

A novel antagonistic role of natural compound icariin on neurotoxicity of amyloid β peptide

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Background & objectives: Amyloid β -peptide ($A\beta$) has been shown to be responsible for senile plaque formation and cell damage in Alzheimer's disease (AD). This study was aimed to explore the role of natural compound icariin on the aggregation and the cytotoxicity of $A\beta$ *in vitro*.

Methods: Thioflavin T (ThT) fluorescence assay and transmission electron microscopy (TEM) imaging were done to determine the influence of icariin on the aggregation of $A\beta_{1-42}$ peptide. MTT assay was used to evaluate the protective effect of icariin on $A\beta_{1-42}$ induced cytotoxicity in neuroblastoma SH-SY5Y cells.

Results: Icariin inhibited $A\beta_{1-42}$ aggregation in a dose-dependent manner. Additionally, icariin also prevented the cytotoxicity of $A\beta_{1-42}$ in SH-SY5Y cells by decreasing the production of peroxide hydrogen during the aggregation of this peptide.

Interpretation & conclusions: The results indicated a novel antagonistic role of icariin in the neurotoxicity of $A\beta_{1-42}$ via inhibiting its aggregation, suggesting that icariin might have potential therapeutic benefits to delay or modify the progression of AD.

Key words Aggregation - Alzheimer's disease (AD) - amyloid β ($A\beta$) - icariin

Alzheimer's disease (AD) is a neurodegenerative disease that mostly affects the elderly. Prevalence studies revealed that there were 25 million persons with AD globally in 2000, and this number is predicted to increase to 114 million by the year 2050 if new preventive or neuroprotective therapies do not emerge^{1,2}. Existing treatments for AD cannot cure the disease, but can offer some people modest improvement in some symptoms with side effects. Therefore, there is a need

for alternative drugs and in particular for that, which has no side effects.

One of the major characteristics of AD is the abnormal aggregation of amyloid β peptide ($A\beta$) into extracellular fibrillar deposits known as senile plaque³. It is accepted that abnormal production and aggregation of $A\beta$ are initial pathogenic events in AD^{1,4-6}. Substantial evidence shows that $A\beta$ -induced

oxidative stress plays a key role in the pathogenesis or progression of AD^{7,8}. Several studies have reported that A β -induced cytotoxicity is caused by intracellular accumulation of hydrogen peroxide (H₂O₂) produced during its aggregation, ultimately leading to the peroxidation of membrane lipids and to a cell death⁹⁻¹¹. Therefore, inhibition of A β aggregation is viewed as a potential method to slow the progression of AD.

Epimedium brevicornum Maxim is a medicinal herb which has been widely used for the treatment of impotence, infertility, osteoporosis, cardiovascular diseases, amnesia, and senile functional diseases¹². The neuroprotective potential of icariin, a flavonoid compound isolated from it, against A β -induced toxicity in PC12 cells had been explored, which demonstrated that treatment with icariin significantly decreased A β ₂₅₋₃₅-induced cytotoxicity and apoptosis rate by inhibiting tau protein hyperphosphorylation¹³. Icariin has been shown to improve the learning and memory abilities in rats with A β ₂₅₋₃₅-induced Alzheimer's disease that can reduce production of insoluble fragments of A β through suppression of β -secretase expression¹⁴. However, the role of icariin in the inhibition of A β accumulation and aggregation has not yet been investigated. The present study was undertaken to explore the anti-amyloidogenic properties of icariin and to examine the effects of icariin on the formation of A β aggregates *in vitro* by using fluorescence spectroscopy with ThioflavinT (ThT) and transmission electron microscopy (TEM). Further, the neuroprotective effect of icariin and its mechanism in A β ₁₋₄₂-treated human neuroblastoma SH-SY5Y cells were also explored.

Material & Methods

A β ₁₋₄₂ was purchased from Anaspec (San Jose, USA). Icariin was isolated from the root of *E. brevicornum* Maxim according to the methods reported by Du *et al.*¹⁵. The purity of Icariin was tested by nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC) (the purity was over 98%). Dulbecco's modified Eagle/F12 medium (DMEM), foetal bovine serum, thioflavinT (ThT), glycine, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were obtained from Sigma-Aldrich, USA.

Preparation of A β ₁₋₄₂ aggregate: The treatment of A β ₁₋₄₂ was done as described earlier^{16,17}.

Cell culture and treatment: SH-SY5Y human neuroblastoma cells were purchased from American Type Tissue Culture (Manassas, USA), cultured in

DMEM/F12 supplemented with 10 per cent heat-inactivated foetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin in a humidified incubator with 5 per cent CO₂ at 37°C. For the cytotoxicity assay, the cells were rinsed once with PBS pH 7.4 and the medium was replaced with phenol red-free and serum-free DMEM before A β treatment. To explore the influence of icariin on the cytotoxicity of A β ₁₋₄₂ aggregations, A β was preincubated with different concentrations of icariin for three days. A β ₁₋₄₂ was dissolved in PBS with a final concentration of 10 mM before used. After the cells were incubated for 48 h in the presence or absence of 5.0 μ M A β ₁₋₄₂ with or without icariin, MTT assay was performed to probe the cell viability, and the same volume of PBS was taken as negative control¹⁸.

ThT fluorescence assay and TMT igaming: ThT is a dye that increases its fluorescence intensity upon binding with the A β aggregates and is, therefore, used to estimate the relative amount of amyloid-like aggregates¹⁹. ThT binding assay was carried out and the solution of A β ₁₋₄₂ peptide was prepared for TEM analysis as described earlier^{16,17}.

*H₂O₂ assay*¹⁶: After SH-SY5Y cells were replaced into 12-well plates at 4 \times 10⁵ cell/well and incubated overnight, the cells were washed once with PBS and the medium was replaced with phenol red-free and serum-free DMEM. Before treated with A β ₁₋₄₂ peptide, the cells were preincubated with indicated concentrations of icariin for two hours, after that, freshly prepared A β ₁₋₄₂ peptide with a final concentration as 5.0 μ M was added and cultured for 24 h. The medium was taken to test for the level of H₂O₂ according to the protocol supplied by manufacturer (Biovision, USA). The collected medium was reacted with oxiRed™ probe for 30 min at room temperature protected from light to produce product with colour, the absorbance value was determined by microplate reader (Varioskan, USA) at 570 nm.

*MTT assay*¹⁶: For the cytoprotective assessment of icariin, before A β ₁₋₄₂ peptide a final concentration of 5.0 μ M icariin was added, the cells were treated with geniposide for 2 h at indicated concentration of icariin, and incubated for 48 h. Cells were incubated for 2 h at 37°C with MTT (0.5 mg/ml final concentration) and dissolved in fresh complete medium, in which metabolically active cells reduced the dye to purple formazan. Formazan crystals were dissolved with DMSO, and the absorbance was measured on a BMG

microplate reader (BMG Technologies, USA), using a reference wavelength of 630 nm and a test wavelength of 570 nm.

Statistical analysis: Statistical analysis was performed using the Student's t test and the ANOVA test followed by Bonferroni correction using the software of Origin version 8.0 (Northampton, USA).

Results

Inhibition of aggregation of $A\beta_{1-42}$ peptide by icariin: To explore the role of icariin on the aggregation of $A\beta_{1-42}$, fluorescence intensity of $A\beta_{1-42}$ aggregate was determined with ThT binding assay. After incubation for three days in the presence or absence of icariin, the extent of $A\beta$ aggregation in the samples was evaluated by taking 10 μ l of $A\beta_{1-42}$ from incubated samples and transferring to a single well of a 96-well plate. To each sample, 200 μ l of 10 μ M ThT in 0.1M glycine buffer (pH 8.9) was added and the plate was read on a microplate reader (Varioskan, Thermo, USA) for fluorescence intensity at Ex/Em 450/482 nm. All ThT fluorescence experiments were performed in triplicate. As shown in Fig. 1, icariin inhibited the aggregation of $A\beta_{1-42}$ in a dose-dependent manner with a calculated IC_{50} (half maximal inhibitory concentration) of 0.4813 μ M (n = 6, $R^2 = 0.9912$).

To visually confirm the effect of icariin on the aggregation of $A\beta_{1-42}$, TEM imaging was done to evaluate the fibril formation of $A\beta_{1-42}$ in the absence

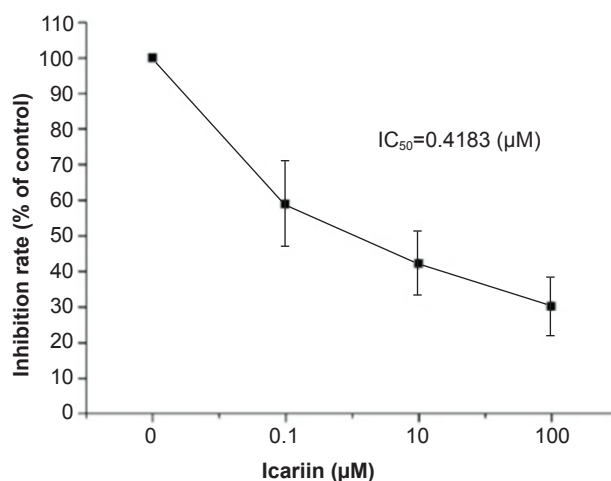


Fig 1. Effect of icariin on the aggregation of $A\beta_{1-42}$ peptide. Data are mean \pm SD from six wells of two independent experiments (n = 6, $R^2=0.9912$).

(Fig. 2A) or presence of icariin (Fig. 2B). Incubation of 50 μ M solution of $A\beta_{1-42}$ in 20 μ M PBS (pH 7.4) for three days at room temperature in the presence of 5.0 μ M icariin markedly inhibited the aggregation of $A\beta_{1-42}$, as much smaller and fewer fibrils were observed in presence of icariin compared to its absence.

Effect of icariin on neurotoxicity of $A\beta_{1-42}$ peptide: The results revealed that icariin prevented cell injury induced by the addition of $A\beta_{1-42}$. The effect was dose dependent and preincubation with 10 μ M icariin was

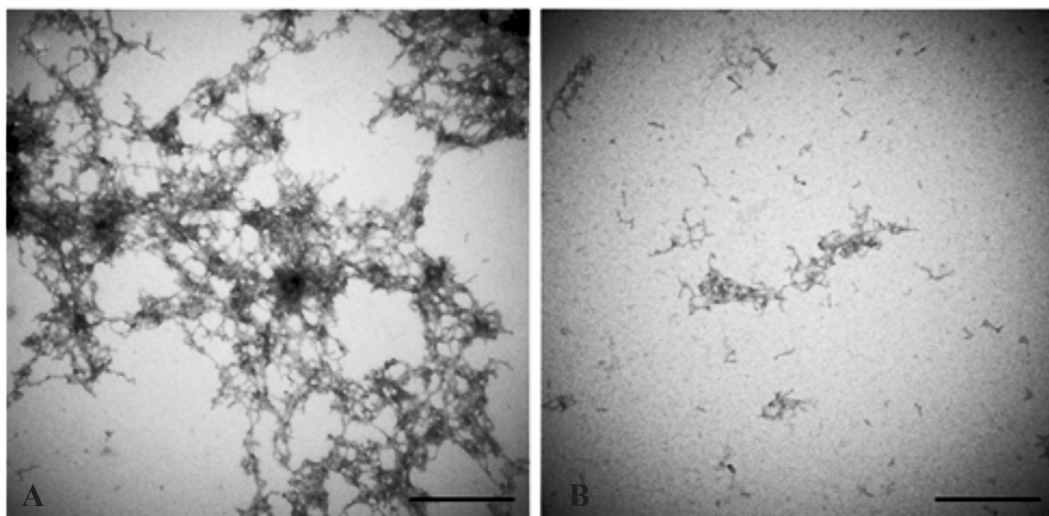


Fig 2. Transmission electron microscopy imaging of $A\beta_{1-42}$ aggregate in the absence (A) or presence (B) of icariin. After incubation for three days without (A) or with (B) icariin at 10 μ M, 2 μ l of each sample was prepared for electron microscopy analysis. Images were acquired using Hitachi 7500 transaction electron microscope at 80 kv. Magnification was 100,000 \times ; Scale bar = 500 nm.

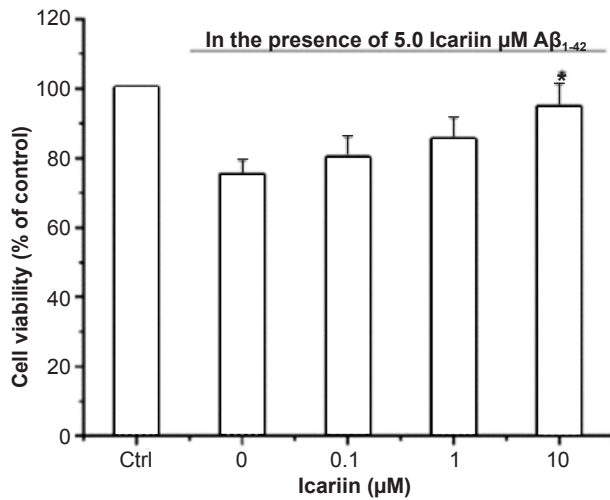


Fig 3. Icariin increased the cell viability of $\text{A}\beta_{1-42}$ -treated SH-SY5Y cells. Data are given as mean \pm SD from three independent experiments ($n = 3$). * $P < 0.05$ vs the group of $\text{A}\beta_{1-42}$ treatment alone.

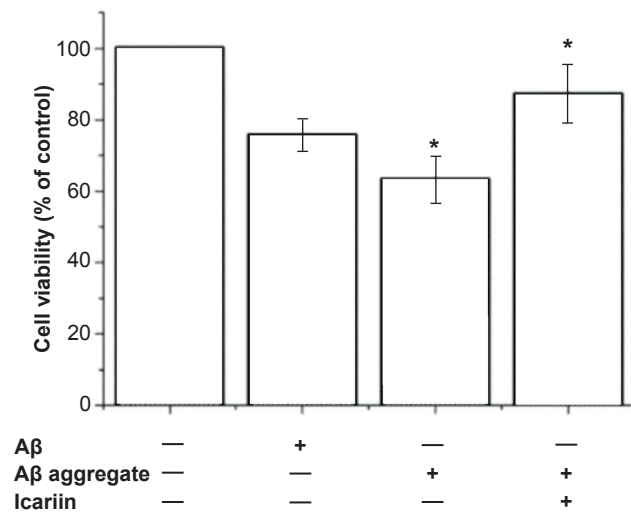


Fig 4. Cytotoxicity of fresh prepared $\text{A}\beta_{1-42}$ and $\text{A}\beta_{1-42}$ aggregates in SH-SY5Y cells. Data are shown as mean \pm SD from three independent experiments ($n = 3$). * $P < 0.05$ vs the group of fresh prepared $\text{A}\beta_{1-42}$ ($\text{A}\beta$) treatment alone; * $P < 0.05$ vs the group of $\text{A}\beta_{1-42}$ aggregate ($\text{A}\beta$ aggregate) treatment alone.

capable of increasing SH-SY5Y cell viability by 22 per cent as compared to the effect of $\text{A}\beta_{1-42}$ alone under control conditions (Fig. 3).

It has been reported that $\text{A}\beta$ in either oligomeric or fibril form has a stronger neurotoxicity than its monomeric form²⁰. To confirm this, we compared the

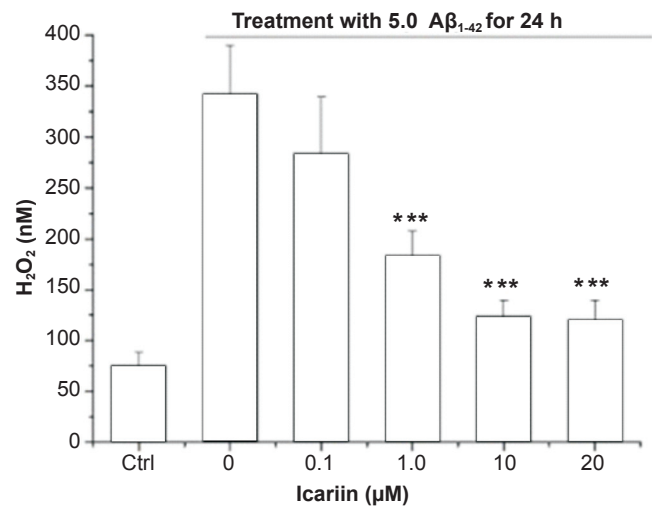


Fig 5. Icariin decreases the level of H_2O_2 in $\text{A}\beta_{1-42}$ -treated SH-SY5Y cells. Data are mean \pm SD from 4 wells of two independent experiments ($n = 4$). ***, $P < 0.001$ vs. the group of $\text{A}\beta_{1-42}$ alone.

cytotoxicity of fresh prepared $\text{A}\beta_{1-42}$ and preincubated $\text{A}\beta_{1-42}$ at 37°C for four hours to obtain the $\text{A}\beta$ aggregates. The results showed that the cytotoxicity of $\text{A}\beta_{1-42}$ aggregate was stronger than $\text{A}\beta_{1-42}$ monomer at the same concentrations, and icariin also inhibited the neurotoxicity of $\text{A}\beta_{1-42}$ aggregate. Preincubation with $10 \mu\text{M}$ icariin for two hours increased the cell viability by about 18 per cent in $\text{A}\beta_{1-42}$ aggregate-treated SH-SY5Y cells (Fig. 4).

Icariin attenuates the level of H_2O_2 in $\text{A}\beta_{1-42}$ -treated SH-SY5Y cells: To confirm the protective mechanism of icariin on $\text{A}\beta_{1-42}$ induced cell damage in SH-SY5Y cells, the level of H_2O_2 was measured in $\text{A}\beta_{1-42}$ -treated cells. The results suggested that incubation with $5.0 \mu\text{M}$ $\text{A}\beta_{1-42}$ peptide increased H_2O_2 levels compared to control ($P < 0.001$), and icariin dose-dependently inhibited the production of H_2O_2 in $\text{A}\beta_{1-42}$ -treated SH-SY5Y cells (Fig. 5).

Discussion

There is evidence linking oxidative damage to the brain of Alzheimer's disease (AD) which is characterized by extracellular amyloid plaques and neurofibrillary tangles⁴. Moreover, $\text{A}\beta$ was found to be responsible for senile plaque formation and cell damage in AD²¹. There are different hypotheses to explain the toxic effect of $\text{A}\beta$, including the formation of ion channels in cell membrane, the spontaneous fragmentation of $\text{A}\beta$ to generate peptidyl radicals, and the direct formation of hydrogen peroxide by

the peptides²². According to the latter hypothesis, A β generates hydrogen peroxide from molecular oxygen through electron transfer interactions involving bound redox-active metal ion. Hydrogen peroxide is readily converted to a highly reactive hydroxyl radical by Fenton reaction and both forms may be responsible for some of the oxidative damage observed during post-mortem examination of AD patient's brain tissue²³.

Icariin exerts multiple therapeutic and preventive effects including antioxidant effect, anti-inflammatory effect, immunoregulation, anti-tumour activity, cardioprotective effect, stimulating osteoblasts, antidepressant-like effect, and regulatory effect on the memory deficits in aluminium-treated rats²⁴. It was also reported that icariin inhibited A β_{1-42} -induced neurotoxicity on cortical neurons by enhancing cocaine and amphetamine-regulated transcript (CART) mRNA and protein levels²⁵. The present study revealed that icariin inhibited the aggregation of A β_{1-42} and prevented neurotoxicity of aggregated A β_{1-42} in SH-SY5Y cell culture. *In vitro* evidence shows that A β as either oligomeric or fibril form has a stronger neurotoxicity than its monomeric form, which may play a critical role in the apoptosis of neurons and the impairment of cognition in AD²⁰. Inhibition of A β aggregation is, therefore, viewed as a potential therapeutic approach to slow or mitigate the progression of AD. All these evidence demonstrates that icariin may be a promising compound to be further tested for the prevention of A β -related AD.

Icariin reduced the production of H₂O₂ in A β_{1-42} -treated SH-SY5Y cells and this effect occurred in a dose-dependent manner. The neuroprotective effects of icariin in A β -stressed SH-SY5Y cells may be due to the inhibition of the A β aggregation process and subsequently reducing the production of H₂O₂ and thus damage due to oxidative stress.

In conclusion, the results indicate towards the neuroprotective mechanism of icariin. Further studies need to be done to see whether the anti-amyloidogenic and neuroprotective effects of icariin can influence A β clearance and be helpful to overcome the memory deficits caused by A β in AD.

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Conflicts of interest: None.

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