Neuroprotective efficacy of thymoquinone against amyloid beta-induced neurotoxicity in human induced pluripotent stem cell-derived cholinergic neurons

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\section*{ABSTRACT}

The natural antioxidant Thymoquinone (TQ) is the most abundant ingredient in the curative plant Nigella sativa seed's oil. An extensive number of studies have revealed that TQ is the most active and most responsible component for the plant's pharmacological properties. It has been documented in several studies that TQ has a wide range of protective activities and many neuropharmacological attributes. Amyloid beta (A\textsubscript{β}) is the major role player peptide in the progression of Alzheimer's disease (AD). Our current study has been implemented to explore the protective possibilities of TQ on A\textsubscript{β1-42} -induced neurotoxicity. To test TQ's effect we used cultured human induced pluripotent stem cell (hiPSC)-derived cholinergic neurons. The obtained results showed that A\textsubscript{β1-42} caused cell death and apoptosis, which was efficiently attenuated by the co-treatment of TQ. Moreover, TQ restored the decrease in the intracellular antioxidant enzyme glutathione levels and inhibited the generation of reactive oxygen species induced by A\textsubscript{β1-42}. Furthermore, using the fluorescent dye FM1-43 we demonstrated that TQ was able to reduce synaptic toxicity caused by A\textsubscript{β1-42}. Thus, the findings of our study suggest that TQ holds a neuroprotective potential and could be a promising therapeutic agent to reduce the risk of developing AD and other disorders of the central nervous system.

\section*{1. Introduction}

As a neurodegenerative disorder, Alzheimer's disease (AD) slowly destroys memory and cognitive function and is known to be the most prevalent cause of dementia in the elderly [1]. In AD patient brain, there are two distinctive histopathological abnormalities: (1) the intracellular neurofibrillary tangles consisting of hyper phosphorylated tau protein, and (2) the extracellularly formed plaques composed of amyloid beta (A\textsubscript{β}) peptide. The cholinergic neurons system has been a major focus in neurodegenerative research and aging for many years since it has a strong correlation with AD [2]. Among the earliest well-established pathological events in AD, is the impairment in function and loss of basal forebrain cholinergic neurons and their cortical cholinergic innervation that strengthen the hippocampus and the neocortex [3,4]. This process of neurodegeneration is triggered by the accumulation of A\textsubscript{β} peptide [5], which have also been hypothesized to induce neurodegenerative changes at cholinergic terminals [6]. Additionally, other studies revealed that oligomeric A\textsubscript{β} induces cell death [7], encourages apoptosis by physically piercing the cell membrane, causes neurotoxic cascade and neurodegeneration that leads to AD [8,9].

Oxidative stress occurs in the early phase of AD and is known to play an essential role in its pathology and development in relation to the presence of A\textsubscript{β} [10]. It is an important feature in AD marked by overproduction of reactive oxygen species (ROS), oxidation in neuronal lipids, proteins, DNA, and RNA leading to the dysfunction and loss of neurons [11]. As previously reported by several studies, AD brain suffers from a significant low content of antioxidant enzymes, which leads the brain to be more susceptible to toxic effects induced by A\textsubscript{β} [12]. Thus, antioxidants have been considered for a long time as an approach to slow down AD progression.

In recent years the herbal medicinal plant Nigella sativa has been actively investigated for its established historical and religion-based remedy for a wide range of health problems and therefore is gaining worldwide attention [13]. Reports have described \textit{Nigella sativa} as possessing many therapeutic effects, including anti-inflammatory, anti-tumor, antimicrobial and immune potentiation in addition to...
antioxidant and neuroprotective effects [14–16]. Maintaining cell health should be considered as one of the most important strategies to prevent damage from oxidative stress, especially in areas vulnerable to oxidative stress such as the brain and that could be achieved by consuming nutrients rich in antioxidants, such as Nigella sativa [17].

The natural antioxidant thymoquinone (TQ) is most the bioactive ingredient of the volatile oil of Nigella sativa seeds. TQ seems promising due to its numerous biological properties, which include antioxidant, anti-inflammatory, and anticancer attributes [18,19] that might be useful in the management of AD. It was demonstrated that in primary rat cortical neurons TQ has a protective role against ethanol-induced neuronal apoptosis [20]. TQ was additionally reported to reduce per-oxidation levels, enhance enzymatic and non-enzymatic antioxidants activities in rats' brain tissue [21], and protects against cytotoxic agents via attenuation of oxidative stress in PC12 cells [22]. Taking into consideration the rising attention in using herbal medicine for the treatment of chronic disorders, the neuroprotective possibilities of TQ seems to be hopeful in the management of neurodegenerative disorders.

Thus, in the current study, we evaluated the effect of TQ and Aβ1-42 on cell viability, caspase 3/7 activities, glutathione (GSH) level, ROS generation, and synaptic activity in human induced pluripotent stem cell (hiPSC)-derived cholinergic neurons.

2. Materials and methods

2.1. Cell culture

Human iPSC-derived cholinergic neurons cell line was obtained from (ReproCell, RCESDA001). 96 micro-well tissue culture plates were coated with poly-β-lysine for 2 h and then treated with (ReproCell) coating solution overnight. Cells were cultured at a density of 3.0 × 10^4 cell/well and were grown at 37 °C in a humid atmosphere of 5% CO2 and 95% air.

2.2. Reagents and treatment

Amyloid β-protein 1–42 (Aβ1-42) was obtained from Peptide Institute Inc., prepared at 1 mM in dimethyl sulfoxide (DMSO, Wako) and stored at ~ 20 °C. A solution of Thymoquinone (TQ) obtained from (Sigma-Aldrich) was freshly prepared on the day of use at 10 mM in DMSO and final concentration was diluted in culture medium.

The concentration of TQ in this study was determined based on a previously established dose curve [23] and the optimal concentration of TQ was selected based on cell viability. When TQ was applied in different concentrations simultaneously with Aβ1-42, it resulted in a striking improvement in cell survival, in a dose-dependent manner; and the maximal rescue occurred at a dose of 100 nM.

Additionally, it was previously established [24] that the treatment of human iPSC-derived neurons with Aβ1-42 (5 μM) resulted in neuronal toxicity when applied to cultures for 48 h. Therefore, in our current study, hiPSC-derived cholinergic neurons were treated with Aβ1-42 (5 μM) and TQ (100 nM). Cultures were treated on day 13 DIV.

2.3. Measurement of cell viability

The protective effect of TQ on cell viability of hiPSC-derived cholinergic neurons was determined by measuring ATP amount which is relevant to the number of live cells in culture using (CellTiter-Glo) assay from Promega. Cultures were treated with Aβ1-42 (5 μM) with or without TQ (100 nM) for 48 h. Cell viability was assessed according to the manufacturer's instructions. Luminescence signals were measured using (TECAN) microplate reader.

2.4. Measurement of caspase-3 and -7 activities

Using the Caspase-Glo 3/7 assay (Promega), the effect of TQ and Aβ1-42 on apoptosis was investigated by measuring Caspase 3 and 7 activities. Aβ1-42 (5 μM) was added to hiPSC-derived cholinergic neurons alone or simultaneously with TQ (100 nM) for 48 h. At the end of the incubation time, assay reagents were prepared according to the manufacturer's instructions and applied to the cells. After 1 h incubation at room temperature, a microplate reader (TECAN) was used to measure luminescence signals.

2.5. Measurement of antioxidant enzyme glutathione

Oxidative stress was assessed through measurement of the glutathione (GSH) using the GSH Glo™ Glutathione assay (Promega). Seeded cells were treated with Aβ1-42 (5 μM) with or without TQ (100 nM) for 48 h. The assay was performed according to the manufacturer's instructions. After 48 h of the treatment, the first prepared reagent was applied to the cells and incubated for 30 min at room temperature. Then, the second prepared reagent of the assay was applied to the cells for 15 min at room temperature. A microplate reader (TECAN) was used to measure luminescence signals.

2.6. Measurement of intracellular ROS

Intracellular ROS level was determined by ROS™ H2O2 assay (Promega). The assay determines ROS level by measuring hydrogen peroxide (H2O2) concentration. Cultures were treated for 48 h with Aβ1-42 (5 μM) with or without TQ (100 nM). After 42 h, the first prepared reagent of the assay, was applied to the cells and the incubation continued for 6 h according to the manufacturer's instructions. Then, the second prepared reagent of the assay was applied to the cells for 20 min at room temperature. Using a microplate reader (TECAN) luminescence signals were measured.

2.7. Measurement of synaptic vesicles recycling activity (FM1–43 assay)

To assess the effect of Aβ1-42 and TQ on synaptic vesicles recycling, we used the fluorescent dye FM1–43 (Molecular Probes) that measures synaptic vesicles recycling. hiPSC-derived cholinergic neurons were treated with Aβ1-42 (5 μM) only or simultaneously with TQ (100 nM) for 48 h on culture day 13. On the final day of treatment, the culture medium was removed, and the neurons were incubated with 1 μg/ml artificial cerebrospinal fluid (ACSF)/FM1–43 solution for 5 min at 37 °C. Ice-cold phosphate buffered saline (PBS) was added to wash the cells three times and then for cell suspension. The excitation wavelength of FM 1–43 fluorescence intake was measured at 480 nm and the emission wavelength at 612 nm with (TECAN) microplate reader.

2.8. Statistical analysis

All data reported are expressed as mean ± SEM. Statistical significance of the results was calculated using one-way ANOVA followed by the Holm-Bonferroni method. The changes in parameters induced by all externally applied chemicals were quantified as a percentage of baseline values.

3. Results

3.1. Effect of Aβ1-42 and TQ on the survival of hiPSC-derived cholinergic neurons

The cytotoxicity of Aβ1-42 and the protective effect of TQ were evaluated using the CellTiter-Glo assay. Fig. 1A shows the effect of Aβ1-42 (5 μM) with or without TQ (100 nM) on hiPSC-derived cholinergic neurons viability. Treatment with Aβ1-42 for 48 h significantly decreased cell viability to 63.5% as compared to the control group (*P < 0.05). However, co-treating the cells with TQ (100 nM) prevented Aβ1-42 induced loss and protected the cells by restoring viability.
3.2. Effect of Aβ1-42 and TQ on caspase 3/7 activities

As illustrated in Fig. 1B, treatment of hiPSC-derived cholinergic neurons with Aβ1-42 (5 μM) induced about 90% increase in the caspase 3/7 activities (**P < 0.01). However, TQ (100 nM) co-treatment restored caspase 3/7 activities to control sample level (**P < 0.01).

3.3. Effect of Aβ1-42 and TQ on level of antioxidant enzyme GSH

In Fig. 2A, Aβ1-42 (5 μM) treatment induced a 53% decrease in GSH level as compared to control group with no significant difference (P = 1). Co-treatment with TQ (100 nM) has abolished Aβ1-42 effect and protected GSH level with a 37.5% increase in GSH level compared to control with no significant difference (P = 0.91).

3.4. Effect of Aβ1-42 and TQ on intracellular ROS level

To clarify the possible antioxidant effect of TQ, the accumulation of ROS was evaluated. In Fig. 2B, the treatment with Aβ1-42 (5 μM) caused a significant increase of 115% in H2O2 concentration compared to control (**P < 0.01). When the cells were treated with both Aβ1-42 and TQ, H2O2 concentration was decreased significantly with 87% decline compared to Aβ1-42 treated group (**P < 0.01).

3.5. Effect of Aβ1-42 and TQ on synaptic vesicles recycling

In Fig. 3, we demonstrated that the addition of Aβ1-42 (5 μM) for 48 h induced a 6-fold increase in the uptake of FM1-43 (**P < 0.01),
which correlates with an increase in the synaptic activity. However, the co-treatment with TQ (100 nM) reduced the increase in the activity by 2.6-fold compared to Aβ1–42 treated sample (**P < 0.01).

4. Discussion

The major neuropathological hallmark of AD is the neurotoxic production and deposition of Aβ [25]. It has been demonstrated by numerous reports that oxidative stress plays an essential part in neuronal toxicity mediated by Aβ which triggers and facilitates neurodegeneration via a large extent of molecular actions that ultimately leads to the death of neurons [26]. Even though the precise mechanisms of Aβ cytotoxicity is still not completely elucidated, lots of studies tried to find how to inhibit the Aβ toxicity on the neurons [27].

TQ, a major active ingredient present in Nigella sativa seed’s oil, has been subjected to a wide range of pharmacological investigations in recent years [28]. Due to its strong antioxidant capabilities, TQ has been demonstrated to protect the brain and the spinal cord from oxidative damage generated by different pathologies induced by a variety of free radicals [29,30]. Moreover, TQ prevented cell death in rat cerebellar granule neurons and attenuated intracellular oxidative stress induced by Aβ in PC12 cells [31,32]. Additionally, TQ was found to effectively ameliorate neurodegeneration [33–35].

Initial cognitive decline observed in AD is strongly related to the cholinergic basal forebrain dysfunction, which forms the basis of the ‘cholinergic hypothesis’ of AD [36]. hiPSC-derived neurons are a great promising tool for they can be applied in human disease modeling, drug evaluation, and how to inhibit the Aβ toxicity on the neurons of the central nervous system. The results strongly suggest the intracellular pathway of TQ to protect against the Aβ1–42 induced neurotoxicity. In conclusion, to the extent of our knowledge, we believe that this work is the first to indicate the neuroprotective effect of TQ against Aβ1–42 induced neurotoxicity in cultured hiPSC-derived cholinergic neurons. The results strongly suggest the intracellular pathway of TQ to protect against the Aβ-induced toxicity on neurons of the central nervous system.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.12.005.

References

[1] T.H. Ferreira-Vieira, L.M. Guimaraes, F.R. Silva, et al., Alzheimer's disease: targeting the cholinergic system, Curr. Neuropharmacol. 14 (2016) 101–115.
[2] R. Schliebs, T. Arrendt, The cholinergic system in aging and neuronal degeneration, Behav. Brain Res. 221 (2011) 555–563.
[3] C. Nyakas, I. Granic, L.G. Halmy, et al., The basal forebrain cholinergic system in ageing and dementia, rescuing cholinergic neurons from neurotoxic amyloid-β/2 with memantine, Brain Res. 221 (2011) 594–603.
[4] R. Goekoop, P. Scheltens, F. Barkhoh, et al., Cholinergic challenge in Alzheimer...
patients and mild cognitive impairment differentially affects hippocampal activation in a pharmacological FMRI study, Brain 129 (2006) 141–157.

[5] A. Baker-Nigh, S. Vahedi, E.G. Davis, et al., Neuronal amyloid-β accumulation within cholinergic basal forebrain in ageing and Alzheimer’s disease, Brain 158 (2015) 1722–1737.

[6] M. Klingner, J. Apelt, A. Kumar, et al., Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse brain with β-amyloid plaque pathology, Int. J. Dev. Neurosci. 21 (2003) 357–369.

[7] B.M. Foidl, P. Do-Dinh, B. Hutter-Schmid, Cholinergic neurodegeneration in an Alzheimer mouse model expressing amyloid-precursor protein with the Swedish-Dutch-Iowa, Neurobiol. Learn. Mem. 136 (2016) 86–96.

[8] K. Heinitz, M. Beck, R. Schliebs, et al., Toxicity mediated by soluble oligomers of beta-amyloid (1–42) on cholinergic SN56.B54G cells, J. Neurochem 98 (2006) 1930–1945.

[9] S. Kar, S.P. Slowikowski, D. Westaway, et al., Interactions between beta-amyloid and central cholinergic neurons: implications for Alzheimer’s disease, J. Psychiatry Neurosci. 29 (2004) 427–441.

[10] T. Jiang, Q. Sun, S. Chen, Oxidative stress: a major pathogenesis and potential therapeutic target of antioxidative agents in Parkinson’s disease and Alzheimer’s disease, Prog. Neurobiol. 147 (2016) 1–26.

[11] C. Cheighjon, M. Tomasz, D. Bonnefont-Rousselot, et al., Oxidative stress and the amyloid beta protein in Alzheimer’s disease, Redox Biol. 14 (2018) 450–464.

[12] G.F. Makheva, S.V. Lushchikina, N.P. Bolneva, et al., 9-Substituted acridine derivatives as acetylcolinesterase and butyrylcholinesterase inhibitors possessing antioxidant activity for Alzheimer’s disease treatment, Bioorg. Med. Chem. 25 (2017) 5981–5994.

[13] M.R. Khadzair, The protective effects of nigella sativa and its constituents on induced neurotoxicity, J. toxicol. 2015 (2015) 8418.

[14] S.S. Ashraf, M.V. Rao, F.S. Kannez, et al., Nigella sativa extract as a potent anti-oxidant for petrochemical-induced oxidative stress, J. chromatogr. Sci. 49 (2011) 521–526.

[15] S. Javid, R.M. Razavi, H. Hosseinzadeh, A review of neuropharmacological effects of Nigella sativa and its main component, thymoquinone, Phytother. Res. 30 (2016) 1219–1229.

[16] W. Kooti, Z. Hasanzadeh-Noohi, N. Sharafi-Alvazi, et al., Phytochemistry, pharmacological, and therapeutic uses of black seed (Nigella sativa), Chin. J. Nat. Med. 14 (2016) 732–745.

[17] M.K. Sahak, A.M. Mohamed, N.H. Hashim, et al., Nigella sativa oil enhances the cholinergic basal forebrain in ageing and Alzheimer’s disease, Brain 158 (2015) 1722–1737.

[18] K. Takahashi, S. Yamanka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.

[19] D. Lauriaut, J. Gide, M. Peschanski, Human pluripotent stem cells in drug discovery and predictive toxicology, Biochem. Soc. Trans. 38 (2010) 1051–1057.

[20] C.D. Alise, R.A. Robinson, J. Cai, et al., Redox proteomics analysis of brains from subjects with amnestic mild cognitive impairment compared to brains from subjects with preclinical Alzheimer’s disease: insights into memory loss in MCI, J. Alzheimer Dis. JAD 2 (2011) 257–269.

[21] J. Kristhal, O. Bragina, K. Metsla, et al., In situ fibrillizing amyloid-beta 1–42 in duces neurite degeneration and apoptosis of differentiated SH-SY5Y cells, PLoS One (2017), https://doi.org/10.1371/journal.pone.0168636.

[22] J. Kim, C.H. Cho, H.G. Hahn, et al., Neuroprotective effects of N-adamantyl-4-methylthiazol-2-amine against amyloid β-induced oxidative stress in mouse hippocampus, Brain Res. Bull. 128 (2017) 22–28.

[23] C. Stadelmann, T.L. Deckwerth, A. Srinivasan, et al., Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer’s disease. Evidence for apoptotic cell death, Am. J. Pathol. 155 (1999) 1459–1466.

[24] M. Anello, M. Sheng, F. Ceconi, Caspase-3 in the central nervous system: beyond apoptosis, Trends Neurosci. 35 (2012) 700–709.

[25] I. Baldeiras, I. Santana, M.T. Proenca, et al., Peripheral oxidative damage in mild cognitive impairment and mild Alzheimer’s disease, J. Alzheimer Dis. 15 (2008) 117–128.

[26] L.O. Soto-Rojas, F. de la Cruz-López, M.A.O. Torres, et al., Neuroinflammation and alteration of the blood-brain barrier in Alzheimer’s disease, in: I. Zerr (Ed.), Alzheimer’s Disease – Challenges for the Future, InTech, United Kingdom, 2015, pp. 48–71.

[27] B. van der Kooi, A. de Vries, E. Altena, et al., Microglia-dendritic cell crosstalk in Alzheimer’s disease, Stem Cell Rev. 10 (2014) 132–143.

[28] E. Abramow, I. Dolev, H. Fogel, et al., Amyloid beta as a positive endogenous regulator of neural activity in hippocampal slices, Neur. Neurosurg. 12 (2009) 1567–1576.

[29] D. Dinner, J. Parodi, F.J. Sepulveda, J. Roa, et al., Beta amyloid causes depletion of synaptic vesicles leading to neurotransmission failure, J. Biol. Chem. 285 (2010) 2506–2514.

[30] M. Kozlowski, M. Luczkowski, M. Remelli, D. Valensin, Copper, zinc and iron in neurodegenerative diseases (Alzheimer’s, Parkinson’s and prion diseases), Coord. Chem. Rev. 256 (2012) 2129–2140.

[31] S.A. Lipton, Paradigm shift in neuroprotection by NMDA receptor blockade: lessons from animal models of neurodegeneration, Trends Pharmacol. Sci. 33 (2012) 250–256.

[32] H. Kozlovska, M. Luckowski, M. Remelli, D. Valensin, Copper, zinc and iron in neurodegenerative diseases (Alzheimer’s, Parkinson’s and prion diseases), Coord. Chem. Rev. 256 (2012) 2129–2140.

[33] S.A. Lipton, Paradigm shift in neuroprotection by NMDA receptor blockade: lessons from animal models of neurodegeneration, Trends Pharmacol. Sci. 33 (2012) 250–256.

[34] I. Kim, C.H. Cho, H.G. Hahn, et al., Neuroprotective effects of N-adamantyl-4-methylthiazol-2-amine against amyloid β-induced oxidative stress in mouse hippocampus, Brain Res. Bull. 128 (2017) 22–28.

[35] C. Stadelmann, T.L. Deckwerth, A. Srinivasan, et al., Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer’s disease. Evidence for apoptotic cell death, Am. J. Pathol. 155 (1999) 1459–1466.

[36] M. Anello, M. Sheng, F. Ceconi, Caspase-3 in the central nervous system: beyond apoptosis, Trends Neurosci. 35 (2012) 700–709.

[37] I. Baldeiras, I. Santana, M.T. Proenca, et al., Peripheral oxidative damage in mild cognitive impairment and mild Alzheimer’s disease, J. Alzheimer Dis. 15 (2008) 117–128.

[38] L.O. Soto-Rojas, F. de la Cruz-López, M.A.O. Torres, et al., Neuroinflammation and alteration of the blood-brain barrier in Alzheimer’s disease, in: I. Zerr (Ed.), Alzheimer’s Disease – Challenges for the Future, InTech, United Kingdom, 2015, pp. 48–71.

[39] B. van der Kooi, A. de Vries, E. Altena, et al., Microglia-dendritic cell crosstalk in Alzheimer’s disease, Stem Cell Rev. 10 (2014) 132–143.

[40] E. Abramow, I. Dolev, H. Fogel, et al., Amyloid beta as a positive endogenous regulator of neural activity in hippocampal slices, Neur. Neurosurg. 12 (2009) 1567–1576.

[41] D. Dinner, J. Parodi, F.J. Sepulveda, J. Roa, et al., Beta amyloid causes depletion of synaptic vesicles leading to neurotransmission failure, J. Biol. Chem. 285 (2010) 2506–2514.

[42] M. Kozlowski, M. Luczkowski, M. Remelli, D. Valensin, Copper, zinc and iron in neurodegenerative diseases (Alzheimer’s, Parkinson’s and prion diseases), Coord. Chem. Rev. 256 (2012) 2129–2140.

[43] S.A. Lipton, Paradigm shift in neuroprotection by NMDA receptor blockade: lessons from animal models of neurodegeneration, Trends Pharmacol. Sci. 33 (2012) 250–256.