Effect of Centella asiatica on Aβ(42) aggregation

Abstract

Amyloid beta (Aβ) is the major pathological and etiological factor implicated in Alzheimer’s disease (AD). Aβ(42) inherently self assembles to form oligomers and fibrils through multifactorial aggregation process. The oligomers and fibrils have toxic effects and may lead to neuronal dysfunction. The inhibition of formation of oligomers and fibrils is a novel strategy in drug development programs for AD. There are limited studies to show that Centella asiatica has a memory enhancing, anti-acetylcholinesterase and antioxidant properties. But there are no studies to insight whether Centella asiatica prevents Aβ fibrils formation from monomers and oligomers and also to understand whether Centella asiatica destabilize the preformed fibrils. Our present study focused on, i) whether the Centella asiatica leaf aqueous extract prevent the formation of oligomers and aggregates from monomer? (Phase I: Aβ(42) + extract co –incubation), ii) Whether the Centella asiatica aqueous extract prevent the formation of fibrils from oligomers (Phase II- extract added after oligomers formation) and iii) whether the aqueous extract dis-aggregates the pre-formed fibrils (Phase III - aqueous extract added to matured fibrils and incubated for 8 days). The aggregation kinetics was studied using thioflavin-T assay and Transmission Electron Microscopy (TEM). The results showed that Centella asiatica aqueous extract could not able to inhibit the Aβ aggregation both from monomer and oligomers and also could not able to dis-integrate the preformed fibrils. These intriguing results are discussed.

Key words: Aβ(42), Fibrils, Aggregation, Thioflavin-T, Transmission Electron Microscopy, Centella asiatica

Introduction

Amyloids are a group of misfolded proteins, implicated in the neurodegenerative disorders such as Parkinson’s disease, Huntington disease, Frontotemporal dementia and Alzheimer’s disease (AD) (1). The amyloid β 42 (Aβ42) is one of the amyloid protein strongly implicated in AD (2). The excessive production and accumulation of Aβ believed to be one of the major risk factor for AD (3-4). Aβ undergoes conformational change and forms deposits in the form of insoluble senile plaques in AD brain (5). The major therapeutic approaches in AD are towards the reduction of Aβ either by decreasing its production or to enhance clearance of the accumulated Aβ [6-12]. The accepted concept is that Aβ oligomers are toxic to neurons and induce cell death (4, 13-14). The therapeutic approaches in AD include acetylcholineesterase inhibitors, antioxidants, anti-inflammatory and anti-amyloidogenic agents as
The anti-amyloidogenic approach is currently active (19, 23-25). There are number of drug targets focused against amyloid load reduction and many of them have not reached the clinical trials (24-28). Now, the studies have been focused on natural products as alternative candidates for evaluating therapeutic potential against AD (21-26). *C. asiatica* has been traditionally used in Asia to cure various ailments. The present study focused in evaluating *C. asiatica* for its anti-β aggregation properties. The dried leaves of Indian penny wort (botanical name: *Centella asiatica*) is mixed with milk and consume as memory improving (29-30) and this is practiced traditionally in selected regions in India (31). There are studies on diverse effects of *C. asiatica* such as acetylcholine-esterase inhibition, antioxidant, neuroprotection, and amyloid load reduction (16, 18, 32-34). However, there are no mechanistic studies to understand whether *C. asiatica* prevents Aβ aggregation. In the current study, we have used Aβ(42), which is the most amyloidogenic peptide, for the formation of oligomers, protofibrils and fibrils. And also, we planned to map whether *C. asiatica* inhibits Aβ aggregation. We used aqueous extract of *C. asiatica* as it is traditionally used by local population in Western Ghats as brain tonic.

**Materials and methods**

Aβ(42) was purchased from EZ Biolabs, USA. Tris buffer, glycine, sodium hydroxide, Hydrochloric acid were from SRL, India. Thioflavin –T was procured from ICN Biomedicals Pvt. Ltd, USA and Copper grids (200 mesh size) were brought from Sigma chemicals, USA and uranyl acetate was procured from BDH Laboratory chemicals Division, India.

The *C asiatica* was procured from local vegetable market, Mysore and it was identified by authenticated botanist (Taxonomic deposit number is 9831).

Flow chart for the preparation of aqueous-leaf extract of *C. asiatica*

The 40 g dried leaves of *C. asiatica* was washed thoroughly in triple distilled water for four times. Two litres of triple distilled water was added to steam extractor, boiled till it become half volume (one liter). The one litre aqueous extract was filtered using Whatman 42 filter paper to get clear solution. The clear solution of the extract was lyophilized to get dry powder. The yield of the extract was 2.5% (w/w).

To evaluate the anti-amyloidogenic property of aqueous extract of *C. asiatica*, the following experiments were designed in vitro using the three-phase study protocol. Phase I: To understand the prevention of Aβ aggregation from monomer. Phase I reaction mixture was as follows: 100μM of Aβ was incubated with 100μg of lyophilized aqueous extracts of *C. asiatica* in a total reaction mixture of 300μl containing 10mM Tris-Cl (pH 7.4) at 0 hrs at 37°C. Aliquots of 20μl (10 μM) were drawn each time from incubated sample at intervals of 0, 6, 20, 72 and 96 hrs. Phase II: To understand the prevention of formation of aggregates from oligomers. Freshly prepared Aβ was allowed to form oligomers till 20 hrs following the
protocol with slight modification [35] and then 100µg of lyophilized extract was added. The aggregation kinetics was studied to follow the formation of aggregates from oligomers as function of time (20-96 hrs) and aliquots were taken at 20, 36, 48, 72 and 96 hrs for thioflavin-T and TEM study. Phase III: To understand the efficacy of extract to dis-integrate the pre-formed fibrils. Freshly prepared Aβ was allowed to form matured fibrils by 96 hrs and then 100µg of lyophilized extract was added and followed the dis-integration of fibrils at 8 days.

Thioflavin-T assay

The thioflavin-T assay was followed to study Aβ aggregation kinetics. Thioflavin-T specifically binds to aggregates but not to soluble monomers. 25µl of (1mM) thioflavin-T was added to 1000µl of total reaction volume containing 5µM Aβ. The thioflavin-T fluorescence was measured at an excitation and emission wavelengths of 446nm and 482nm, respectively using a F4500 Hitachi Fluorescence Spectrometer. The background thioflavin-T fluorescence intensity was subtracted from the experimental values. The thioflavin-T fluorescence data was analyzed for standard error using origin 6.0.

Transmission Electron microscopy (TEM) study

Transmission Electron Microscopy (TEM) study was conducted to detect presence or absence of aggregates. 10µL of incubated sample was placed on carbon coated copper grid (200 mesh size) and allowed for one min and excess sample was wicked off with lens paper and then negatively stained by transferring the grid face down to a droplet of (2% (w/v) uranyl acetate for one min before wicking off the solution. Then the grids were air dried for an hour. Four individual experiments were carried out for each sample. The grids were completely dried to avoid moisture and then scanned under JOEL 1010 TEM.

Results

The effect of C. asiatica on the Aβ aggregation is analyzed as follows.

Phase I: Inhibition of the formation of aggregates from monomers: Fig 1A shows three phases of aggregation kinetics of Aβ as monitored by thioflavin-T fluorescence as a function of time (0-96 hrs). The aggregation kinetics followed a sigmoidal curve. The thioflavin –T data indicated a lag period upto 20 hrs, where thioflavin-T fluorescence intensity is static indicating the presence of monomers only. After 20 hrs, there is an intermediate phase from where oligomers and other intermediate forms form till 48 hrs. The thioflavin –T fluorescence steeply increases in this phase indicating formation of misfolded intermediates. The other phase is the saturated phase where fully matured fibrils are formed. This phase is from 48 to 96 hrs. The thioflavin –T fluorescence is higher and static in nature indicating matured fibril formation. In the presence of the aqueous extract, the sigmoidal pattern of Aβ aggregation kinetics is followed a similar pattern with no significant reduction in fluorescence intensity. This indicates that C. asiatica could not be able to prevent the Aβ aggregation from monomers. Fig 1B shows the thioflavin-T fluorescence at 0, 6, 20, 72 and 96 hrs time intervals. For Aβ alone, as the time increases, the thioflavin-T fluorescence increases. And in the presence of aqueous extract, thioflavin-T fluorescence do not significantly altered; supporting the concept that extract could not able to prevent Aβ aggregation. The samples represented in Fig 1B are taken for TEM study. Fig 1C shows results of TEM study for the presence or absence of aggregates at different time intervals (0, 6, 20,72 and 96 hrs). The TEM data clearly shows that when Aβ alone is allowed to aggregate from 0 to
96 hrs, the formation of fibrils is in time dependent aggregation kinetics. There are no aggregates from 0 to 20 hrs. The aggregates start growing from 72 and 96 hrs (Fig 1C). Even in the presence of C. asiatica, both at 72 and 96 hrs the aggregates are seen indicating that the extract could not able to totally prevent the formation of fibrils (Fig 1C). Both thioflavin-T and TEM data clearly supports that the extract could not able to totally prevent the formation of aggregates.

**Phase II:** Inhibition of the formation of aggregates from oligomers: Fig 2A shows the
results of phase II. Thioflavin-T fluorescence data of Aβ is analyzed with and without extract at different time intervals (20, 36, 48, 72 and 96 hrs). Aβ is allowed to form oligomers for 20 hrs, and at 20 hrs, extract is added and the Aβ aggregation process is monitored at different time intervals. The thioflavin-T fluorescence has increased from 20 to 96 hrs indicating the formation of fibrils from oligomers stage. In the presence of C. asiatica, the thioflavin-T fluorescence values do not decrease significantly indicating that the extract could not inhibit the formation of fibrils from oligomers. Fig 2B shows the results of TEM of phase II. The formation of fibrils increased with time from 20 to 96 hrs in Aβ alone and in the presence of C. asiatica, also the fibrils are seen but less in number. This indicates that C. asiatica could not be able to totally prevent fibril formation from oligomers (Fig 2B).

**Phase III: Dis-integration of pre-formed fibrils:** Fig 3A shows the results of thioflavin-T fluorescence assay of phase III. The matured fibrils after 96 hrs are allowed to further grow till 8 days. The fluorescence has increased from 96 to 8 days in Aβ alone and in the presence of C. asiatica extract no significant reduction in thioflavin-T fluorescence was observed. This indicates that extract did not dis-integrate the pre-formed fibrils even after 8 days of incubation. Fig 3B shows the results of TEM of phase III. At 96 hrs, there are matured fibrils with extensive branching in sample having Aβ alone. The extract is added to 96 hrs-matured fibrils and then incubated for 8 days. The extract could not dis-integrate totally the pre-formed fibrils even after 8 days of incubation.

**Discussion**

Alzheimer’s disease is a progressive neurodegenerative disease affecting millions of people worldwide. The etiological factors include oxidative stress, inflammation, Aβ over expression, elevation in metals etc. (2, 36-37). Among the risk factors implicated, Aβ is strongly associated with AD [3, 38]. The therapeutic approaches in AD include, reducing the amyloid production or enhancing the clearance of amyloid load in AD [39-40]. The ancient Indian system of medicine, Ayurveda has described traditional use of herbal medicinal therapies for the treatment of dementia (29). In particular, C. asiatica has been...
listed in ancient Indian Ayurveda medical text Caraka Susmita as a treatment for dementia. The leaves of *C. asiatica* is used as a memory booster in some regions of India [30-31]. The animal studies have shown that extracts of *C. asiatica* improves memory in rats (32-34). The *C. asiatica* also found to improve memory, behavior and performance tests of mentally retarded children (41). Kumar and Gupta (42) have shown that *C. asiatica* prevents streptozotocin induced cognitive deficits in rats. Subathra et al. (43) reported that *C. asiatica* reduced the protein carbonyls in the aged rat brain. Further, Kumar et al. (34) have demonstrated that *C. asiatica* significantly decreases the acetylcholine esterase activity in colchicine induced cognitive impairment and oxidative stress. Nalini et al. (44) showed that *C. asiatica* showed improvement in the avoidance task in rats. Rao et al. (45) reported that *C. asiatica* able to improve the brain function of mice if treated during postnatal period. Recently, Dhanasekaran et al. (16) have reported that *C. asiatica* moderately decreased Aβ(40) and Aβ(42) load both in cortex and hippocampus region of PSAP Alzheimer’s disease mice model. But the reduction of Aβ(40) and Aβ(42) did not improve the Y-maze and open field behavior tests. The mechanism of reduction in the amyloid load is not clearly understood. There are no studies to show the effects of *C. asiatica* on Aβ aggregation kinetics. Our results showed that *C. asiatica* aqueous extract could not significantly inhibit the Aβ aggregation either from monomer and oligomers and also not be able to dis-integrate the pre-formed fibrils. Based on our data and literature findings, we propose the following mechanism of action. Aβ exists in monomer form and monomers will be in random coil conformation. Aβ in suitable condition self-aggregates into fibrils. The aggregation process will pass through different conformation in the following order: random coil, misfold, β-sheet/β-turn or in combination of all these conformations. The fully aggregated long fibrils will be either in β-sheet/β-turn conformation. The aqueous extract of *C. asiatica* could not stabilize random coil of monomers hence could not prevent fibril formation. However, we further propose that *C. asiatica* extract may be acting by other possible pathways such as (i) enhancing the α-secretase pathway of APP processing or inhibiting β-secretase activity; (ii) may be acting as anti-oxidant, so that oxidative stress will be reduced. And Oxidative stress enhances Aβ expression; (iii) may enhance clearance mechanism of accumulated amyloid; or (iv) may be acting as anti-inflammatory candidate. All these events together may be helpful for *C. asiatica* as brain tonic or memory or cognitive function enhancer. Further work is needed to understand more on the efficacy of *C. asiatica* as a therapeutic intervention molecule.

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**References**


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