Asian Pac J Trop Biomed 2015; 5(11): 948–954

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2015.07.024

Decreasing α-synuclein aggregation by methanolic extract of *Centella asiatica* in zebrafish Parkinson's model

Husnul Khotimah[1*], Mulyohadi Ali[2], Sutiman Bambang Sumitro[3], Mochamad Aris Widodo[2]

[1]*Medical Faculty, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia*

[2]*Laboratory of Pharmacology, Medical Faculty, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia*

[3]*Department of Biology, Faculty of Science, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia*

ARTICLE INFO

Article history:
Received 22 May 2015
Received in revised form 24 Jun, 2nd revised form 27 Jun, 3rd revised form 3 Jul, 4th revised form 4 Jul 2015
Accepted 15 Jul 2015
Available online 22 Aug 2015

Keywords:
α-Synuclein
Centella asiatica
Rotenone
Parkinson's disease
Zebrafish

ABSTRACT

Objective: To observe the effects of *Centella asiatica* (*C. asiatica*) methanolic extract on α-synuclein aggregation and its expression in rotenone-exposed zebrafish.

Methods: Zebrafish (*Danio rerio*) were exposed to 5 μg/L rotenone for 28 days and co-incubated with 2.5, 5.0 and 10.0 μg/mL of *C. asiatica* methanolic extract. The medium was changed every 48 h for maintain the concentration of rotenone and extract. After 28 days zebrafish were sacrificed on the ice block and protein was isolated from zebrafish brain for ELISA of dopamine and Western blotting of α-synuclein. Immunohistochemistry was conducted to observe the α-synuclein expressions from histopathological preparation of zebrafish brain. The head were soaked in 10% formaline for less than 24 h and embedded onto paraffin block, then sliced for immunohistochemistry using anti α-synuclein antibody. We also measured zebrafish motility for 5 min in each week.

Results: *C. asiatica* has important bioactive compounds such as asiaticoside that has anti-inflammatory and antioxidant properties. It may inhibit cascade reaction due to oxidative stress induced by rotenone. Decreasing reactive oxygen species proposed probability of radical attack to α-synuclein protein that caused aggregation and increase of its expression. The motility of zebrafish was also maintained in *C. asiatica* groups due to the increasing dopamine level in rotenone-induced zebrafish. High level of reactive oxygen species inactivated enzyme for dopamine synthesis such as tyrosine hydroxylase, and oxidized dopamine itself. Oxidized dopamine increased α-synuclein aggregation. Thus, the dopamine level decreased in rotenone-induced zebrafish, but *C. asiatica* increased dopamine level.

Conclusions: *C. asiatica* has a potential to be developed as an anti-Parkinson's disease treatment due to its capability for minimized the sign of Parkinson's such as α-synuclein aggregation and expression, increasing motility and dopamine as well.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused by genetic and environmental factors. PD is also the most prevalent neurodegenerative movement disorder of adults and occurs sporadically in more than 90% of cases. While the primary motor deficits in PD arise from progressive death of dopaminergic substantia nigra neurons, the pathology of PD begins preclinically in lower brainstem nuclei with the appearance of small α-synuclein containing aggregates (Lewy neurites) [1]. α-Synuclein is an abundant presynaptic protein that binds to negatively charged phospholipids [2,3], functions as a soluble *n*-ethylmaleimide-sensitive factor attachment protein receptor complex chaperone 3 and contributes to PD pathogenesis [4,5]. α-Synuclein is a 140 amino acid protein, mainly localized in presynaptic terminals in the brain. α-Synuclein is normally an unstructured and soluble protein [6]. Although the detailed physiological functions of α-synuclein are still not clear, recent studies suggest that it plays a key role in synaptic functions [7]. Clinical diagnosis of PD, in both clinic treatment and research, relies upon characteristic symptoms and cardinal signs. The diagnosis of PD presently depends upon observation of bradykinesia, "lead pipe" rigidity, resting tremor, and subsequent loss of postural reflexes [8]. The

*Corresponding author: Husnul Khotimah, Doctoral Student of Medical Faculty, Brawijaya University, Jl. Veteran, 65145 Malang, Indonesia.
Tel: +628113646739
E-mail: husnul_farmako.fk@ub.ac.id

Peer review under responsibility of Hainan Medical University.
Foundation Project: Supported by DPP-SPP Medical Faculty of Brawijaya University and LPPM Brawijaya University with Grant No. 023.04.2.414989/2014.

action of dopamine on the aggregation of unstructured α-synuclein protein may be linked to the pathogenesis of PD. Dopamine and its oxidation derivatives may inhibit α-synuclein aggregation of non-covalent binding [9].

One valuable type of animal model for PD is established by treating animals with PD inducing neurotoxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, rotenone, and paraquat [10]. These neurotoxins are thought to inhibit mitochondrial complex I activity leading to oxidative stress, impaired energy metabolism, proteasome dysfunction, and, eventually, dopamine neuronal loss [11]. Rotenone is a pesticide that inhibits mitochondrial complex I activity, thus creating an environment of oxidative stress in the cell [12].

One of the newest model for PD is the zebrafish or *Danio rerio* [13]. According to studies, 70% of protein-coding human genes are related to zebrafish genes, and 84% of the genes known to be associated with human disease have a counterpart in the zebrafish genome. These findings highlight the importance of the zebrafish model in human disease research [14]. Although the molecular mechanisms involved in PD are not fully understood, much progress has been made in identifying some pathogenic pathways, such as inflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress, that might be involved in ischemic neuronal death [15]. *Centella asiatica* (*C. asiatica*) (local name: pegagan) is a herbaceous plant that might also have medicinal value. It is being used in Ayurvedic and traditional medicine preparations to improve learning and memory [16]. Published data suggest that the plant extract has nootropic effects [17], protects the brain from age-related oxidative damage [18], and promotes nerve growth and neuronal dendritic arborization [19]. Our previous findings showed that *C. asiatica* has anti-inflammatory effects in lipopoly saccharide-treated neuronal cells of rats [20].

In this research we evaluated the expression of α-synuclein and aggregation formation in rotenone-induced zebrafish treated by *C. asiatica* extract. We also analyzed zebrafish motility and dopamine levels in the brain.

2. Materials and methods

2.1. Subjects

Adult male and female zebrafish were obtained from commercial suppliers from Tulungangung, East Java, Indonesia. Zebrafish were identified in Hydrology Laboratory of Fishery Faculty of Brawijaya University. Before treatment, zebrafish were housed in semi-static 60 L tank and reared under standard procedure [21]. Fish were fed three times daily (Tetra Bit and Color Tropical Flakes, Tetra Sales, Blacksburg, Germany), and kept on a 14:10 light–dark cycle. Water temperature was maintained between 24 and 26.5 °C. All procedures were approved by the Committee of Medical Faculty of Brawijaya University (No. 253/EC/KEPK/03/2014).

2.2. Collection of plant material and extraction

C. asiatica was gained from Materia Medica, Batu, Malang, Indonesia. The aerial part (leaves and branches above ground) was washed and dried. Dried powder of *C. asiatica* (100 g) was diluted in 900 mL of 96% methanol (maceration) and evaporated in 67 °C. Active compound composition is very important to know the potential of herbs. One of active compound that used as both standard quality and biomarker for *C. asiatica* is asiaticoside. The asiaticoside level in the extract was then measured as one of biomarker of *C. asiatica* by liquid chromatography-mass spectrometry (Thermo Scientific, Accela).

2.3. Rotenone and C. asiatica treatment

The used concentrations of rotenone (Sigma 8875) were based on explorative experiment. We used 2 μg/L rotenone and had no significantly effect on adult zebrafish. We used 2, 5 and 10 μg/L rotenone for 28 days exposure. Finally, we found that the appropriate concentration was 5 μg/L. Rotenone concentration at 2 μg/L was found to have no effects on an explorative zebrafish motility and rotenone 10 μg/L caused fish death after 48 h (data not shown). So we used 5 μg/L concentration because it had significant effects and the zebrafish were still alive until 4 weeks. Five fish were placed in 3 tank (25 cm × 16 cm × 12 cm) for each group, fed three times daily and the medium was changed every 48 h. All fish were reared under a photoperiod of 14:10 (dark: light). Methanolic extract of *C. asiatica* was added in various concentrations (2.5, 5.0 and 10.0 μg/mL) in the same time with rotenone for 28 days.

2.4. Motility observation

Dysfunction of locomotor activity is clinical syndrome for PD. One of them is bradykinesia (decreasing locomotor activity). The locomotor activity of adult zebrafish was assessed in a 2 L tank (L × W × H: 25.0 cm × 16.5 cm × 12.5 cm) filled with 2 L water system. Normal behavior of fish is to swim back and forth along the length of the tank. Simple observation was used to determine the locomotor activity of adult zebrafish. Three vertical lines were drawn on the tank at equal distances, dividing the tank into four zones (the length of each zone was 6.25 cm). Locomotor activity was measured for 5 min by counting the number of lines that adult zebrafish crossed. Therefore, the total distance that the adult zebrafish traveled was in direct proportion to the total number of lines that the fish crossed. The locomotor activity was calculated by the total number of lines that the zebrafish crossed, divided by time, and expressed in the number of crossed lines/5 min (modified [22]).

2.5. Dopamine measurement by ELISA

Zebrafish were euthanized using the standard National Institutes of Health recommended methods by submersion in ice water (5 parts ice/1 part water, 0–4 °C) for at least 10 min following cessation of opercular (*i.e.*, gill) movement. The head part was then extracted to get the protein and dopamine level was measured by ELISA method (Fast Track procedure base on LDN's technology). The samples of each group were gained from three heads of zebrafish.

2.6. Western blotting

2.6.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

α-Synuclein protein isolated from heads of zebrafish was added by reducing sample buffer and heated at 100 °C for 3 min. Cooled samples were injected into the wells which is 20 μL for each well. The samples (20 μg) were ran in 30 mA and 130 V until reach 0.5 cm from the bottom of the plate. The gel was stained with EtBr and destained for 20 min. After transferring the gel to nitrocellulose, the membrane was rinsed and washed