beta_{25-35}$ treatment leads to significant alterations on the activities of antioxidant enzymes (GPx and GR) and AChE in PC 12 cells as compared to control ($*p < 0.05$, $A\beta_{25-35}$ vs. control). Pretreatment of TQ significantly attenuated activities of these enzymes as compared to $A\beta_{25-35}$-treated group ($^{\#}p < 0.01$ TQ [1, 2, and 4 μM] vs. $A\beta_{25-35}$)

2 μM ($p < 0.01$) and 4 μM ($p < 0.01$) concentrations significantly decreased the toxicity induced by Aβ_{25-35}.

Effect of thymoquinone on NO level

Figure 8 shows significant increase ($p < 0.01$) of NO level in Aβ_{25-35}-treated cells as compared to control group. All three concentrations of TQ significantly protected ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) the toxicity induced by Aβ_{25-35} with maximum protection at the highest dose.

Effect of thymoquinone on iNOS expression

The up regulation of iNOS is linked with AD pathogenesis. iNOS expression was increased in PC 12 cells treated with Aβ_{25-35} (Fig. 9). In TQ-treated cells, the expression was decreased as compared to Aβ_{25-35}-treated cells. Only TQ did not show any remarkable effect on the cells as compared to control group (data not shown).

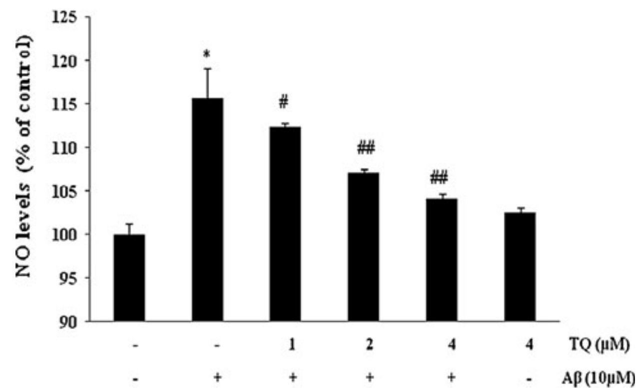


Fig. 8 Effect of TQ pretreatment on NO level in PC 12 cells exposed to Aβ_{25-35}. Data are presented as mean ± SEM of three independent experiments, each in triplicate. NO level was significantly increased in Aβ_{25-35} group as compared to control group (*$p < 0.01$ Aβ_{25-35} vs. control). TQ pretreatment significantly decreased NO level as compared to Aβ_{25-35}-treated group ($^{\#}p < 0.05$ TQ [1 μM] vs. Aβ_{25-35}, $^{\#\#}p < 0.01$ TQ [2 and 4 μM] vs. Aβ_{25-35})

Discussion

The neurotoxic activity of Aβ, which is associated with senile plaques formed in the AD brains, is attributable to the amino acids located in positions 25–35 of the full length. The exact mechanism of Aβ_{25-35}-mediated neurodegeneration is not completely elucidated, but several studies have shown that Aβ is associated with ROS generation, which leads to mitochondrial dysfunction, lipid peroxidation, and apoptosis [31, 32]. Thus, removal of

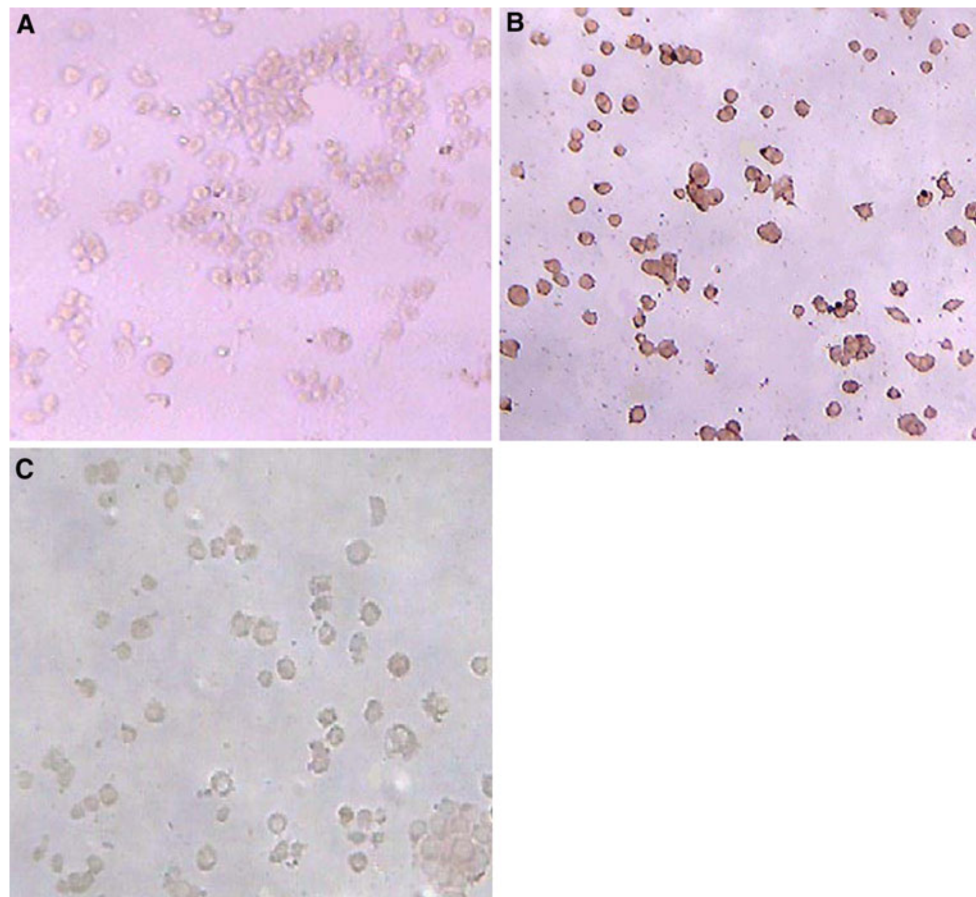


Fig. 9 Effect of TQ pretreatment on iNOS expression. The profound expression of iNOS was observed in Aβ_{25-35} group (**b**) compared to control group (**a**), while Aβ_{25-35} group pretreated with TQ (**c**) has shown a moderate staining of iNOS. However, the control group has shown almost negligible staining

excess ROS or suppression of their generation by antioxidants may be effective in preventing oxidative cell death. TQ, a major active ingredient present in *Nigella sativa* oil has been reported to be a strong antioxidant [11, 12]. In this study, we investigated the protective role of TQ against $A\beta_{25-35}$-induced oxidative stress.

The IC_{50} of TQ after 24 h was about 5.4 μM, so for further studies we selected the preventive doses below 5.4 μM. $A\beta_{25-35}$ caused a decrease in MTT reduction in differentiated PC 12 cells which was partly restored by the addition of TQ. The normal plasma membrane is impermeable to LDH, but the damaged cell membrane become permeable and causes the leakage of LDH into the extracellular fluid [27]. LDH assay also revealed that exposure to $A\beta_{25-35}$ resulted in PC 12 cells injury which was restored significantly by TQ.

Increased oxidative stress is a key feature of neurodegenerative diseases and mitochondria are a major source of ROS. However, a high level of oxidative stress finally inflicts critical damage to the oxidative phosphorylation machinery and mitochondrial DNA (mtDNA). Mutations in mtDNA and oxidative stress both contribute to aging, which is the greatest risk factor for age-related neurodegenerative diseases including AD. Lipids are the main constituents of the brain and probably the first target of free radicals [20, 33]. Accumulating data from experimental and human studies indicate that oxidative stress is an important causative factor in the development and progression of AD [33–35]. Specifically, $A\beta_{25-35}$-treated differentiated PC 12 cells have been shown to accumulate ROS, which can be eliminated by antioxidants, suggesting that antioxidants may protect against $A\beta_{25-35}$-induced cytotoxicity [36, 37]. In agreement with this notion, $A\beta_{25-35}$-induced oxidative cell death was suppressed by pretreatment with TQ. The primary defense against oxidative damage in tissues rests with antioxidants such as the tripeptide glutathione (GSH), which is consumed in order to counteract the effects of oxidative stress. The loss of GSH and formation of protein–glutathione mixed disulfide (PrSSG) in the brain results to various membrane dysfunctions [38]. GSH with the help of GPx reduces H_2O_2 and organic peroxides and gets converted to GSSG [33, 39]. Due to decrease activity of GPx or GR, the redox cycles of GSSG is inhibited, which further increase the toxicity of $A\beta_{25-35}$. The activities of GPx and GR were protected significantly by TQ.

It has also been shown that enhancement of AChE activity induced by $A\beta_{25-35}$ is mediated by oxidative stress in cultured retinal cells, and this effect can be reversed by antioxidants [40]. Moreover, the abnormal expression of cholinergic enzymes is a consequence of a disturbance of calcium homeostasis [40] which is related to the mitochondrial dysfunction. We speculated from the current study that AChE activation by $A\beta$ was restored by TQ which is in accordance with the previous studies [41, 42].

The decreased Na^+/K^+-ATPase activity implied the collapse of the mitochondrial membrane, which might consequently cause apoptotic insult and cell death [43]. Our present study found that $A\beta_{25-35}$ treatment markedly reduced Na^+/K^+-ATPase activity in PC 12 cells, which indicate that mitochondrial membranes of these cells lost their function for Na^+/K^+ exchange.

The mitochondrial respiratory chain, which consumes 85–90 % of cellular oxygen, is the main source of oxygen free radicals. A recent discovery indicates that $A\beta_{25-35}$ can interact directly with an enzyme termed $A\beta$-binding alcohol dehydrogenase (ABAD) in the mitochondrial matrix, promoting leakage of ROS, mitochondrial dysfunction and cell death [44]. In this study, we found that $A\beta_{25-35}$ significantly decreased mitochondrial membrane potential (MMP) and TQ treatment ameliorated MMP. The findings suggest that disruptions to mitochondrial function and dissipation of MMP may be involved in the mechanisms of $A\beta_{25-35}$-induced cell death in differentiated PC 12 cells [45].

Nitric oxide (NO), an intercellular messenger generated from L-arginine by the action of nitric oxide synthase (NOS), plays an important role in various pathological processes [46]. Our study has shown that $A\beta_{25-35}$ increased the level of NO and iNOS expression. PC 12 cells treated with TQ have down regulated the iNOS expression along with NO level which was increased manifold in $A\beta_{25-35}$-treated cells. Our results are parallel to previous reports that TQ has inhibitory effect on some inflammatory mediators [5, 47].

Conclusion

In conclusion, TQ could mitigate $A\beta_{25-35}$-induced oxidative stress associated inflammation in PC 12 cells. The protective effects of TQ were not only related to modulate endogenous antioxidant status and inflammation-related protein expression, but also to restore the abnormal MMP and ROS level. As far as we know, this is the first report to demonstrate that TQ has the neuroprotective effects against $A\beta$-induced toxicity in PC 12 cells. The protection effects of TQ against neurotoxins may help to provide the pharmacological basis of its clinical usage in the treatment of neurodegeneration in AD.

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