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Thymoquinone protects cultured rat primary neurons against amyloid β-induced neurotoxicity

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ABSTRACT

Thymoquinone (TQ) is the main constituent of the oil extracted from *Nigella sativa* seeds, which is known to be the active constituent responsible for many of the seed antioxidant and anti-inflammatory effects. The present study was designed to investigate whether TQ can protect against Alzheimer's amyloid-β peptide (Aβ) induced neurotoxicity in rat primary neurons. Cultured hippocampal and cortical neurons were treated with $A\beta_{1-42}$ and TQ simultaneously for 72 h. Treatment with TQ efficiently attenuated $A\beta_{1-42}$-induced neurotoxicity, as evidenced by improved cell viability. TQ also inhibited the mitochondrial membrane potential depolarization and reactive oxygen species generation caused by $A\beta_{1-42}$. In addition, TQ restored synaptic vesicle recycling inhibition, partially reversed the loss of spontaneous firing activity, and inhibited $A\beta_{1-42}$ aggregation *in vitro*. These beneficial effects may contribute to the protection against Aβ-induced neurotoxicity. In conclusion, our results suggested that TQ has neuroprotection potential against $A\beta_{1-42}$ in rat hippocampal and cortical neurons and thus may be a promising candidate for Alzheimer disease treatment.

1. Introduction

Alzheimer disease (AD) is the most common form of dementia in the elderly. It is a slowly progressive neurodegenerative disorder of the central nervous system, characterized by profound impairment of cognitive function and memory [1]. Pathologically it is characterized by progressive brain atrophy, accumulation of cortical senile plaques, and neurofibrillary tangles. A fibrillary amyloid substance is deposited in senile plaques, formed by the aggregation of the 4.2-kD amyloid beta peptide (Aβ) [2]. Aβ is derived by proteolytic cleavage from a transmembrane amyloid beta precursor protein (AβPP) by β-site APP-cleaving enzyme-1 (BACE1), followed by γ-secretase [3]. This cleavage is imprecise and produces Aβ variants, which include those ending at residues 40 (Aβ40) and 42 (Aβ42). The Aβ42 is deposited earliest and most abundantly in plaques [4]. The progressive accumulation of Aβ42 aggregates is widely believed to be fundamental to the initial development of neurodegenerative pathology and to trigger a cascade of events, such as mitochondrial dysfunction through free radical generation, which results in oxidative stress [5], impairment of synaptic plasticity [6], and chronic inflammatory reactions [7]. It has been also suggested that neurotoxic amyloid aggregates may lead to synaptic dysfunction and loss [8], leading to neuronal death [9], which contributes to the progression of AD. Therefore, many therapeutic efforts focus on reducing Aβ production, including inhibiting secretase, increasing Aβ clearance with amyloid vaccines, or blocking Aβ aggregation (with antibodies, peptides, or small organic molecules that selectively bind and inhibit Aβ aggregate and fibril formation) [10].

The seeds of *Nigella sativa* L., commonly known as black seed or black cumin, has been used for medicinal purposes for centuries, both as herb and pressed into oil, in Asia, Middle East, and Africa. It has been traditionally used for various conditions and treatments related to respiratory health, stomach and intestinal health, kidney and liver function, circulatory and immune system support, and for general well-being [11]. The seeds contain fixed and essential oils, proteins, alkaloids, and saponin. Much of the biological activity of the seeds has been shown to be due to thymoquinone (TQ), the major component of essential oil, which is also present in the fixed oil [12]. TQ is known to be the active principle responsible for many of the seed antioxidant and anti-inflammatory effects [13], which is frequently used in herbal medicine. It has various beneficial properties including anti-inflammatory actions [14], neuroprotection [15], and suppression of oxidative stress-induced neuropathy [16]. TQ also causes morphological improvements and prevents neurodegeneration by chronic toluene exposure [17], shows significant anti-anxiety-like activity through possible modulation of NO and GABA [18], and suppresses nuclear factor kappa B (NF-KB) activation in brain and spinal cord [19].

Based on these findings, our aim was to explore the protective effect of TQ against $A\beta_{1-42}$ induced neurotoxicity in rat hippocampal

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and cortical neurons. Cell viability was assessed using the CellTiter-Glo assay. Then, mitochondrial membrane potential and intracellular reactive oxygen species level was determined by Rhodamine 123 and DCFH–DA assays, respectively. Because synaptic degeneration is one of the mechanisms underlying the neurotoxicity of Aβ [20], we investigated the role of TQ against Aβ-induced synaptic vesicle recycling inhibition using FM1–43 dye. Aβ can also cause functional toxicity by inhibiting spontaneous firing of hippocampal neurons [21]. Therefore, we investigated the effect of TQ against Aβ-induced spontaneous firing inhibition using a multi-electrode array. To further clarify our results, we investigated the role of TQ in the cellular defense against $A\beta_{1-2}$ using a thioflavin T binding assay.

2. Materials and methods

2.1. Primary hippocampal and cortical cultures

The brains in Wistar rat embryos (E18) were dissected, and primary cultures were prepared as reported previously [22]. Dissociated cells were collected in MACS Neuro Medium (MACS Media) supplemented with L-glutamate, B-27 supplement, penicillin–streptomycin (all from GIBCO). Cultures were grown at 37 °C in an atmosphere of 5% CO_2/95% air.

2.2. Preparation of $A\beta_{1-42}$ and TQ

Amyloid β-protein 1–42 (Peptide Institute Inc.) was dissolved in dimethyl sulphoxide (DMSO; Wako) and stored at −20 °C. Thymoquinone solution (10 mM; Sigma–Aldrich) was prepared immediately before the experiment by dissolving 1.642 mg of TQ in a 1 ml of solution made of DMSO and culture medium. Final concentrations of TQ were prepared in the culture medium. $A\beta_{1-42}$ was administrated to cell cultures with or without TQ on day 13 *in vitro* for 72 h.

2.3. CellTiter-Glo assay

Cell viability of cultured primary hippocampal neurons was assessed using the CellTiter-Glo luminescent cell viability assay (Promega). Hippocampal neurons were prepared as indicated, and cultured on a poly-D-lysine (PDL; Sigma–Aldrich)-coated 96-micro-well plate (Nunc) at a density of 2×10^4 cells/well. First, $A\beta_{1-42}$ was applied to cultures in a range of concentrations (2, 5, 10 μM). Then, to investigate the effect of TQ on the survival rate of hippocampal neurons, cultures were treated only with TQ (0.1, 1, 10, 100 nM). To investigate the neuroprotective potential of TQ against $A\beta_{1-42}$ cytotoxicity, cultures were treated with $A\beta_{1-42}$ (10 μM) and TQ (100 nM) simultaneously. On the day of the experiment, the plate and its contents were equilibrated to room temperature for approximately 30 min. A volume of CellTiter-Glo reagent equal to that of the culture medium was added to the cells and mixed gently for 2 min; cells were then incubated at room temperature for 10 min to allow luminescent signals to stabilize. Then, luminescence signals (relative light units) were measured using a microplate reader (TECAN).

2.4. Rhodamine 123 assay

Rhodamine 123 was used to assay mitochondrial membrane potential ($\Delta\psi m$). Primary hippocampal neurons cultured on a PDL-coated 96-micro-well plate were treated with $A\beta_{1-42}$ (10 μM) and TQ (100 nM) simultaneously. On the day of the experiment, the medium was removed, neurons were incubated with (1 nM) Rhodamine 123 (Sigma–Aldrich) for 15 min at 37 °C, and washed in triplicate with phosphate-buffered saline (PBS) to remove the extracellular Rhodamine 123. Cells were then suspended in PBS. The fluorescence intensity (relative fluorescence unit) was measured using a fluorescence microplate reader at the excitation wavelength of 485 nm and emission wavelength of 535 nm. The data of the treatment groups were expressed as a percentage of the Rhodamine 123 fluorescence generated in control cells under identical incubation conditions.

2.5. DCFH–DA assay

Intracellular reactive oxygen species (ROS) level was determined using a dichlorofluorescin diacetate (DCFH–DA; Sigma–Aldrich) assay. Primary cortical neurons cultured on PDL-coated 96-micro-well plates were treated with $A\beta_{1-42}$ (10 μM) and TQ (100 nM) simultaneously. On the day of the experiment, the medium was removed, cortical neurons were incubated with (100 μM) DCFH–DA for 30 min at 37 °C, and the cells were washed to remove extracellular DCFH–DA. Cells were then suspended in PBS. Fluorescence signals were measured using a fluorescence microplate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm.

2.6. FM1–43 assay

The fluorescent styryl dye FM1–43 (Molecular Probes) that is readily taken up into synaptic recycling vesicles was used to determine synaptic activity as previously described [23]. Primary cortical neurons cultured on PDL-coated 24-micro-well plates (IWAKI) were treated with $A\beta_{1-42}$ (2 μM) only or concurrently treated with TQ (100 nM). On the day of the experiment, cortical neurons were incubated with 1 μg/ml FM1–43 and 1 μM high potassium (K^+) for 10 min, washed 5 times in ice cold PBS, and solubilized in methanol at 1×10^6 neurons/ml. Soluble extracts were transferred into 96-micro-well plates and fluorescence was measured with a TECAN microplate reader at an excitation wavelength of 480 nm and emission wavelength of 612 nm.

2.7. Microelectrode array

Therefore, the effect of $A\beta_{1-42}$ and TQ on the action potential of hippocampal neurons was studied using a microelectrode array (MEA) system (ALPHA MED SCIENTIFIC, Japan). MEA probes (MED-P530A, ALPHA MED SCIENTIFIC, Japan) were PDL-coated for 24 h at 37 °C. After rinsing PDL, the MEA probe was dipped into solid state 2.25% (w/v) agarose (BM Bio, Japan) and spread using a spin-coater (MIKASA CO. LTD) at 2500 rpm for 20 s to form a 5-μm-thick agarose layer. To obtain the best recording results, the portion of agarose layer upon each electrode was etched by spot heating using a 1064-nm infrared laser to form microchambers [24,25]. Hippocampal neurons were cultured at a density of 1×10^6 neurons/mm^2. The MEA probe was placed in sterile-petri dish, and then incubated at 5% CO_2, at 37 °C. To detect the spontaneous activity of the neurons attached to the electrodes, after 24 h, the culture medium was washed out and filtered to remove any suspended cells outside the microchambers. On culture day 13, hippocampal neurons were exposed to $A\beta_{1-42}$ (2 μM) only or to $A\beta_{1-2}$ and TQ (100 nM). Recordings were obtained for 72 h using Mobius software. Cultures were kept in the incubator during recording sessions.

2.8. Thioflavin T

Aβ aggregation into insoluble amyloid fibrils occurs through a number of intermediate structural forms, such as soluble oligomers or protofibrils. All these Aβ species may differentially affect

neuronal function and viability *in vitro* and *in vivo* [26].We investigated the effect of TQ on $A\beta_{1-42}$ amyloid formation using thioflavin T (ThT)-induced fluorescence [27]. $A\beta_{1-42}$ diluted in PBS (10 μM) was incubated in an Eppendorf tube at 37 °C for 72 h in the presence or absence of TQ (100 nM). Samples were then vortexed, and the contents of the tubes were then transferred to a 96-micro-well plate. ThT (100 nM; Sigma) diluted in 50 mM glycine buffer (pH 8.5) was added to each well. Fluorescence was measured using a TECAN microplate reader with an excitation wavelength at 450 nm and emission wavelength at 535 nm.

3. Results

3.1. Effect of $A\beta_{1-42}$ and TQ on the survival of primary hippocampal neurons

The CellTiter-Glo assay was used to determine the effect of various concentrations of $A\beta_{1-42}$ on viability, and the protective effects of TQ against $A\beta_{1-42}$-induced cell death in cultured hippocampal neurons. As shown in Fig. 1A, Aβ-induced a decrease in hippocampal cell viability in a dose-dependent manner, inducing maximal cell death at a dose of 10 μM ($P < 0.05$). Subsequent exposure of hippocampal cells to different concentrations of TQ had no significant effect on the survival rate of hippocampal neurons (Fig. 1B). However, applying TQ simultaneously with $A\beta_{1-42}$ resulted in a striking improvement in cell survival, in a dose-dependent manner; maximal rescue occurred at a dose of 100 nM TQ, with a total cell loss of 15% compared with a 50% cell loss in cultures subjected to $A\beta_{1-42}$ alone ($P < 0.0003$; Fig. 1C).

3.2. TQ inhibited Aβ1–42–induced Δψm collapse

Δψm is an important parameter of mitochondrial function used as an indicator of cell health. Δψm was determined using the fluorescent cationic dye, Rhodamine 123. As shown in Fig. 2A, treatment with $A\beta_{1-42}$ (10 μM) for 72 h decreased Rhodamine 123 fluorescence intensity by 30% compared with control samples, which is related to Δψm collapse ($P < 0.01$). When compared with the $A\beta_{1-42}$-exposed group, we found that cotreatment of cultured hippocampal neurons with 100 nM TQ significantly inhibited $A\beta_{1-42}$-induced Δψm collapse (15% decrease) compared with control samples ($P < 0.0007$).

3.3. TQ reduced intracellular ROS level in neurons treated with Aβ1–42

To examine whether the protective effect of TQ on the toxicity of $A\beta_{1-42}$ is mediated by antioxidant ability, the level of intracellular ROS was determined using the fluorescent probe DCFH–DA. When cortical neurons were exposed to 10 μM $A\beta_{1-42}$, the intracellular ROS level was significantly increased to 2.5-fold the normal level (Fig. 2B) revealing that $A\beta_{1-42}$ enhanced ROS generation in cultured cortical neurons ($P < 0.003$). However, treatment with TQ (100 nM) effectively reduced intracellular ROS levels to 1.65 times that of control levels in neurons treated with $A\beta_{1-42}$ ($P < 0.001$).

3.4. TQ reduced Aβ-induced inhibition of synaptic vesicle recycling

In order to further clarify the protective effect of TQ, we investigated its effect against $A\beta_{1-42}$–induced inhibition of

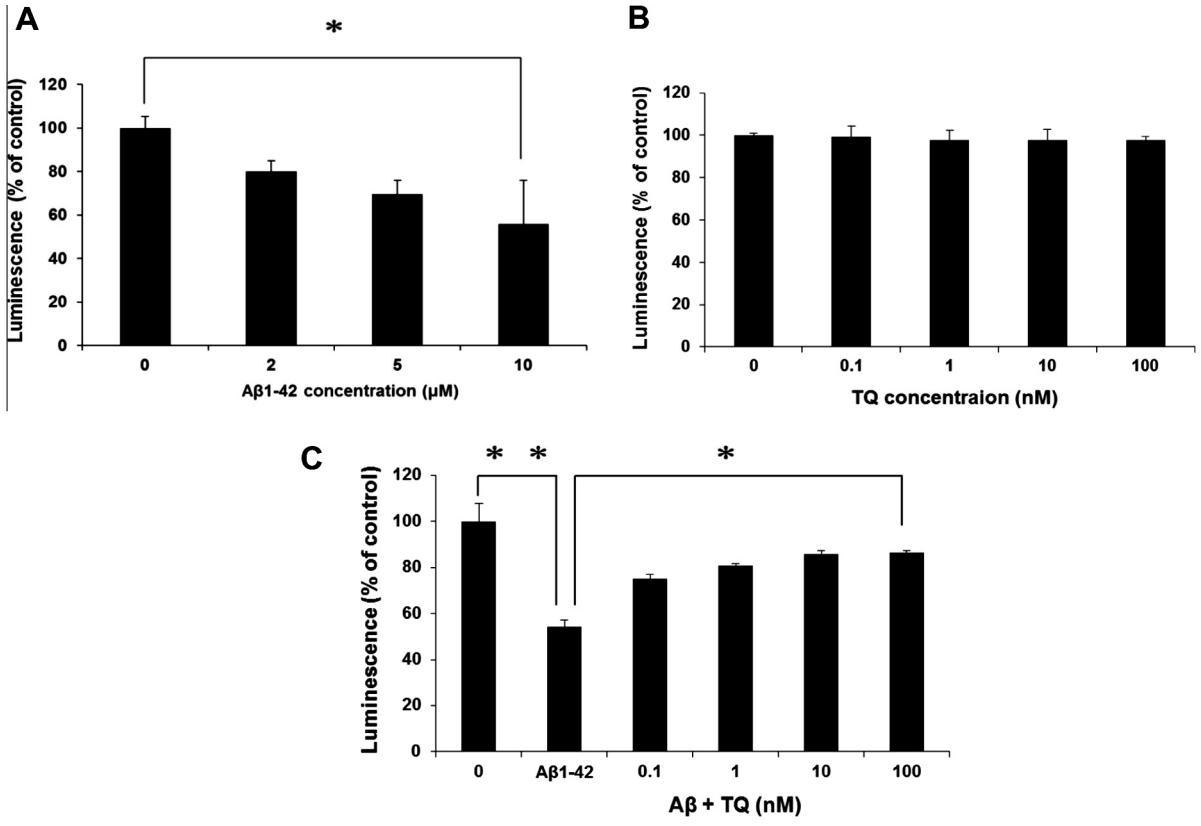

Fig. 1. Protective effects of TQ against $A\beta_{1-42}$-induced neurotoxicity in cultured hippocampal neurons. (A) Treatment with $A\beta_{1-42}$ (2, 5, 10 μM) for 72 h induced decrease in viability of hippocampal neurons in a dose-dependent manner. $^{*}P < 0.05$ vs. control. (B) Treatment with TQ (0.1, 1, 10, 100 nM) for 72 h had no significant effect on the survival rate of hippocampal neurons. (C) Hippocampal neurons were exposed to $A\beta_{1-42}$ (10 μM) for 72 h in the absence or presence of varying amounts of TQ (0.1, 1, 10, 100 nM). Cell viability was identified by CellTiter-Glo assay. Values shown are the mean %luminescence (where 100% = luminescence in control hippocampal neurons) ± SD, $n = 6$ ($^{*}P < 0.0003$ vs. Aβ alone; $^{**}P < 0.0003$ vs. control).

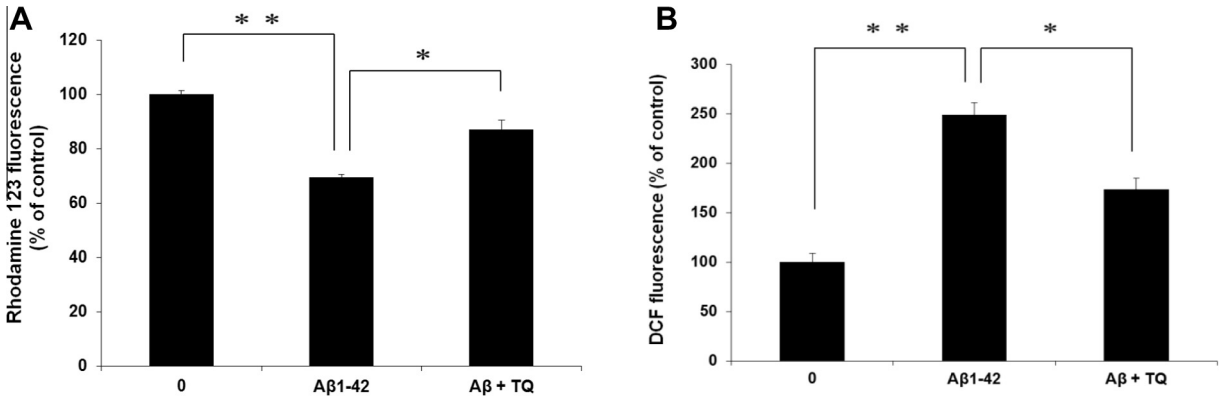

Fig. 2. Effect of TQ on mitochondrial membrane potential ($\Delta\psi m$) changes and ROS generation induced by $A\beta_{1-42}$. Neurons were treated for 72 h with $A\beta_{1-42}$ (10 μM) in the presence or absence of TQ (100 nM). (A) TQ attenuated $A\beta_{1-42}$-induced $\Delta\psi m$ depolarization. $\Delta\psi m$ was measured as Rhodamine 123. V alues shown are the mean %fluorescence (where 100% = fluorescence in control hippocampal neurons) ± SD, $n = 5$ ($^{*}P < 0.01$ vs. Aβ alone; $^{**}P < 0.0007$ vs. control). (B) Effect of TQ on alterations in ROS generation induced by $A\beta_{1-42}$. TQ inhibited $A\beta_{1-42}$-induced ROS generation. Values shown are the mean %fluorescence (where 100% = fluorescence in control cortical neurons) ± SD, $n = 5$ ($^{*}P < 0.001$ vs. Aβ alone; $^{**}P < 0.003$ vs. control).

synaptic vesicle recycling. The uptake of the fluorescent dye FM1–43 into synaptic vesicles was used as a measure of synaptic vesicle recycling, and thus neurotransmission. The addition of $A\beta_{1-42}$ (2 μM) affected synaptic vesicle recycling, as indicated by a 50%-reduction in FM1–43 uptake, compared with control samples ($P < 0.02$). However, co-administration of TQ (100 nM) enhanced the uptake of FM1–43 by 15% compared with control levels in cortical neurons and thus enhanced neurotransmission ($P < 0.001$; Fig. 3A).

3.5. Partial functional activity could be recovered by administration of TQ

We investigated neuronal activity using *in vitro* extracellular electrophysiological recording of the temporal electrical activity of cultured hippocampal neurons using MEA. Fig. 3B shows the pattern of spontaneous firing activity of hippocampal cells before and after the simultaneous administration of $A\beta_{1-42}$ (2 μM) and TQ (100 nM). TQ enabled the cells to maintain their spontaneous firing frequency even 72 h after administration.

Moreover, addition of $A\beta_{1-42}$ (2 μM) abolished spontaneous firing activity of hippocampal neurons; no spontaneous action potential was recorded in the exposed cells at 72 h after exposure. However, when we co-administrated TQ (100 nM), cells were able to maintain approximately 60% of their baseline firing activity, as opposed to the complete loss of functionality that resulted from treatment with $A\beta_{1-42}$ only (Fig. 3C).

3.6. TQ inhibited Aβ1–42 aggregation in vitro

While there is currently no effective treatment for AD, substances that can efficiently inhibit amyloid formation have been sought as drug candidates for treating AD. Therefore, to further clarify our previous results and to understand the mechanism by which TQ inhibited the neurotoxicity of Aβ, we investigated its effect on $A\beta_{1-42}$ amyloid formation.

Induced fluorescent change was observed for 72 h in the presence of $A\beta_{1-42}$ (10 μM), with or without TQ (100 nM). Compared with the control ThT sample, the sample incubated with $A\beta_{1-42}$ increased ThT fluorescence to 2.6-fold ($P < 0.0007$). However, the sample co-incubated with TQ reduced the fluorescence increase to 1.6-fold compared with the control sample, indicating that fewer cross β-sheets formed in this sample ($P < 0.001$; Fig. 4).

4. Discussion

The Aβ cascade hypothesis proposes its central role in the pathogenesis of AD. Aβ peptide induces neurodegeneration of cortical and hippocampal neurons through oxidative stress, secondary excitotoxicity, and a wide range of molecular events that disturb neuronal homeostasis. Thus, considerable attention has been focused on identifying naturally occurring antioxidants that are able to protect against Aβ-mediated oxidative damage. TQ, the most active constituent of *Nigella sativa* L. seed oil, was reported to display potent antioxidant and neuroprotective properties. The antioxidant properties of TQ appeared to play an important role in rescuing THir neurons against MPP+ and rotenone-induced cell death in dopaminergic cell cultures relevant to Parkinson's disease [15]. It has also been reported that TQ significantly reduced neuronal cell death in the hippocampal CA1 region from ischemia-induced brain injury [28], and caused morphologic improvement on neurodegeneration in the hippocampus in rats after chronic toluene exposure [17].

Oxidative stress is recognized as an early event in the neurodegenerative process in AD and plays a key role in Aβ-induced cell death. Several studies have shown that addition of Aβ to cultured neurons causes oxidative stress [29]. Excessive ROS, which mainly derives from electron leakage from the mitochondrial respiratory chain complexes, causes free radical attack of membrane phospholipids, leading to loss of mitochondrial membrane potential [30]. In the present study we demonstrated that TQ prevented $A\beta_{1-42}$-induced cell death in primary hippocampal neurons. Moreover, we found that TQ significantly inhibited $A\beta_{1-42}$-induced mitochondrial membrane potential collapse in cultured hippocampal neurons. We also tested the intra-cellular ROS levels and showed that the increased ROS level produced by incubation of primary cortical neurons with $A\beta_{1-42}$ is markedly reduced in cells subjected to TQ. Based on these findings, we proposed that the anti-oxidative action of TQ may contribute to its protection against Aβ-induced oxidative damage.

It is now accepted that diverse forms of Aβ are responsible for producing synaptic failure. Previous findings have demonstrated that soluble Aβ can also cause functional toxicity; it inhibits spontaneous firing of hippocampal neurons without causing significant cell death at low concentrations. This toxicity will eventually also lead to loss of the synapse [31]. We report here that the uptake of FM1–43 dye in cortical neurons was reduced following the addition of $A\beta_{1-42}$, but not after the addition of TQ. Therefore, TQ can

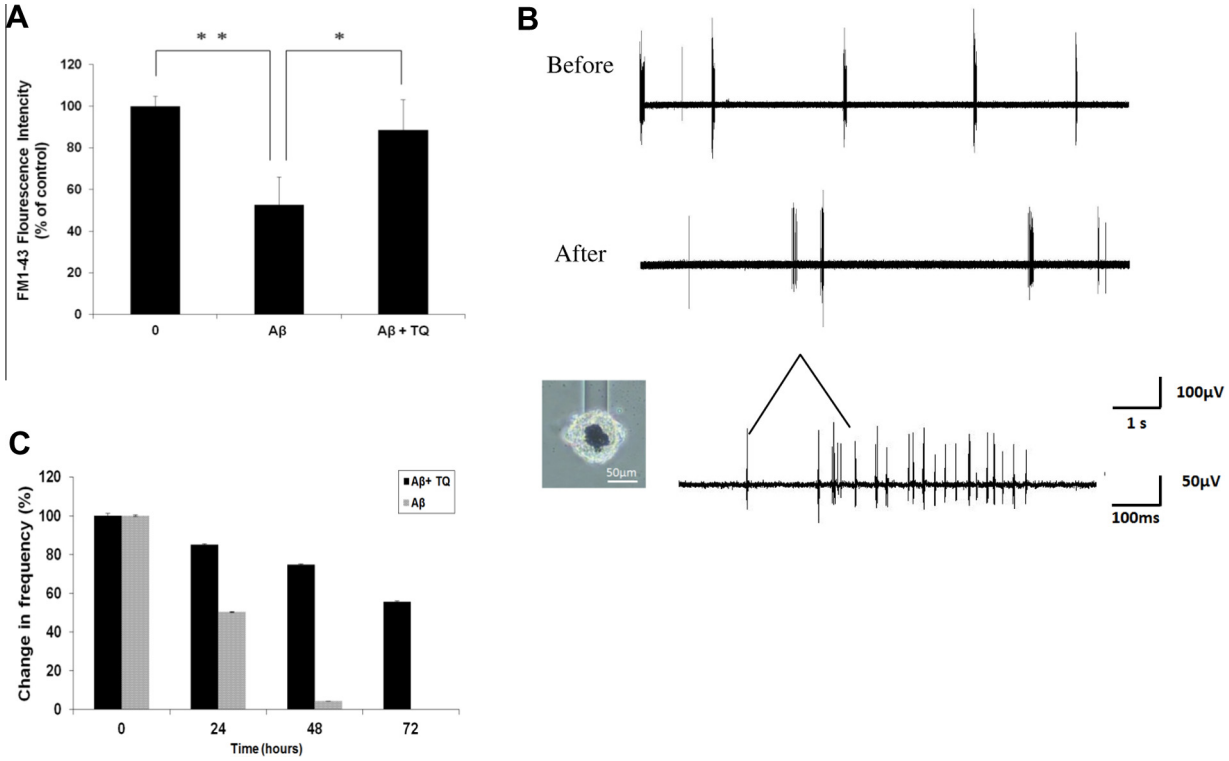

Fig. 3. Protective effects of TQ against $A\beta_{1-42}$-induced inhibition of synaptic vesicle recycling and inhibition of spontaneous firing activity. Neurons were treated for 72 h with $A\beta_{1-42}$ (2 μM) in the presence or absence of TQ (100 nM). (A) Effect of $A\beta_{1-42}$ and TQ on synaptic vesicle recycling. The co-administration of TQ increased the uptake of FM 1–43 dye and decreased the inhibitory effect of $A\beta_{1-42}$ upon synaptic vesicle recycling. Values shown are the mean %fluorescence (where 100% = fluorescence in control cortical neurons) ± SD, $n = 4$ ($^{*}P < 0.001$ vs. Aβ alone; $^{**}P < 0.02$ vs. control). (B) Spontaneous firing activity pattern before and after the simultaneous treatment of cells with $A\beta_{1-42}$ and TQ. The firing activity of hippocampal neurons had not disappeared by 72 h after the exposure. The phase contrast image shows hippocampal neurons (13 DIV) cultured inside the agarose microchamber and attached to the microelectrode probe of MEA 72 h after treatment with $A\beta_{1-42}$ and TQ. (C) Reversal of the effect of $A\beta_{1-42}$ on the firing frequency of hippocampal neurons by TQ. Time course of the effect of TQ on spontaneous firing frequency when co-administrated with $A\beta_{1-42}$. When the cells were exposed to $A\beta_{1-42}$, spiking activity started to decline 24 h after the exposure and the firing ceased after 72 h, $n = 29$. However, co-administration of TQ partially reversed the loss of spontaneous firing activity even 72 h after the exposure, $n = 51$. (100% implies baseline values before exposure to Aβ).

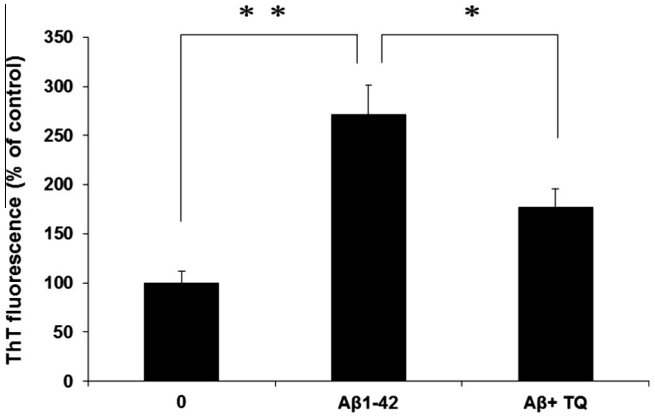

Fig. 4. Effect of TQ on $A\beta_{1-42}$ aggregation. The formation of fibrillary aggregates of $A\beta_{1-42}$ was measured by thioflavin T binding. Aβ (at a concentration of 10 μM in PBS) was incubated at 37 °C for 72 h in the presence or absence of TQ (100 nM) and peptide aggregation was measured by ThT fluorescence assay. Values shown are the mean %fluorescence (where 100% = fluorescence) ± SD, $n = 4$ ($^{*}P < 0.001$ vs. Aβ alone; $^{**}P < 0.0007$ vs. control).

reduce the inhibition of synaptic vesicle recycling caused by $A\beta_{1-42}$. We also report here that treatment of hippocampal neurons with $A\beta_{1-42}$ abolished spontaneous activity completely, while co-administration of $A\beta_{1-42}$ and TQ partially reversed this loss of spontaneous activity. Therefore, this functional deficit can be reversed through use of TQ, as proved by MEA.

Furthermore, using ThT fluorescence, we showed that TQ can inhibit $A\beta_{1-42}$ aggregation. What is the molecular mechanism used by TQ to inhibit $A\beta_{1-42}$ self-assembly? The most likely reason is that soluble Aβ undergoes a conformational change to high β-sheet content, rendering it prone to aggregation into soluble, and larger, insoluble, high-molecular weight assemblies. TQ treatment affected these dynamics. Alternatively, there may be multiple pools of Aβ, and TQ treatment affected the pool that is destined to become high-molecular-weight aggregates [32]. Although the precise mechanism of TQ neuroprotection remains unclear, the antioxidant ability, inhibition of functional toxicity, and disaggregation of $A\beta_{1-42}$ may be involved in the neuroprotective effects of TQ.

In conclusion, the study demonstrated for the first time that TQ can inhibit spontaneous aggregation and neurotoxicity of $A\beta_{1-42}$ in primary hippocampal and cortical neurons, providing evidence for TQ as a promising therapeutic agent for AD.

Acknowledgments

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