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Zebrafish Parkinson's Model: Rotenone decrease motility, Dopamine, and increase α-synuclein Aggregation and Apoptosis of Zebrafish Brain

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Abstract: Rotenone is a pesticide that is widely used to kill insects and nuisance fish in lakes. The mechanism of toxicity of rotenone is primarily mediated by its potent mitochondrial complex I inhibition. In this study, we examined the motility, dopamine, α-synuclein and apoptosis as hallmark of PD in rotenone-induced zebrafish (*Danio rerio*). We used adult zebrafish (8 months) and exposed to 5 μg/L rotenone for 28 days. Motility observed for 5 minutes in each week. Measurement dopamine (DA) by ELISA, apoptosis by immunohistochemistry and α-synuclein by immunohistochemistry and westernblotting of zebrafish brain. The results showed that rotenone significantly ($p < 0.05$) decreased motility start at day 14, decreased dopamine, increasing α-synuclein expression and aggregation that seems both of that leading to apoptosis of neuronal cells. Together, these data suggest that sub chronic exposure (28 days) of rotenone 5 μg/L caused Parkinsonism due to decreases dopamine level, locomotor activity, increasing α-synuclein expression and aggregation and apoptosis of dopaminergic neuron as well.
Key words : Zebrafish, Rotenone, motility, dopamine, *α-synuclein.*

Introduction

Parkinson Disease (PD) is the second most chronic neurodegenerative disorder in the world, after Alzheimer´s Disease (AD), and is estimated to affect about 2% of the population over 60 years of age. It is characterized by the clinical triad of rigidity, bradikinesia and tremor, and by the neuropathological loss of dopaminergic neurons (DNs) caused by the disruption of dopaminergic neurotransmission in the basal ganglia, which causes a reduction in the numbers of dopaminergic neurons in the substantia nigra with typical intra cytoplasmatic ubiquitin and α-synuclein-positive inclusions, the Lewy bodies[1].

Idiopathic PD is clinically defined by both the development of extrapyramidal motor disturbances, such as bradykinesia, resting tremors, rigidity, and a later loss of postural reflexes, and a good response to l-dihydroxyphenylalanine (l-dopa) treatment.

The main pathological hallmark of idiopathic PD is a progressive loss of neuromelanin-containing dopaminergic neurons from the *substantia nigra pars compacta* (SNpc; about 5% of cell loss per year), a midbrain structure. Dopaminergic cell loss is associated with the presence of eosinophilic intraneuronal inclusions, called Lewy bodies (LB), composed of neurofilaments and ubiquitin[2]. At present, it is widely accepted that α-synuclein may play a central role in several neurodegenerative disorders because of the presence of insoluble α-synuclein as the major fibrillar component of inclusion bodies[3].

α-Synucleins natively unfolded and predominantly non-phosphorylated in vivo[4], but in aging human brains[5] and synucleinopathies, a significant fraction of aggregated α-synuclein is phosphorylated at Ser 129 (p-Ser 129)[4,6]. p-Ser 129 was initially reported to accelerate the oligomerization and fibrillization of α-synuclein[4,7,8] as well as accumulation and aggregation of α-synucleinto form LB in animal models of synucleinopathies[9,10]. There is evidence that inhibition of α-synuclein aggregation process is ascosiated with a decrease of α-synuclein toxicity[11,12] and overexpression of α-synuclein cause progressively neuronal loss[13] which means the neuronal cells dealing with apoptosis process.

Neurotrophins play a key role in the neuroprotection of the dopaminergic phenotype[14]. Brain-derived neurotrophic factor (BDNF) is a potent dopaminergicneurotrophin and its decrease is reported in the substantianigra of PD patients, especially in the pars compacta[15-17]. Therefore, it is highly likely that BDNF down- regulation might play an important role in the pathogenesis of PD. Another research proved that a-synucleinoverexpression was decreased the expression of BDNF, and also to suppress the transactivation of nuclear factors of activated T-cells (NFAT) and cAMP response element binding protein (CREB), both of which regulate BDNF expression[18].

Since rotenone, a complex I inhibitor, can cause many of the pathological features of PD in rats and complex I dysfunction has been associated with PD in humans. Rotenone used as agent for PD model due to inhibit mitochondrial complex I (c-I), decreasing endogenous antioxidants and generating oxidative stress from complexes I and III (c I, c III, respectively) which leads to oxidation of macromolcules. Additionally, cytochrome c (Cyt c) is released from the intermitochondrial space, activating caspase signaling and subsequent apoptotic cell death[19].

Zebrafish is becoming an increasingly attractive model organism for understanding biology and developing therapeutics, because as a vertebrate, it shares considerable similarity with mammals in both genetic compositions and tissue/organ structures, and yet remains accessible to high throughput phenotype-based genetic and small molecule compound screening[20]. Zebrafish models have significantly contributed to our understanding of vertebrate development and, more recently, human disease. The growing number of genetic tools available in zebrafish research has resulted in the identification of many genes involved in developmental and disease processes[21]. In this research we observed the rotenone-induced Zebrafish PD model through the expressions of α-synuclein, BDNF, caspase-3, caspase-9 and apoptosis of adult zebrafish (*Daniorerio*) brain.

Materials and Methods

Subject

Adult male and female zebrafish were obtained from commercial suppliers from Tulungangung, East Java, Indonesia. Zebrafish identified at Hydrology Laboratory of Fisheries Faculty Brawijaya University. Before treatment zebrafish adapted in semi-static 60 L tank and rear as standard procedure[22]. Fish 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 28±1 °C. All procedures were approved by the Ethical Committee of Medical Faculty Brawijaya University (No. 253/EC/KEPK/03/2014).

Rotenone Treatment

Rotenone (Sigma 8875) concentration based on explorative experiment. Recent research used 2 μg/L rotenone and had no significantly effect on adult zebrafish[23]. We used 2, 5 and 10 μg/L rotenone for 28 days exposure. Finally we found appropriate concentration was 5 μg/L. Rotenone concentration 2 μg/L had no effect on zebrafish motility and rotenone 10 μg/L caused fish died after 48 hours (data not shown). Five fish placed in 25x16x12 cm tank for each group in 2 L water, fed 3 times daily and change the medium every 48 hours.

Motility Assessment

The locomotor activity of adult zebrafish was assessed in a 2L tank (LxWxH: 25x16.5x12.5 cm) filled with 2 L system water. Five fish placed in each tank. As the 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 recorded and measured for 5 min by counting the number of lines that adult zebrafish crossed. Therefore, the total distance that the adult zebrafish traveled was indirect 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 were expressed in number of crossed lines/5 min[23] (Modified).

Dopamine Measurement by ELISA

Zebrafish were euthanized using the standard NIH recommended methods by submersion in ice water (5 parts ice/1 part water, 0-4º C) for 30 seconds following cessation of opercular (i.e., gill) movement. The head part then extracted to get the protein for dopamine ELISA (Fast Track procedure base on LDN). The samples of each group gained from 3 heads of zebrafish.

Immunohistochemistry

After 28 days zebrafish were sacrificed to obtain the brain by decapitation of head in cold ice. The head immediately immersed in buffer formalin before prepared for paraffin block. Head was sliced (without decalcification) 0.4 μM thick and prepare for immunohistochemistry. Slide was deparaffinization and stain based on vendor manual procedure. Antibodies we used were BDNF (SantaCruz), α-synuclein (Sigma), apoptosis (ApopTag @Peroxidase), caspase-9 (AnaSpec 55379) and Caspase-3 (Abcam ab13847). The expression of BDNF, α-synuclein, caspase-3, caspase-9 and apoptosis were observe at the midbrain area of zebrafish brain 4 times observation each slide in 1000 times magnification.

Results and Discussion

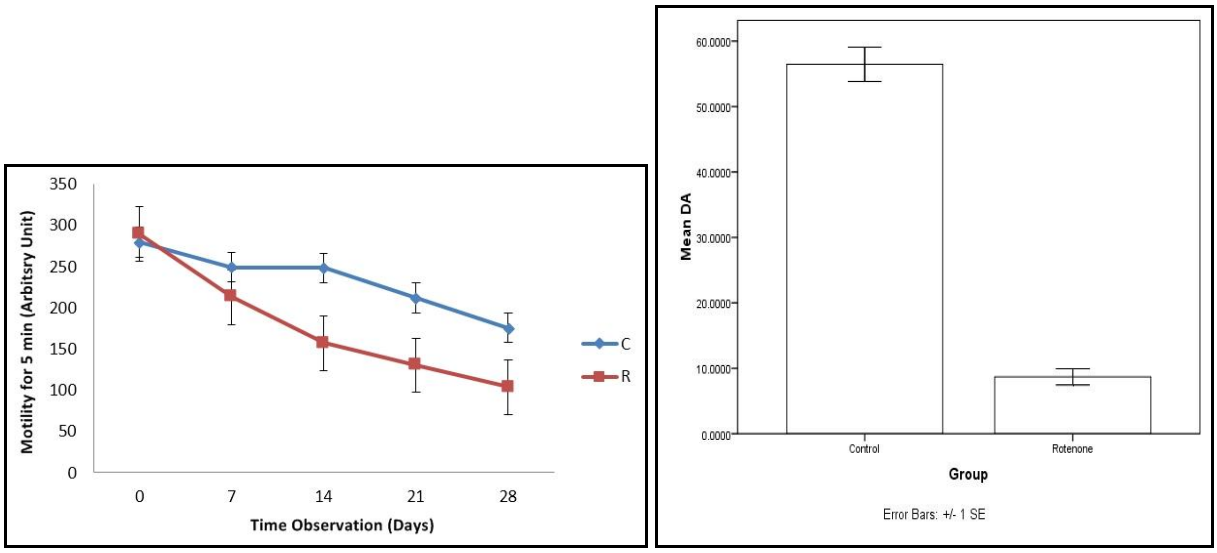
Rotenone and Locomotor Activity

Rotenone, a potent retinoid which is used as a pesticide and insecticide, has been shown to cause systemic inhibition of mitochondrial complex I activity, with consequent degeneration of dopaminergic neurons within the substantianigra and striatum, as observed in Parkinson's disease[24]. A strong link between mitochondrial dysfunction and PD is supported by the findings that neurotoxins affecting respiratory complex I induce specific death of DNs, and by the discovery that a number of causative genes in familial forms of PD encode mitochondrial proteins. Remarkably, emerging pathogenic pathways in PD are related to an impaired mitochondrial stability. Recently, an intrastriatal rotenone infusion approach was found to produce a useful Parkinsonian model, as shown by behavioural, immuno-histochemical and biochemical analyses of nigrostriatal function[25].

Rotenone toxicity may result from oxidative stress. Brains of PD patients show evidence of oxidative stress, including decreased levels of reduced glutathione and oxidative modifications to DNA, lipids, and proteins[26-29], and oxidative damage is hypothesized to contribute to the neurodegenerative process in PD[30].

Since its discovery as a prominent chemical neurotransmitter in the vertebrate nervous system, dopamine (DA) is recognized to have many important physiological functions including the control of movement, cognition, affect, as well as neuroendocrine secretion[31]. DA neurons exhibit overall conserved organization and function across vertebrates (Smeets and Reiner, 1994). In adult zebrafish DA neurons are conspicuously absent from the ventral midbrain, the ventral forebrain DA neurons ascending to the striatum (where ventral midbrain DA neurons in mammals project) are likely the functional counterpart of the mammalian midbrain DA neurons[32].

Decreasing locomotor activity of zebrafish seems due to mitochondrial dysfunction as powerhouse of the cells that caused depleting ATP production, disruption of mitochondrial permeability, increasing Ca^{2+} and overproduction of reactive oxygen species. These condition can lead to autooxidation of dopamine or its enzyme (tyrosine hydroxilase), therefore decreasing dopamine as neurotransmitter for motility (Figure 1). In the other hand, stress oxidative by mitochondrial dysfunction lead to releasing caspasefamiliy protein and apoptosis of dopaminergic neuron in substantianigra (neuronal loss).



(a) **(b)**

Figure 1. (a) Motility assessment and (b) Dopamine level. Rotenone 5 μg/L exposure comparing to control group motility observed each week (4 weeks) for 5 minutes recording and ELISA. Data showed that rotenone significantly decrease zebrafish motility and dopamine level. Pre-treatment motility (W0) showed that fishes had same motility (homogen). But rotenone gradually decrease the motility until 28 days (n=5).

Rotenone increased α-Synuclein, Caspase-9 and Caspase-3

Subsequent studies found that the rotenone model accurately recapitulates many other features of PD[33], including: accumulation and aggregation of endogenous, wild-type α-synuclein; α-synuclein and polyubiquitin positive Lewy bodies and Lewy neuritis, apomorphine responsive behavioral deficits, early and sustained activation of microglia, oxidative modification and translocation of DJ-1 into mitochondria in vivo, impairment of the nigral ubiquitin proteasome system, accumulation of iron in the substantia nigra through a mechanism involving transferrin and transferrin receptor 2[34].

Neurons critically rely on mitochondrial function and oxygen supply, since most neuronal ATP is produced by oxidative phosphorylation. High ATP levels are required to sustain axonal transport of macromolecules and organelles such as mitochondria, to maintain ionic gradients and the membrane potential, to load synaptic vesicles with neurotransmitters and to release neurotransmitters into the synaptic cleft. Moreover, synaptic mitochondria are exposed to extensive Ca^{2+} influx and have a key role to buffer the cytosolic Ca^{2+} concentration[1].

Oxidative stress due to mitochondrial dysfunction could mutated α-synuclein which is easier to form aggregation. Ubiquitin proteasome system also inhibit by oxidative stress that caused increasing accumulation of misfolding protein such as α-synuclein. This accumulation can lead to cell death because its very toxic. Inspite of α-synuclein accumulation, mitochondrial dysfunction also loss of its permeability and release cytochrome family caspase-9. Caspase-9 activate pro-caspase3 became caspase3 as apoptotic executor. Our results showed increasing toxic protein aggregation α-synuclein (Figure 2). In the other hand we proved that rotenone mechanism through mitochondrial dysfunction by increasing caspase-9 and caspase-3 (Figure 3 and 4). As the end point point of those events was apoptosis known as neuronal lost.

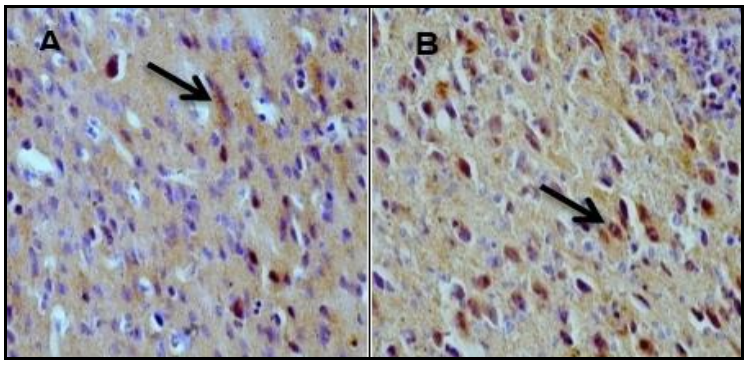


Figure 2. Expression of α-Synuclein in zebrafish midbrain. T-test analysis showed sub-chronic exposure of rotenone significantly increased α-synuclein expression in zebrafish midbrain (B) compare to control (A) (p = 0.000) n=5; (1 bar = 0.01 mm)

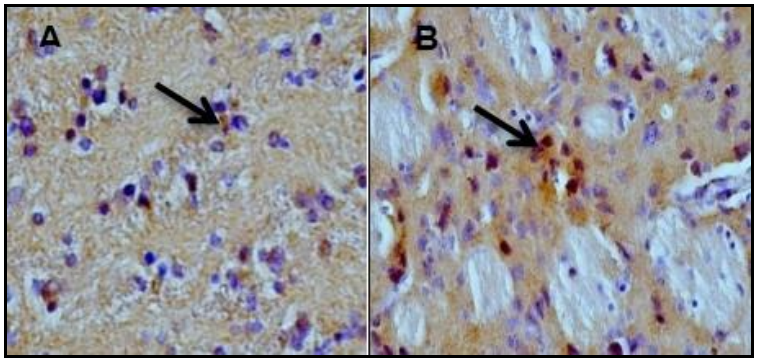


Figure 3. Expression of caspase-9 in zebrafish brain. T-test analysis showed sub-chronic exposure of rotenone significantly increased caspase-9 expression in zebrafish midbrain (B) compare to control (A) (p = 0.000) n=5; (1 bar = 0.01 mm)

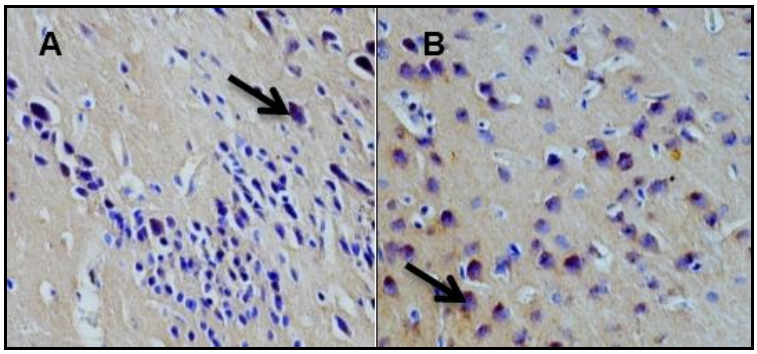


Figure 4. Expression of caspase-3 in zebrafish brain. T-test analysis showed sub-chronic exposure of rotenone increased caspase-3 expression in zebrafish midbrain (B) compare to control (A) (p = 0.005) n=5; (1 bar = 0.01 mm)

Rotenone decreased BDNF expression

Brain-derived neurotrophic factor (BDNF) is directly involved in neurite outgrowth and regulates the survival, differentiation, and maintenance of function in dif- ferent neuronal populations[35]. The level of BDNF in PD patient known decreased[36].

A novel intra striatal rotenone model of Parkinson's disease was used to examine the neuroprotective effects of valproic acid (VPA), which is known to upregulate neurotrophic factors and other protective proteins in the brain[24]. Base on the data we gained that expression of BDNF in zebrafish midbrain exposed to rotenone was decrease (Figure5 and Figure 6).

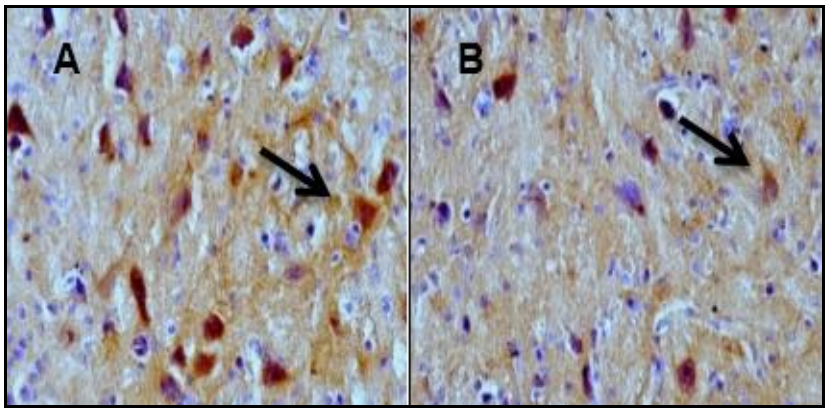


Figure 5. Expression of BDNF in zebrafish brain. T-test analysis showed sub-chronic exposure of rotenone significantly decreased BDNF expression in zebrafish midbrain (B) compare to control (A) (p = 0.001) n=5; (1 bar = 0.01 mm)

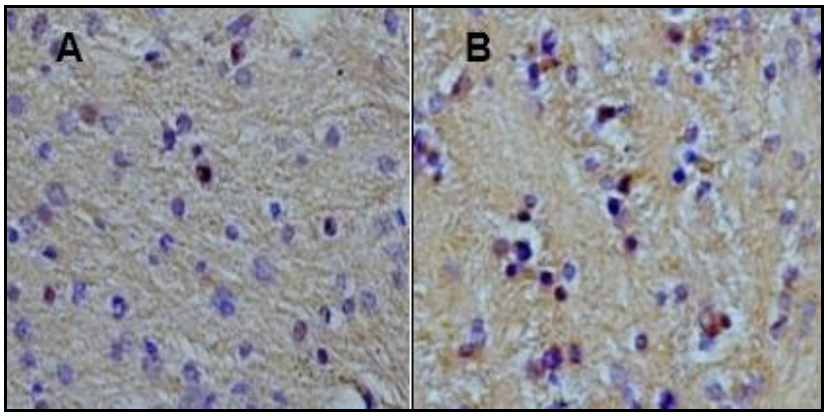


Figure 6. Expression of Apoptosis (TUNEL) in zebrafish brain. T-test analysis showed sub-chronic exposure of rotenone significantly increased apoptosis in zebrafish midbrain (B) compare to control (A) (p = 0.000) n=5; (1 bar = 0.01 mm)

Conclusion

Rotenone 5 μg/L exposure for 28 days showed Parkinsonism signal such as increasing α-synuclein expression, caspase-9, caspase-3 and apoptosis of zebrafish midbrain, decreasing neurotrophic factor BDNF as well.

Acknowledgement

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