Anti-aggregation effects of thymoquinone against Alzheimer’s β-amyloid in vitro

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Beta amyloid peptide (Aβ) aggregation has been identified as one of the major causes of neurodegenerative processes in Alzheimer’s disease (AD). Despite recent improvements in the symptomatic therapy of cholinergic drugs; development of an effective therapeutic approach, that may interfere directly with Aβ aggregation in the central nervous system, is desperately required. Thymoquinone (TQ) is a bioactive compound isolated from Nigella sativa, which has been reported to be possessing various pharmacological attributes. The effects of TQ on the formation of Aβ1-40 were studied spectrophotometrically, over a duration of 16 days at pH 7.2 and 37°C. The inhibitory properties of TQ were compared with tannic acid (TA). Thymoquinone and TA, with IC50 of 0.1 and 29 µM at day 0 (within 5h), respectively, inhibited Aβ aggregation in a dose-dependent way at different concentrations, that is 1, 10 and 50 µM. However, at day 16, IC50 of TQ and TA were found to be 0.2 and 0.01 µM, respectively. In electron microscopic study, TQ, co-incubated with Aβ1-40, reduced the numbers of fibrils in some degree with shorter fibrils and small amorphous aggregates. Pretreated TQ protected cytotoxic effects of Aβ1-40 on primary cultured cerebellar granule neurons (CGNs). Therefore, TQ might have a direct interaction with Aβ resulting in prevention of Aβ aggregation and mediating its neuroprotective effects; thus may have potential in the therapeutic development of AD.

Key words: Anti-aggregation, β-amyloid, thymoquinone, tannic acid, Alzheimer’s disease.

INTRODUCTION

Alzheimer’s disease is ranked among the major causes of dementia among the elderly population (Sudhir, 2004). So far, only four drugs, that is, acetylcholinesterase (AChE) inhibitors; tacrine (Cognex®), donepezil (Aricept®), rivastigmine (Reminyl®) and memantine (Namenda®), have been approved by U.S. Food and Drug Administration for the treatment of Alzheimer’s disease (AD) patients. But these drugs can only treat the cognitive and behavioral symptoms of AD (Anekonda and Reddy, 2005), causing some side effects such as hepatotoxicity and peripheral cholinergic effects (Benzi and Moretti, 1998). The main pathological hallmarks of
AD are senile plaques and neurofibrillary tangles in patient brain (Yankner, 1996; Selkoe, 1999). The major constituent of senile plaques is Aβ peptide with 39 to 43 amino acids, which is the by-product of amyloid precursor protein (Hardy and Higgins, 1992). Aβ1-40, Aβ25-35 and Aβ1-42 have been reported to be neurotoxic to neuronal primary culture and cell lines (Irie and Keung, 2003; Jeong et al., 2005; Yu et al., 2005; Lai et al., 2006). Their toxic effects have been contributed due to aggregation of Aβ in the forms like oligomers (Roher et al., 1996), protofibrils and fibrils (Ward et al., 2000). Amyloid fibrils and oligomers, which are formed via self-assembly of peptide and protein monomers, have been found to be associated with a crucial process in the pathogenesis of AD (Harper and Lansbury, 1997; Hensley et al., 1994).

Although there are no effective treatments for AD at the moment, but many therapeutic target molecules have been reported to be directly interfering with the formation, aggregation, deposition and clearance of beta amyloid peptide (Aβ) (Ono et al., 2006a). Among the candidate molecules, non-steroidal anti-inflammatory drugs (Hirohata et al., 2005) and anti-Parkinsonian agents (Ono et al., 2006a) are quite common. Since significant studies have demonstrated that oxidative stress may play a key role in neurodegeneration of AD (Grundman and Delaney, 2002), number of antioxidative compounds have been determined to protect neurons from Aβ toxicity in vitro (Kumar and Gupta, 2003), like lyophilized red wine (De Ruvo et al., 2000), grape polyphenols (Sun et al., 1999), quercetin (Shutenko et al., 1999) and (+)-catechin (Inanami et al., 1998). Moreover, many antioxidant compounds have also been reported to be possessing the direct inhibitory properties towards Aβ fibril formation, some of these identified compounds include polyphenols such as resveratrol and piceid (Riviere et al., 2007), curcumin and its analog rosmarinic acid (Ono et al., 2004a), myricetin (Ono et al., 2003), nordihydroguaiaretic acid and rifampicin (Naiki et al., 1998). Nevertheless, resveratrol diglucoside, piceatannol, astringine and viniferin exerted less inhibition in comparison to curcumin (Riviere et al., 2007).

Intake of wine containing polyphenols has been reported to be responsible for lowering the risks of AD (Luchsinger and Mayeux, 2004). On the other hand, lipophilic antioxidants, vitamin A (Ono et al., 2004) and coenzyme Q10 (Ono et al., 2005), α-lipoic acid and its metabolic product, that is dihydrolipoic acid (Ono et al., 2006b) exhibited the formation of Aβ fibril. Tannins are commonly found in plants and herbs and are reported to be exhibiting much stronger inhibitory effects on lipid peroxidation as compared to vitamin E (Okuda et al., 1983). Previous studies have indicated that tannic acid (TA) prevented the development of AD through both modes; by scavenging the reactive oxygen species and inhibiting the deposition of fibril Aβ in the brain (Ono et al., 2004c).

Thymoquinone (TQ) is a bioactive constituent of *Nigella sativa* and is well-known for its different biological activities such as antioxidant, anti-inflammatory, anti-microbial, anti-tumor, immunomodulatory, hypertension, anti-nociceptive, uricosuric, choleretic, anti-fertility, anti-diabetic, and anti-histaminic (Salem, 2005). But no report describing the inhibition potential of TQ for Aβ fibril formation and aggregation has been presented so far. In view of the fact that increasing number of people across the globe are switching towards use of herbal medicines as supplements for treatment of different diseases, it would be of immense significance to explore the potential of a well documented plant based bioactive compound or herbal medicine as a neuroprotective agent (Lai et al., 2006).

In the present study, inhibitory effects of TQ in comparison to tannic acid (TA) on Aβ fibril formation have been investigated. UV-visible spectroscopic measurements and electron microscopy have been employed at pH 7.2 and 37°C for 16 days in vitro. The neuroprotective effects of TQ were examined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, lactate dehydrogenase (LDH) release assay and the caspases-3, -8 and -9 activations. This work will contribute a new source of botanical origin for preventing the aggregation of Aβ to the existing database.

**MATERIALS AND METHODS**

**Chemicals**

Aβ1-40 peptide, dimethyl sulfoxide (DMSO), TQ, TA and other chemicals were purchased from Sigma-Aldrich (St.Louis, MO, USA).

**Preparation of Aβ1-40 peptide, TQ and TA solutions**

Stock solution of Aβ1-40 peptide (0.23 mM) was prepared by solubilizing the lyophilized peptide in phosphate buffered saline (PBS), pH 7.2. All the steps were carried out at 4°C to prevent the polymerization of Aβ1-40. Thymoquinone was dissolved in dimethyl sulfoxide (DMSO), while TA in distilled water to prepare respective stock solutions of 0.9 mM. The stock solution was further diluted to different concentrations of 1, 10 and 50 μM and stored at -20°C. To study the kinetics of Aβ1-40 aggregation, experiments were carried out using a solution mixture containing 80 μl of 0.8 mM PBS (pH 7.2) and 10 μl of 5, 10, 25, 50 or 100 μM Aβ1-40. This solution mixture was sonicated for 1 min to ensure that no peptide aggregation was formed. Ten microliters of 0.1% DMSO were added to this solution to create the same conditions as in presence of TQ, which was solubilized in 0.1% DMSO. To study the inhibitory activity of TQ and TA on Aβ aggregation, the mixture solution was prepared by adding 80 μl of 0.8 mM PBS (pH 7.2), 10 μl of 50 μM Aβ1-40 and 10 μl of TQ or TA at 1, 10 and 50 μM. All the preparations were carried out at 37°C. This experiment was performed according to Riviere et al. (2007) with some modifications such as...
temperature, final concentration of PBS and Aβ sequences.

**Measurement of Aβ aggregation and inhibitory activity by UV-visible spectroscopy**

Aβ aggregation and inhibitory activity was measured using a Mini UV-VIS 1240 spectrophotometer (Shimadzu, Japan). Initially, the UV spectra of TQ and TA were recorded over the range of 190 to 500 nm at 37°C in order to control any artifacts due to its own aggregation effects. Then the optimal measurements of Aβ1-40 were recorded within 190 to 500 nm at 37°C. The anti-aggregation effects of Aβ1-40, in presence of TQ or TA were monitored from 0 to 16 days at 220 nm; to observe the absorption of peptide bond. To rule out any influence due to compounds absorbance, their UV-visible spectra were subtracted from Aβ1-40 absorption spectra.

**Observation of transmission electron microscopy (TEM)**

After 16 days of incubation at 37°C, 20 μl of Aβ1-40, in presence of TQ or TA were viewed under TEM. The samples were prepared on continuous carbon support films, followed by glow discharging and negative staining of 2% aqueous uranyl acetate (pH 4.5) by the single droplet procedure. The Aβ1-40 in presence of TQ or TA was adsorbed onto the carbon film for 5 min, washed with 2 μl droplets of water and uranyl acetate solution, each and were viewed under a TEM Philips HMG 400 at 30,000 and 100,000× magnifications. A representative assessment of Aβ fibril formation was made at several positions (~10) across each EM grid, to avoid the inadvertent production of a biased/subjective data selection.

**Neurotoxicity assays (MTS and LDH)**

The MTS and LDH release assays, as an indicator for cell viability and cell death, respectively were performed according to our previous studies (Ismail et al., 2008).

**Caspases-3, -8 and -9 activations**

The caspase-3 activity was determined using Ac-DEVD-pNA, as a colorimetric specific substrate (Caspase™ Assay System, Promega) labeled with chromophore p-nitroaniline (pNA). Briefly, after treatment, the cells were harvested by centrifugation at 450 g for 10 min at 4°C. The cell pellets were kept on ice followed by resuspension in cell lysis buffer. The supernatant fraction (cell extract) was collected and 2 μl of DEVD-pNA substrate (10 mM stock) were added to each sample. The absorbance of caspase-3 activity was measured after 4 h incubation at 37°C by microplate reader at 405 nm. Caspase-8 and -9 activities were measured using Kits, Colorimetric, from Sigma-Aldrich (St Louis, MO, USA) and Chemicon Inc., (Pennsylvania, USA), respectively. The substrates used were Ac-IETD-pNA and Ac-LEHD-pNA for caspase-8 and -9, respectively. The samples preparation was similar to caspase-3 procedure.

**RESULTS AND DISCUSSION**

**Aggregation of Aβ by UV-visible spectroscopy**

The Aβ1-40 spectra showed maximum absorbance at 220 nm (Figure 1A). The electromagnetic spectrum range of 190 to 220 nm was corresponded to peptide bond, whereas 280 nm for protein. Aβ1-40 showed characteristics of sigmoidal curve only at 50 and 100 µM (Figure 1B). Thus, 50 µM was chosen for further studies. 100 and 200 µM Aβ25-35, incubated at 15°C, showed appreciable absorbance (Riviere et al., 2007). However, the absorption decreased at 50 µM and lost at 10 µM. The maximum decrease in absorbance was observed as incubation time was increased from 0 to 5 h, finally leading to equilibration after 5 h. In the present study, samples were incubated at 37°C, however no significant changes could be noticed within 1 h interval during first 5 h. The maximum absorbance only decreased significantly on prolonging the span of incubation up to 3 days and finally to equilibration after 6 days (Figure 1B). The decrease in absorbance may be due to hidden peptide bond in macromolecular structures (Riviere et al., 2007). The differences between these two conditions may be due to different sources and peptide sequences of Aβ used in the experiments. In addition, our samples were incubated at 37°C as this temperature was similar to incubation of Aβ for toxicity study in cell culture instead of 15°C used by Riviere et al. (2007). Moreover, studies by Ono et al. (2006a, 2004a, 2003, 2004, 2005, 2006b, 2004c) and Gilead et al. (2006) used 37°C as the incubation temperature for Aβ1-40 and the equilibrium level was achieved after 6 days incubation.

**TA and TQ effects on Aβ1-40 aggregation by UV-Visible spectroscopy**

*N. sativa* has been demonstrated to have protective effects against Aβ-induced toxicity in neuronal cells (Ismail et al., 2008). However, disaggregation effects of its bioactive constituent, that is, TQ on Aβ aggregates have not been studied yet. In this study, TA was used as a reference since it showed anti-amyloidogenic effects (Ono et al., 2004c) in comparison to TQ. To verify the dose- and time-dependent effects on 50 µM Aβ1-40 fibril inhibitions by 1, 10 and 50 µM TA and TQ, the relative absorbance variations at 0, 5 and 16 days were measured. Percent inhibition (I %) by TA and TQ on Aβ fibril formation were calculated according to Riviere et al. (2007). Inhibition percentages of Aβ fibril formation increased with the increase in concentrations of TA and TQ in a dose-dependent manner, with small inconsistency as function of time (Figure 2). At day 0 and 5, all the TQ concentrations showed more than 50% inhibition of Aβ. However, 50 µM TQ showed more than 50% inhibition while 1 and 10 µM TA did not inhibit more than 40%. Nevertheless, at day 16, TA at all the concentrations showed 100% inhibition of Aβ as compared to TQ, which exhibited 100% inhibition only at
Figure 1. A representative of absorbance spectra of $\text{A}\beta_{1-40}$ versus time at $37^\circ\text{C}$ from 0-96 h at 190-500 nm. Reaction mixture containing 50 µM of $\text{A}\beta_{1-40}$, 0.8 mM phosphate buffer, pH 7.2 and 10 µl DMSO (A). A recording from 0-9 days at 220 nm shows the typical lag phase required for nucleation (B).

Figure 2. Inhibitory effects of tannic acid (TA) and thymoquinone (TQ) on $\text{A}\beta_{1-40}$ aggregation at 1, 10 and 50 µM concentrations within day 0, 5 and 16 of incubation at $37^\circ\text{C}$. Reaction mixtures containing 50 µM of $\text{A}\beta_{1-40}$, 0.8 mM phosphate buffer, pH 7.2 and 10 µl of TA or TQ. Means and SD of two independent experiments are shown. *Significantly different from the TA inhibitory effect (p<0.05).
groups from Aβ. The same interactions have been suggested for resveratrol (Riviere et al., 2007). In addition, the hydrophobic regions of Aβ could interact with lipophilic chain of TQ, as occurred between Aβ and rifampicin (Ono et al., 2006b). On the other hand, TA also showed anti-amyloidogenic activity in the present study, as indicated by Ono et al. (2004c) previously. This anti-amyloidogenic effect may depend on their molecular structure. Tannic acid is a flexible bulk molecule with rings containing numerous hydroxyl groups (Figure 4). The number of hydroxyl groups of these polyphenols maybe responsible for its inhibitory effect on Aβ fibril formation (Ono et al., 2003). However, a large molecule of tetracycline possessed a weak anti-amyloidogenic activity (Ono et al., 2004c).

On the other hand, resveratrol and curcumin were among the small molecules that were effective as anti-amyloidogenic agents (Riviere et al., 2007; Ono et al., 2004a). The molecular weight of TQ was much smaller than resveratrol and curcumin. Thymoquinone may have other mechanisms in inhibition of Aβ fibrils formation since its structure does not contain any hydroxyl groups and it is smaller in size than resveratrol and curcumin. Aromatic ring of TQ may also be suspected to be responsible for inhibition. Thus, TQ may have some properties in such a way to prevent Aβ aggregation.

### Protective effects of TQ on Aβ1-40-induced toxicity (MTS and LDH assays)

Exposure of CGNs to aged 10 μM Aβ1-40 at 37°C showed inhibition of cell viability by MTS assay to 54 ± 0.98% (p < 0.01) for 4 days of profibrils formation (Figure 5). As stated by Durairajan et al. (2008), the death rate increases with the aging time of Aβ fibrils. In this study, Aβ1-40-induced toxicity was attenuated by pretreatment with TQ in a dose-dependent way. Thymoquinone at 0.1 and 1 μM restored the cells viability of CGNs against aged Aβ1-40-induced toxicity by 99 ± 2.31 and 91 ± 1.92%, respectively (p < 0.01). Treatment of TQ alone did not affect the growth of CGNs within tested concentrations. To further confirm the MTS finding, LDH assay was performed. Exposure of CGNs to 10 μM Aβ1-40 increased LDH activity by 117.00 ± 0.74% as compared to control (Figure 5). Pretreatment with 0.1 and 1 μM TQ significantly protected the neurotoxicity induced by Aβ1-40 (61 ± 2.05 and 65 ± 3.09%, respectively) compared to Aβ1-40 alone. Thus, the findings from MTS and LDH assays showed that TQ, even at low doses, was able to protect the cells against toxicity of Aβ1-40.

### TQ reduced the activation of caspasces-3, -8 and -9 on Aβ1-40 exposure

Figure 6 shows an increment of caspase-3 activity after

### Table 1. EC<sub>50</sub> of tannic acid (TA) and thymoquinone (TQ) on the formation of Aβ1-40 fibrils.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; of TQ (µM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; of TA (µM)</th>
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<tbody>
<tr>
<td>Day 0</td>
<td>0.1</td>
<td>29</td>
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<tr>
<td>Day 5</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>Day 16</td>
<td>0.2</td>
<td>0.01</td>
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EC<sub>50</sub> is defined as the concentrations of TA and TQ that inhibit the formation of Aβ<sub>1-40</sub> by 50% of the control value, which is calculated from the sigmoidal curve fitting of the data. The reaction mixtures containing 50 μM Aβ<sub>1-40</sub>, 0.8 mM phosphate buffer, pH 7.2 and 10 μl tannic acid (TA) or thymoquinone (TQ) at concentrations of 1, 10 and 50 μM were incubated at 37°C for 0, 5 and 16 days.

50 μM. Meanwhile, 1 and 10 μM TQ inhibited Aβ fibrils formation by 60 and 70%, respectively. A little inconsistency needs to be probed, as mechanism is still unclear. But, it may be hypothesized that 10 μM TA might have inhibited slowly due to some structure-activity relationships following stoichiometric balancing at the said concentration. The effective concentrations at 50% value (IC<sub>50</sub>) of TA and TQ are shown in Table 1. IC<sub>50</sub> is defined as the concentrations of TA and TQ, which inhibited the formation of Aβ<sub>1-40</sub> fibrils by 50% of the control value, calculated from the sigmoidal curve fitting in data.

### Observation on TA and TQ effects on Aβ1-40 aggregation under TEM

After incubation of 50 μM Aβ1-40 at 37°C for 16 days, extensive formations of fibrils were observed under TEM (Figure 3G). Protofibrils were formed during first week of Aβ1-40 incubation before mature fibrils were generated (Harper et al., 1999). Therefore, in the present study, we observed mature fibrils formation after incubation of 2 weeks. This result was supported by Cohen et al. (2006) and Gilead et al. (2006), who stated that Aβ should be incubated at 37 °C for at least 14 days to achieve a steady state (maximal) level of fibrillation. When 50 μM Aβ1-40 were co-incubated with 1, 10 and 50 μM of TA or TQ, the number of fibrils were reduced in some degree with shorter fibrils and small amorphous aggregates (Figure 3A to F).

Our findings showed that TQ could be a promising Aβ inhibitor. Its inhibitory effects were slightly similar to TA at day 16 (Table 1). TQ is a non-polar molecule which was dissolved well in organic solvent (acetone). Therefore, it is postulated that the binding between TQ and Aβ<sub>1-40</sub> could be induced by hydrophobic interactions of TQ and hydrophobic region of Aβ. Thus blocking the associations between Aβ molecules and thereby inhibiting the fibril formation. These interactions could be reinforced by the H-bond acceptor group of TQ (Figure 4) with some donor
exposure to 10 µM Aβ1-40 for 24 h. Pre-treatment of CGNs with 0.1 µM TQ for 5 h before exposure to 10 µM Aβ1-40 was able to reduce caspase-3 activity from 115 ± 1.09 to 87 ± 5.76%, significantly. Incubation of TQ alone had no effect on caspase-3 activity. Hence, the efficiency of TQ in reducing caspase-3 activation was further examined on its ability to inhibit caspase-8 and -9 activations.

Caspase-8 was localized at the top of hierarchy of caspase cascade and a member of the upstream or initiator family of caspases. Caspase-8 activated downstream caspases (3, 6, and 7) that cleaved key cellular substrates and led to apoptotic cells death. Figure 6 shows significant differences in caspase-8 activation (111 ± 6.82%) of Aβ exposed to CGNs compared to control. However, 0.1 µM TQ was able to reduce the caspase-8 activity to 83 ± 5.08%. Aβ induced apoptosis

**Figure 3.** Electron micrographs of Aβ1-40 fibrils. The reaction mixtures containing 50 µM Aβ1-40, 0.8 mM phosphate buffer, pH 7.2 and 10 µl tannic acid (TA) or thymoquinone (TQ) incubated at 37°C. (A) 50 µM Aβ1-40 + 1 µM TA (B) 50 µM Aβ1-40 + 10 µM TA (C) 50 µM Aβ1-40 + 50 µM TA (D) 50 µM Aβ1-40 + 1 µM TQ (E) 50 µM Aβ1-40 + 10 µM TQ (F) 50 µM Aβ1-40 + 50 µM TQ (G) Aβ1-40 alone. The bar represents 200 nm.
Figure 4. Structure of TQ and TA.

Figure 5. Neuroprotective effect of TQ against Aβ₁₋₄₀. The CGNs were pretreated for 5 h with thymoquinone (TQ) at concentrations of 0.1 and 1 μM before being exposed to 10 μM Aβ₁₋₄₀ for 24 h and measured using MTS and LDH assays. The percentage of DMSO was at 0.01% (v/v). Values represent means ± S.E.M. *P<0.01 versus Aβ₁₋₄₀, #P<0.01 versus control.

In the AD brain led to caspase-8 activation by cross-linking and activating the death-receptor. Rohn et al. (2001) stated that many cell types were sensitive to receptor-mediated apoptosis followed by oligomerization of receptors on cell surface. Ivins et al. (1999) showed Aβ toxicity induced apoptosis by causing receptor oligomerization and activated the caspase-8. The neurotoxic action of Aβ involved the fibrillar features of Aβ (Cribbs et al., 1997). Fibrillar Aβ may prompt neuronal cell death associated with AD by induction of apoptosis.
followed by cross-linking of death-receptors and concomitant activation of caspase-8 and caspase-3.

An alternative pathway of Aβ-induced apoptosis, in neuron culture, also involved the caspase-9 activation. Exposure of CGNs to 10 μM Aβ1-40 for 24 h activated the caspase-9 (148 ± 8.32%). Thymoquinone (0.1 uM) reduced caspase-9 activation to 95 ± 5.34%, significantly (p < 0.01) (Figure 6). Oxidative damage is the key feature of normal aging but more extensive in the AD brain (Smith et al., 2000). Extensive oxidative insults accumulated by neurons may have selectively undergone apoptosis (Lu et al., 2000). Mitochondria were particularly vulnerable to oxidative damage, as it produced major sources of oxidants through normal metabolic processes. Mitochondrial dysfunction has been observed in AD brain (Hirai et al., 2001), and as a consequence, stimulation of mitochondrial pathway of apoptosis may be elicited through oxidative damage leading to release of cytochrome c. Released cytochrome c interacted with Apaf-1 and this complex recruited caspase-9 activations (Kuida, 2000).

In this study, 10 μM Aβ1-40 was found capable to activate apoptosis via both extrinsic and intrinsic pathways. Caspase-8 activation was slightly increased, whereas caspase-9 showed marked activations as compared to control. These findings indicate the potential of TQ in reducing the activation of both caspase-8 and -9. In conclusion, these results indicate the protective effects of TQ on CGNs may be due to its contribution in disaggregating the neurotoxic Aβ accumulation, thus protecting cells from cell death.

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