Effect of *Centella asiatica* Extract on Locomotor Activity and Hsp60 Expression in Zebrafish models of Parkinsons

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**Abstract** Parkinson's disease characterized by a decrease in motor activity is a progressive neurodegenerative disorder caused by the degeneration of dopaminergic neurons. *Centella asiatica* is suspected of having a neuroprotectant effect and is not yet known how *Centella asiatica* role in the prevention of Parkinson's disease. The study was conducted to prove the effect of *Centella asiatica* extract on the expression of Hsp60 and locomotor activity zebrafish models of Parkinson's with rotenone exposure. The study was conducted using 25 zebrafish divided into various groups. *Centella asiatica* extract and rotenone exposure have given for 28 days, observed locomotor activity on days 0, 7, 14, 21 and 28. The expression of Hsp60 measured using immunohistochemical techniques. There is a significant difference between locomotor activity at various doses of *Centella asiatica* (p<0.05) with a very strong correlation (r=0.929; p<0.01) where the higher doses of *Centella asiatica*, the higher locomotor activity. Found a significant difference between the reduced expression of Hsp60 to various *Centella asiatica* dose group (p<0.05) but no correlation between the expression of Hsp60 with *Centella asiatica* dose groups and locomotor activity. *Centella asiatica* extract is able to increase locomotor activity and decrease the expression of Hsp60 in zebrafish models of Parkinson's.

**Introduction** Parkinson's disease is a progressive neurodegenerative disorder that has clinical characteristics such as tremor, rigidity, bradykinesia and postural instability. Such motor abnormalities result from the loss of dopaminergic neurons in the substantia nigra pars compacta. The pathological abnormality that arises is the presence of Lewy bodies, a cytosolic inclusion body largely dominated by α-synuclein proteins (Surendran & Rajasankar, 2010; Fitzmaurice & Bronstein, 2011).

Mitochondrial dysfunction, oxidative stress, and inflammation occurring in neurodegenerative conditions are the underlying cause of damage to dopaminergic neurons (Przedborski, 2007). Although genes have been found to be associated with familial Parkinson's disease, few cases are found in comparison with cases of sporadic Parkinson's disease, so environmental factors are thought to play an important role in the pathogenesis of Parkinson's disease (Betarbet et al., 2000; Fitzmaurice & Bronstein, 2011).

The progressive neurodegenerative nature of Parkinson's disease arises from exposure to toxins from persistent environmental ingredients resulting in the incidence of cell death cascades. One environmental material that is toxic to neurons is rotenone, a natural pesticide which is a specific inhibitor of complex 1 mitochondrial role in oxidative phosphorylation, a high affinity and is highly hydrophobic, so it is able to cross biological membranes easily and does not depend on the dopamine transporter to enter the cytoplasm.
Effect of Centella asiatica Extract on Parkinson's Disease

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In a previous study, rotenone was known to cause ROS formation, inhibited VMAT2 activity in the cytosol and induced apoptosis from dopaminergic cell culture (Watabe, 2007, 2008).

One of the triggers for the emergence of protein metabolism dysfunction in Parkinson's disease is oxidative stress due to ROS that will be associated with protein misfolding and α-synuclein aggregation (Przedborski, 2007). Hsp60 is a chaperone in charge of folding misfolding proteins to maintain healthy mitochondria. Hsp60 in the brain is endogenously expressed in astrocytes, neurons, microglia, oligodendrocytes and ependymal cells. Most of the Hsp60 protein is in the mitochondria (Stetler et al., 2010). Previous research on Hsp60 is still much in contention, a study by Sonia et al. (2012), shows up-regulation of Hsp60 seen in cerebellum, striatum, substantial nigra and cerebral cortex in rotenone-induced brain than in the control group, but Hsp60 is known to decrease significantly in transgenic mice given rotenone and increase α-synuclein aggregation (George et al., 2010).

Management of Parkinson's disease until now only a symptomatic therapy. The success rate of dopamine agonist therapy is only about 46% (Shulman et al., 2000). Lack of therapy that can be used for Parkinson's disease led to many studies being developed about new therapies for Parkinson's disease.

Centella asiatica is widely used in research in neurodegenerative disorders because of its ability not only as antioxidant and anti-inflammatory but also useful as neuroprotectant (Rao et al., 2008; Xu et al., 2013). It is interesting to note further considering the anti-inflammatory and neuroprotective effects of Centella asiatica are thought to inhibit free radicals, so it may be possible to inhibit the effects of chronic rotenone exposure. In this study will be proved the effect of giving Centella asiatica extract to the expression of Hsp60 and increased activity of locomotor Zebra Fish (Danio rerio) Parkinson model with rotenone exposure.

Materials and Methods

Reagents and Antibodies

Rotenone pesticide from Sigma (R8875), with ≥95% purity. 20 mg of rotenone was dissolved in 1 ml of DMSO (dimethyl sulfoxide). Dilution was performed for preparation of 2x105 μg / l stock solution, 10 μl of stock solution 2x105 μg / l added aquades 990 μl (DMSO level 1%). To obtain a concentration of 5 mg / l, administration of rotenone on stock 2 x 105 g / l of 50 mL (DMSO concentration of 0.25 ppm) into a 2-liter tank and replaced every 2 days. The primary anti-Hsp60 antibody from Abcam (ab31115) with a ratio of 1: 150.

Centella asiatica Extraction

Parts of leaves and stems above ground was washed and then cut 1-2 cm, put in a temperature oven 400 degrees Celsius until dry. Centella asiatica mashed with a blender weighed as much as 100 grams, soaked with methanol solution in an erlenmeyer glass until the volume to 1 liter. Shaken ± 30 minutes, then settled 1 night to settle and filtered using filter paper. The solution of the extraction process is then evaporated. The baths are obtained approximately ¼ of dry matter. An apparatus extract solution was stored for the stock at a concentration of 10 g / L (10 mg/ml) in an aqueous solvent.

Calculation of Locomotor Activity

Locomotor activity is calculated from the motion activity of the zebrafish measured in 2 liter aquarium volume and the length of x width x height = 25 x 16.5 x 6.5 cm which is given 3 lines at the bottom of the aquarium vertically on the long side, divide the aquarium into 4 equal parts (the distance of each section 6.25 cm). Zebrafish are allowed to move actively,
observed and recorded using a video camera for 5 minutes. The movement of the fish is calculated from the recorded video, which is calculated is the movement of fish through the striped area (Bretoaud et al., 2004).

**Sample Preparation of the Zebrafish Brain**

Zebrafish taken and placed on ice pack. After the zebra fish do not show spontaneous movement, performed surgery to take brain tissue, by separating the fish head from the fish’s body. The fish head is inserted into a bottle containing 10% formalin solution, the bottle is tightly closed and stored at -20°C. Furthermore, shedding the brain tissue and making a slide with paraffin block.

**Immunohistochemistry examination**

Slides that have been through the process of deparafinisasi done retrieval antigen by using decloaking chamber for 30 minutes with a temperature of 97°C. Immunohistochemically cleansing slides were sterilized with 4% H2O2 in methanol and incubated for 15 min, then a non-specific blocking of proteins was performed, the slides were split with a Background sniper (Biocare Startrex) for 30 min at room temperature.

The incubation process of primary antibody with Hsp60 primary antibody dissolved in buffer PBS + 2% FBS + 0.25% Triton X-100 (1: 150) and incubated for 2 hours at room temperature (25-27 degrees Celsius). Slides were washed subsequently drip with secondary antibody (Trekkie Universal Link Biocare) 50 mL and incubated for 60 min at room temperature. Then stamped with SA-HRP (Trek Avidin - HRP Radis Peroxidase) 50 μl for 40 min at room temperature.

Chromagen application does DAB (DAB chromogen: DAB buffer = 1:40), incubation of 5 minutes at room temperature. The slides were stained by counterstain deposition with Mayer’s Hematoxilen (Mayer’s Hematoxilen: aquades comparison 1:20) incubated for 5 min at room temperature and rinsed with aquades. The last step of immunohistochemical staining is the process of mounting with Entellan, then the slide is dried and observed under a microscope.

**Statistical analysis**

The data were analyzed statistically using one-way Anova test, with Tukey Post Hoc Test, followed by Pearson correlation test. For data that did not meet the requirements of the Anova test, the data will be analyzed using Kruskal-Wallis test, with Mann-Whitney Post Hoc Test. Statistical test results are considered significant when p<0.05.

**Results and Discussion**

**Zebrafish Locomotor Activity**

The average of locomotor activity on days 0, 7, 14, 21 and 28 between the treatment group using ANOVA test, there was a significant result (p<0.05). To know in which groups there was a significant difference in the post-hoc Tukey analysis (Table 1).

The homogeneity test of locomotor activity on day 0 has a significance value of 0.023 (p<0.05) so that it does not have any homogeneous data variance, after the logarithmic transformation has no homogeneous data variance, so Kruskal-Wallis of nonparametric test and post hoc Mann-Whitney were used. From the Kruskal-Wallis test, there was a significant difference (p<0.05).

The result of Pearson correlation test to determine correlation between dose of Centella asiatica with locomotor activity effect showed significant value (p<0.01) with correlation coefficient value 0.929 which showed there was significant correlation between locomotor activity with Centella asiatica dose with very strong correlation. There was a positive correlation where the higher the dose of Centella asiatica, the higher the activity of locomotor.
To know whether there is difference of exposure time with activity of locomotor then tested using repeated Anova at each treatment group. In the Centella asiatica group dose 2.5 µg / ml obtained the distribution of abnormal data, so that nonparametric test Friedman followed by post hoc Wilcoxon test (Table 2).

**Table 1. Comparison of Various Time Locomotor Activities with Various Doses of Centella Asiatica**

<table>
<thead>
<tr>
<th>Observation Time</th>
<th>Number of Locomotor Activity every 5 minutes (±SD)</th>
<th>Sign. (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K (-)</td>
<td>K (+)</td>
</tr>
<tr>
<td>Day-0</td>
<td>114.8 ±23.32</td>
<td>151 ±12.45</td>
</tr>
<tr>
<td>Days-7</td>
<td>142.6 ±13.72</td>
<td>149.4 ±11.06</td>
</tr>
<tr>
<td>Days-14</td>
<td>196.4 ±10.64</td>
<td>137.6 ±7.86</td>
</tr>
<tr>
<td>Days-21</td>
<td>149 ±22.63</td>
<td>111.4 ±17.53</td>
</tr>
<tr>
<td>Days-28</td>
<td>181.2 ±36.29</td>
<td>148.2 ±12.05</td>
</tr>
</tbody>
</table>

**Table 2. Test Result Differences Time Exposure with Locomotor Activity**

<table>
<thead>
<tr>
<th>Locomotor activity</th>
<th>Locomotor activity Days-7</th>
<th>Locomotor activity Days-14</th>
<th>Locomotor activity Days-21</th>
<th>Locomotor activity Days-28</th>
<th>Sign. (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control Group</td>
<td>151 ±12.45</td>
<td>149.4 ±11.06</td>
<td>137.6 ±7.86</td>
<td>111.4 ±17.53</td>
<td>148.2 ±12.05</td>
</tr>
<tr>
<td>Rotenone + Centella asiatica 2.5 µg/ml Group</td>
<td>191.8 ±20.07</td>
<td>215 ±18.18</td>
<td>180.2 ±18.21</td>
<td>197.2 ±19.33</td>
<td>172.6 ±15.48</td>
</tr>
<tr>
<td>Rotenone + Centella asiatica 5 µg/ml Group</td>
<td>253.8 ±35.02</td>
<td>244.4 ±24.05</td>
<td>217.2 ±10.50</td>
<td>193.6 ±35.99</td>
<td>211.4 ±9.37</td>
</tr>
<tr>
<td>Rotenone + Centella asiatica 10 µg/ml Group</td>
<td>238.2 ±21.83</td>
<td>203.6 ±15.90</td>
<td>238.8 ±22.96</td>
<td>221.8 ±28.56</td>
<td>235.8 ±19.45</td>
</tr>
</tbody>
</table>

**Hsp60 Expression Examination of the Zebrafish Brain**

Hsp60 expression is checked after 28 days exposure. The expression of Hsp60 protein in brain tissue of zebrafish was measured in brain tissue by immunohistochemical method using anti-Hsp60 antibody (Figure 1). Cell counting was done in brain tissue of zebra fish in microscope in 10 field of view with magnification of 40x (hotspot method). The result of the data obtained is the average number of cells expressed in 10 viewing field (Table 3).
Normality test has normal data distribution but homogeneity test does not have the same data variance, so it is necessary to do data transformation so that the data variance is same. In the test of data variance the result of transformation obtained value of significance of 0.088 (p>0.05) or already has the same data variance, so that ANOVA test is valid.

**Figure 1.** An expression of Hsp60 brain expression of zebra fish with immunohistochemical techniques in the control group and treatment group on day 28 (400× magnification).

Annotation: (A) Hsp60 expression (arrow) in negative control group; (B) Hsp60 expression (arrow) in positive control group (rotenone); (C) Hsp60 expression (arrow) in rotenone + Centella asiatica 2.5 µg/ml group; (D) Hsp60 expression (arrow) in rotenone + Centella asiatica 5 µg/ml group; (E) Hsp60 expression (arrow) in rotenone + Centella asiatica 10 µg/ml group.

**Table 3. Average Number Expression of Hsp60 in the Treatment Group**

<table>
<thead>
<tr>
<th>Treatment Group (±SD)</th>
<th>K (-) (±12.85)</th>
<th>K (+) (±28.29)</th>
<th>P1 (2.5 µg/ml) (±5.72)</th>
<th>P2 (5 µg/ml) (±21.79)</th>
<th>P3 (10 µg/ml) (±17.82)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp60</td>
<td>48.37</td>
<td>75.46</td>
<td>43.89</td>
<td>33.78</td>
<td>57.35</td>
</tr>
</tbody>
</table>

Annotation : K(-): negative control group, K(+): positive control group (rotenone), P1: rotenone + Centella asiatica 2.5 µg/ml group, P2: rotenone + Centella asiatica 5 µg/ml group, P3: rotenone + Centella asiatica 10 µg/ml group

The result of ANOVA test showed that there was a significant difference between Hsp60 expression and treatment group (p<0.05). From Tukey post hoc test there was a significant difference of Hsp60 expression significantly in rotenone group compared to rotenone + Centella asiatica group of 5 µg/ml (Table 4).

To determine the correlation between the dose of Centella asiatica and the expression of Hsp60 then tested the correlation between the treatment group with the average of Hsp60 expression that has been transformation. Pearson correlation test results obtained significance value 0.463 (r=-0.165; p>0.01) indicating that there is no significant relationship between Hsp60 expression with Centella asiatica dose.

To know the correlation between locomotor activity with expression of Hsp60 hence correlation test between mean variable of activity of lokomotor day 28 with average of expression of Hsp60 which have been transformation. Pearson correlation test results showed no correlation between locomotor activity with Hsp60 expression (r=-0.395; p=0.069).
Table 4. One Way ANOVA Hsp60 Analysis Results

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (±SD)</th>
<th>Sign. (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>6.91 ± 0.92</td>
<td>0.040</td>
</tr>
<tr>
<td>Positive Control</td>
<td>8.57 ± 1.61</td>
<td></td>
</tr>
<tr>
<td>Rotenone + Centella asiatica 2.5 µg/ml</td>
<td>6.61 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>Rotenone + Centella asiatica 5 µg/ml</td>
<td>5.60 ± 1.78</td>
<td></td>
</tr>
<tr>
<td>Rotenone + Centella asiatica 10 µg/ml</td>
<td>7.50 ± 1.19</td>
<td></td>
</tr>
</tbody>
</table>

Annotation: Tukey HSD Post-Hoc Test: Values of Positive Control Group vs Group of Rotenone + Centella asiatica 2,5 µg/ml are p=0.025 (p<0.05)

Rotenone is a pesticide that inhibits complex I of the mitochondrial electron transport chain, selectively working on dopaminergic neurons and inducing Parkinsonism (Fitzmaurice & Bronstein, 2011). In addition to causing direct damage to dopaminergic neuron cells, rotenone can also cause dopaminergic neuron cell damage through microglia activation. Research conducted by Gao et al. (2002), using pure dopaminergic neuron cell culture and mixed cell neuron-glial cultures showed that the damage to rotenone-induced neurons was further enhanced by the presence of glial cells especially microglia through O2-production. Rotenone also distributes its toxicity through activated microglia, and the mechanism may be related to the NFkB signaling pathway (Yuan et al., 2013).

Chronic administration of rotenone will lead to the emergence of symptoms of Parkinsonism gradually including decreased motor activity and increased muscle tone or rigidity (Sonia et al., 2012). The use of rotenone in zebrafish will induce motion impairment or locomotor activity in larvae and adult fish (Breudaud et al., 2004). In this study, we observed changes in the locomotor activity of the Parkinson’s zebrafish using rotenone exposure between the control groups and the groups using various doses of pegagan extract.

After 28 days of rotenone exposure, it was found that there was a change in locomotor activity between groups. The average rotenone group activity (positive control) was lower than the mean of negative control group activity, although the statistical test showed no significant difference between the two groups (p=0.129), it can be assumed that rotenone would decrease the locomotor activity of the zebrafish.

The emergence of Parkinsonism symptoms induced by rotenone exposure is a high oxidative stress activity and leads to dopaminergic neuron cell death (Ahmadi et al., 2008). Centella asiatica (L) which has Asiatic acid as an active ingredient has several neuroprotective effects on oxidative stress induced by MPTP exposure in Parkinson’s mice (Gohil et al., 2010; Md, 2013). Another study conducted by Xiong et al. (2009), suggested Asiatic acid protective effect against H2O2 and mitochondrial dysfunction in SH-SY5Y-induced rotenone neuroblastoma cell culture.

The results of this study indicate that there is a significant difference between the locomotor activity of zebrafish exposed to rotenone with the dose of Centella asiatica extract (p<0.05). The correlation test showed that there was a significant correlation between locomotor activity and Centella asiatica dose (p<0.01) with very strong correlation coefficient, so it can be concluded that there is a dose-effect relationship between locomotor activity with the dose of Centella asiatica, where
the higher the dose of *Centella asiatica*, the higher also locomotor activity caused.

Previous studies using Asiatic acid in rat brain showed no significant difference in locomotor activity in the Asiatic acid group compared with the normal control group of saline (Nasir *et al.*, 2012). However, another study conducted by Xu *et al.* (2012), showed that groups of mice treated with asiaticoside had better locomotor activity compared to those using MPTP alone in open field and ladder walking tests. Further studies of Xu *et al.* (2013), using madecassoside in mice given MPTP also showed improvement in locomotor activity versus MPTP group.

In the early observation or locomotor activity on day 0, there was a significant difference between negative control group and positive control group, *Centella asiatica* group dose 2.5 μg/ml, *Centella asiatica* group dose 5 μg/ml, and *Centella asiatica* group dose 10 μg/ml. This may be due to the difference in the recording time of the locomotor activity in each group after aquarium replacement, causing differences in the rate of absorption of the treatments in each group.

In this study, we also performed time series checks on each group on days 0, 7, 14, 21 and 28 to see the effect of prolonged exposure on locomotor activity. From the repeated ANOVA test results, no significant difference between exposure time with locomotor activity in each group of rotenone and group of *Centella asiatica* dose 10 ug/ml, it can be assumed that locomotor activity change happened since day 0 and relative remain until observation day to 28. In the *Centella Asiatica* group dose 2.5 μg/ml, there was a significant difference between locomotor activity on day 0 with day-to-day locomotor activity and between day 7 activity and locomotor activity on day 21 and 28 days, group *Centella asiatica* dose 5 μg/ml found a significant difference between locomotor activity on day 0 with activity of locomotor day 21 and between activity of locomotor on day 7 with activity of day-to-day locomotor 28. In both group got activity of locomotor which tends to lower at the final observation (day 21 or 28) compared with observations at early (day 0 or 7), but the average locomotor activity is still higher than the average rotenone group activity alone. From these data, it can be assumed that dosing of *Centella Asiatica* extract dose 2.5 μg/ml and 5 μg/ml has not been effective enough to prevent the decrease in locomotor activity due to degeneration of dopaminergic neurons that continue to be exposed by rotenone.

Hsp60 or chaperonin is the best chaperone candidate in conjunction with Parkinson's disease, as it is the main folding machine of the mitochondria and more expressed in the substantia nigra that is the pathological location of Parkinson's disease (Broer *et al.*, 2011; Sonia *et al.*, 2012). Hsp60 in the brain expressed endogenously in astrocytes, neurons, microglia, oligodendrocytes and ependymal cells. Most of the Hsp60 protein resides within the mitochondria, and about 15-20% are located in extra mitochondrial locations such as the cytosol and the surface of non-nerve cells (Stetler *et al.*, 2010). Hsp60 is an inducible stress protein and is known to have interaction with caspase 3 by accelerating the activation of caspase 3 thus stimulating apoptotic pathways (Kitamura & Nomura, 2003; Stetler *et al.*, 2010).

Previous research on the role of Hsp60 is controversial, Hsp60 is known to have decreased significantly in transgenic mice given rotenone and increased α-synuclein aggregation (*George et al.*, 2010). Another study conducted by Broer *et al.* (2011), it suggests that yeast cells under normal Hsp60 conditions tend to accumulate failed folding proteins, resembling the α-synuclein aggregation. On the other hand, research conducted by Sonia *et al.* (2012) shows up-regulation of Hsp60 seen in the cerebellum, striatum, substantial nigra and
cerebral cortex in the rotenone-induced brain than in the control group. This powerful Hsp60 expression reflects the burden of the mitochondria and is an indication of mitochondrial dysfunction or oxidative stress due to rotenone.

No previous research has assessed the relationship between Centella asiatica extract and Hsp60, so this study aims to see the effect of Centella asiatica extract on Hsp60 expression. In this study, there was a significant difference of Hsp60 expression on various treatment groups (p<0.05), there was an increase in Hsp60 expression in the zebrafish brain of positive control group compared with the negative control group after chronic exposure, but the increase was not significant (p=0.353). In the group treated with Centella asiatica, Hsp60 expression decreased, a significant difference was found between Hsp60 expression between rotenone group and Centella asiatica group of dose 5 ug/ml (p=0.025). But there was no significant difference from Hsp60 expression from each treatment group given Centella asiatica extract. The correlation test also showed that there was no significant relationship between Hsp60 expression and Centella asiatica extract dose.

From these results, it can be assumed that there is a possibility of increased Hsp60 expression in the rotenone group is a response of oxidative stress due to rotenone exposure as evidenced by a decrease in locomotor activity in the rotenone group, and in the group treated with Centella asiatica extract helped to decrease the activity of oxidative stress characterized by the decrease of Hsp60 expression.

Oxidative stress will induce Hsp60 synthesis that will protect cells both in vitro and in vivo, which will then be associated with a decrease in cellular protein oxidation and this indicates the antioxidant function of Hsp60. This increase in Hsp60 secretion will also cause the opening of the transition pores of the mitochondrial membrane permeability so that Hsp60 is considered a pro-apoptotic protein. However, in some studies, Hsp60 in mammals also exhibited anti-apoptotic and pro-apoptotic function (Sarangi et al., 2013).

Hsp60 plays an important role in mitochondrial protein homeostasis, and whether the role of Hsp60 is pro-survival or pro-apoptosis is controversial. Research shows that the acceleration of cytotoxicity in response to rotenone correlates with increased production and translocation of Hsp60 from mitochondria into the cytoplasm. The theory proposed by researchers is that there is a cross-talk between mitochondrial oxidative stress and Hsp60 in response to the pharmacological of OXPHOS in tumor cells (Sarangi et al., 2013)

References


