The effect of centella asiatica extract in Zebrafish (*Danio rerio*) larvae as a Chronic Constant Hypoxia (CCH) model

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ABSTRACT

**Background:** Chronic Constan Hypoxia (CCH) are able to occur in cyanotic congenital heart disease and bronchopulmonary dysplasia (BPD). *Centella asiatica* has antioxidant and neuroprotective effects in experimental animals. This study aims to observe the effect of *Centella asiatica* ethanol extract on Zebrafish in chronic hypoxic conditions

**Method:** A laboratory experimental study with a randomized post-test controlled group design was conducted among 20 Zebrafish (*Danio rerio*) 3-8 day post fertilization (dpf) as samples per tube. The total group treatment was five with inclusion and exclusion criterias. Variables assessed in this study included Average body length, locomotor activity, and the relationship between average body length and locomotor activity. Data were analyzed using SPSS version 18 for Windows.

**Result:** There was a moderate significant correlation between *Centella asiatica* concentration of 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml with an enhancement of body length (r=0.431) and locomotor activity (r=0.262) in zebrafish larvae CCH model (p<0.05).

**Conclusion:** It can be concluded that chronic constant hypoxia (CCH) inhibit the growth of body length and decrease locomotor activity in Zebrafish larvae. *Centella asiatica* treatment can avoid the CCH by using various concentration.

**Keywords:** Hypoxia, Centella asiatica, Zebrafish, CCH


INTRODUCTION

The first 1000 days of life are critical periods for growth disturbances, including short stature and stunting.1 According to WHO, the prevalence of short stature toddlers becomes a public health problem if the prevalence is 20% or more. 29% of Indonesian children under five years old are included in the short stature category, with the highest percentage in East Nusa Tenggara and West Sulawesi province. Therefore the percentage of short stature children under five years old in Indonesia is still high and that is a health problem that must be addressed.2

According to the study of Pichavant et al (2001),3 a state of hypoxia is a key factor causing short stature because of its effect on lowering the metabolic rate. Chronic constan hipoxia (CCH), can occur in patient with cyanotic congenital heart disease and bronchopulmonary dysplasia (BPD). While, Chronic intermittent hypoxia (CIH) can occur in patient with obstructive sleep apnea (OSA), sickle cell anemia and asthma.4

In hypoxia condition, the production of reactive oxygen species (ROS) also increase which can cause damage and death to neuron cells, thus can affect sensory and motor disorders.5 Sensory and motor disorders can be assessed by locomotor activity. Along with locomotor activity, the motility of an organism can be observed to know the developmental disturbance.

The Zebrafish larvae (*Danio rerio*) has high genetic homology similarity (70-80% gene similarity). Their organ structure and function similar to mammals, additionally it can absorb chemicals that dissolved in water ease.6 Sorribes (2013) study was comparing the analogy of zebrafish age with humans based on the ontogeny of the wake-up cycle showed that the age of 4-10 days of zebrafish larvae was equivalent to the age of children aged 2-8 years.6

An alternative effort to reduce cell damage due to hypoxia-ischemic complications is using an herbal compound. In biomedical research, the use of herbs is an alternative considered, in addition to the use of drugs that have been widely produced. *Centella asiatica*, known as pegagan, is one of the medicinal plants that can be found in subtropical and tropical regions like Indonesia. *Centella asiatica* has antioxidant and neuroprotective effects in experimental animals.7,8,9 The active ingredients
of *Centella asiatica* are *asiaticoside*, *madecassocide*, and *flavonoids* which have antioxidant effects can protect cells from reactive oxygen species (ROS).\(^\text{10,11}\)

The objective of this study was to observe the effect of *Centella asiatica* ethanol extract on zebrafish in chronic hypoxic conditions.

**METHODS**

This study used an experimental laboratory with a randomized post-test control group design. It was conducted in 2019 in the Pharmacology Laboratory of the Faculty of Medicine and the Fisheries Reproduction Laboratory of the Faculty of Fisheries and Maritime Affairs, Universitas Brawijaya, Malang. The sample of this study was zebrafish larvae (*Danio rerio*) aged 3-8 dpf (day post fertilization) with 20 larvae per tube (duplo) in each group treatment. The total group treatment was five with inclusion and exclusion criterias. The Inclusion criterias those were 1.) Zebrafish larvae aged 3 dpf, 2.) Healthy zebrafish larvae (normal motion activity) and are not disabled. The exclusion criterias were 1.) Zebrafish larvae which were dead during the experiment, 2.) Zebrafish larvae that have used for other experiments.

The *Centella asiatica* ethanol extract was obtained from the leaves and stems through maceration method with 98% ethanol solvent. The *Centella asiatica* ethanol extract was divided into three concentrations (1.25 μg / ml, 2.5 μg / ml, and 5 μg / ml) which was a modification of the results study by Khotimah, et al (2015).\(^\text{12}\) This *Centella asiatica* originates from Balai Materia Medika Batu Malang.

Chronic constant hypoxia (CCH) is a chronic hypoxia treatment by providing 2 L / min nitrogen gas flow for 9 minutes 48 seconds. It was given to zebrafish larvae from the age of 3 dpf to 8 dpf.\(^\text{13}\)

The Zebrafish larval body length is the length of the body measured from the snout of the mouth to the end of the body in millimetres (mm).\(^\text{14}\) The Measurements were done by the optilab software and image raster on days 4, 6, 8 dpf during straight and non-movement position.

The Locomotor activity is zebrafish larvae swimming activity. It was observed in wells that have been given a circular pattern. This activity was measured for 5 minutes, calculated by adding up the lines that were passed by zebrafish larvae.\(^\text{12,14,15}\)

The Negative controls are samples that were not given chronic constant hypoxia (CCH) treatment and *Centella asiatica* ethanol extract. While, positive controls are samples that were treated with chronic constant hypoxia (CCH) and were not given *Centella asiatica* ethanol extract. This research has been conducted an ethical test and has been declared ethical by the Faculty of Medicine, Universitas Brawijaya research ethics.

The data of this study were statistically analyzed with SPSS 18.0 software for windows. The independent t-test and paired test used to compare differences in body length and locomotor activities. Normality test used one-sample Kolmogorov-Smirnov test. The homogeneity test used Levene test followed by Least Significant Difference (LSD). The Kruskal Wallis test used if the normality and homogeneity were not fulfilled and continued to Mann Whitney U test. If the requirements are fulfilled, the One Way Anova test was done. The statistically significant was conducted if the p-value <0.05.

**RESULT**

Chronic hypoxic constant condition (CCH) in this study was carried out by giving 2 L/min nitrogen gas flow into the tube and measured the concentration of dissolved oxygen in water using a Dissolved Oxygen meter (DO meter). The decreased dissolved oxygen concentrations reached a state of hypoxia at 2 mg / L in 9 minutes 48 seconds (Figure 1).

At the 4 dpf, the Mann-Whitney test results were obtained to compared average body length and found that the average body length of the positive control group, was 0.42 mm shorter than the negative control (p <0.05). At the age of 6 dpf, the average body length of the positive control group was 0.43 shorter mm than negative controls (p <0.05). Similar to the age of 8 dpf, the average length of the positive control group was shorter 0.39 mm.
## Table 1. Comparison of Average Body Length (mm) Between Negative and Positive Control Groups in Zebrafish Larvae Ages 4, 6 and 8 dpf

<table>
<thead>
<tr>
<th>Age (dpf)</th>
<th>Group</th>
<th>Control -</th>
<th>Control +</th>
<th>Control -</th>
<th>Control +</th>
<th>Control -</th>
<th>Control +</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Mean ± SD</td>
<td>3.36 ± 0.20</td>
<td>2.94 ± 0.11</td>
<td>3.44 ± 0.13</td>
<td>3.01 ± 0.08</td>
<td>3.51 ± 0.12</td>
<td>3.12 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

## Table 3. Comparison of Average Body Length (mm) of Zebrafish Larvae between the Positive Control Group and the Treatment Group

<table>
<thead>
<tr>
<th>Age (dpf)</th>
<th>Group</th>
<th>Control +</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Mean ± SD</td>
<td>2.94 ± 0.11</td>
<td>3.06 ± 0.15</td>
<td>3.14 ± 0.12</td>
<td>3.21 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.000</td>
<td>0.01</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>6</td>
<td>Mean ± SD</td>
<td>3.01 ± 0.06</td>
<td>3.31 ± 0.14</td>
<td>3.40 ± 0.09</td>
<td>3.42 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.01</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>8</td>
<td>Mean ± SD</td>
<td>3.12 ± 0.23</td>
<td>3.33 ± 0.06</td>
<td>3.41 ± 0.21</td>
<td>3.47 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>
mm compared to the negative control (Table 1).
Mann-Whitney test results between the control groups at ages 4, 6 and 8 dpf obtained the average locomotor activity in the positive control group was lower than the negative control (p < 0.05) (Table 2).
Centella asiatica ethanol extract concentrations of 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml were given to zebrafish larvae together with chronic constant hypoxia (CCH) exposure from 3 – 8 dpf. Evaluation of the effects of Centella asiatica extract of various concentrations was carried out through body length measurements and observation of the locomotor activity of zebrafish larvae at ages 4, 6 and 8 dpf. Kruskal-Wallis statistical test results at the age of 4, 6, and 8 dpf have p < 0.05 (Table 3). Moreover, in Table 4 it can be seen that Centella asiatica doses 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml have a significant effect on increasing body length at ages 4, 6 and 8 dpf.
Locomotor activity in each centella asiatica treatment group on 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml, both at 4, 6 and 8 dpf were higher when compared to the positive control group, with a p value 0.000 (p < 0.05) (Table 5).
The spearman test results between Centella asiatica concentrations 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml with body length on zebrafish larvae of CCH models aged 8 dpf, were moderate (r=0.431, p<0.05). While the results of the Spearman test between Centella asiatica concentrations of 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml with locomotor activity shower low correlation (r=0,262, p<0,05) (Table 6).

**DISCUSSION**

The treatment of chronic constant hypoxia (CCH) and Centella asiatica extract in this study were given since zebrafish hatching, the CCH condition can occur from birth. Therefore, the administration of Centella asiatica extract was also given since born. The observations were carried out periodically on zebrafish larvae aged 4, 6 and 8 dpf which are analogous to ages of 2-8 years children. The basic observations were made starting at the age of 4 dpf because of stunting growth problems seen at the age of 2 years. Normal oxygen concentration in aquatic ecosystems is > 6.5 mg / l. The species of fish that routinely experience mild hypoxia (O2 concentration <4 mg / l) to severe (O2 concentration <1 mg / l) will trigger a series of the organism, cellular and genetic responses.

The CCH condition in this study was made at dissolved oxygen (DO) 2 mg / l which is categorized as moderate (moderate) hypoxia to determine the concentration of lethal dissolved oxygen (DO) in fish embryos. The study found the association between dissolved oxygen concentration and mortality of fish embryos after 144 hours, the percentage of embryos dead were 95%, 31%, 16% and 12% respectively, at DO levels of 0.5 mg / l, 1, 2, and 6 mg / l. The highest mortality (p <0.05) was observed in the severe hypoxia group with DO levels of 0.5 mg / l. There is no significant difference in viability (p> 0.05) between the normoxia group (6 mg / l) and 2 mg / l. Based on that the dissolved oxygen concentration of 6 mg / l (normoxia) and 2 mg / l (hypoxia) was used in this study.
The positive control group has a shorter body length when compared to the negative control group. This condition shows that the condition of chronic constant hypoxia (CCH) can inhibit body length growth in zebrafish larvae. The results of this study are in line with previous studies, which said

### Table 2. Zebrafish Larva Locomotor Activity In The Control Group

<table>
<thead>
<tr>
<th>Group</th>
<th>4 dpf</th>
<th>6 dpf</th>
<th>8 dpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control -</td>
<td>56.17 ± 5.12</td>
<td>63.17 ± 6.64</td>
<td>63.83 ± 1.43</td>
</tr>
<tr>
<td>Control +</td>
<td>2.17 ± 0.52</td>
<td>23.50 ± 3.26</td>
<td>7.33 ± 1.35</td>
</tr>
</tbody>
</table>

### Table 4. The Result of Kruskall-Wallis for Each Centella asiatica Dose Group 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml

<table>
<thead>
<tr>
<th>Group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 (1.25 ug/ml extract +CCH since 3-8 dpf)</td>
<td>0,000</td>
</tr>
<tr>
<td>C2 (2.5 ug/ml extract +CCH since 3-8 dpf)</td>
<td>0,000</td>
</tr>
<tr>
<td>C3 (5 ug/ml extract +CCH since 3-8 dpf)</td>
<td>0,000</td>
</tr>
</tbody>
</table>

### Table 5. Average Zebrafish Larva Locomotor Activity In Control And Treatment Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>4 dpf</th>
<th>6 dpf</th>
<th>8 dpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control -</td>
<td>56.17 ± 5.12</td>
<td>63.17 ± 6.64</td>
<td>63.83 ± 1.43</td>
</tr>
<tr>
<td>Control +</td>
<td>2.17 ± 0.52</td>
<td>23.50 ± 3.26</td>
<td>7.33 ± 1.35</td>
</tr>
<tr>
<td>C1</td>
<td>30.67 ± 4.20</td>
<td>55.50 ± 3.70</td>
<td>41.00 ± 4.43</td>
</tr>
<tr>
<td>C2</td>
<td>36.17 ± 4.63</td>
<td>57.17 ± 2.08</td>
<td>43.33 ± 4.00</td>
</tr>
<tr>
<td>C3</td>
<td>46.17 ± 4.37</td>
<td>68.00 ± 3.99</td>
<td>56.67 ± 3.70</td>
</tr>
</tbody>
</table>

### Table 6. Relationship Test Results between Centella asiatica Concentration with Body Length and Locomotor Activity in Zebrafish Larvae Model CCH Ages 8

<table>
<thead>
<tr>
<th>Variable</th>
<th>r-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centella asiatica extract with body length</td>
<td>0.431</td>
<td>0.00</td>
</tr>
<tr>
<td>Centella asiatica extract with locomotor activity</td>
<td>0.262</td>
<td>0.00</td>
</tr>
</tbody>
</table>
that the factors that cause short stature is the state of hypoxia. Meanwhile, according to Matozel (2009), hypoxic conditions disrupt the development of the digestive system.

In hypoxic conditions, cellular respiration chains and mitochondrial dysfunction occur. Inhibition of the respiration chain of the mitochondrial complex will cause a decrease in ATP production that is indispensable for the body's metabolism increased production of ROS including the superoxide anion O2− will be converted to hydrogen peroxide (H2O2) and eventually to hydroxyl radicals (OH-). High ROS can trigger oxidative stress. Oxidative stress can inhibit the activation of growth hormone and growth factors, such as IGF-1 (Insulin-Like Growth Factor-1), BMP2 (Bone Morphogenic Protein 2), TGF β (Transforming Growth Factor β) and BDNF (Brain-Derived Neurotropic Factor). The body needs all of these growth factors for cell proliferation and differentiation. Inhibition of proliferation and differentiation of chondrocyte cells (chondrogenesis) will cause bone cells cannot grow longer and is known as stunting or linear growth. 

One way to assess sensory and motor disorders are by observing the locomotor activity. From this research, it was found that the positive control group showed significantly lower locomotor activity compared to the negative control group at ages 4, 6 and 8 dpf. This event means that the condition of chronic constant hypoxia (CCH) can reduce locomotor activity in zebrafish larvae. The results of this study are in line with study by Marks et al. (2005) which showed that zebrafish maintained in hypoxic condition exhibits less aggressive and has avoidance behavior than zebrafish maintained in normoxic conditions. Zebrafish that is kept in hypoxic condition has a slower swimming speed than zebrafish that is maintained in normoxic conditions.

Centella asiatica concentrations of 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml are known to have a significant effect on the addition of larval body length at ages 4, 6 and 8 dpf, and this is possible due to the administration of Centella asiatica ethanol extract which has a function as an antioxidant. If antioxidants in the body increase, oxidative stress due to hypoxic conditions will decrease so that the body's metabolic processes can continue to run and prevent further cell damage. This result is in line with the study of Darwiti et al. (2018) who concluded that Centella asiatica increases body length in rotenone-induced zebrafish larvae. In the study, there was a decrease in malondialdehyde (MDA) levels, an increase in superoxide dismutase (SOD) and catalase levels followed by a significant increase in body length in the Centella asiatica treatment group compared with the rotenone group. Centella asiatica ethanol extract 5 µg / mL have been shown to reduce the activity of reactive oxygen species (ROS) by reducing MDA, increasing SOD and catalase levels in zebrafish larvae induced by rotenone. So this ethanol extract from Centella asiatica can increase body length through modulation of oxidative stress.

At ages 4, 6 and 8 dpf the positive control group in this study also showed lower locomotor activity when compared to the negative control group and the Centella asiatica group concentration of 1.25 µg / ml, 2.5 µg / ml and 5 µg / ml. In the Centella asiatica group, concentrations of 1.25 µg / ml and 2.5 µg / ml showed an increase in locomotor activity at 6 dpf, but decreased at 8 dpf, and this was significantly different from the p-value of 0.000 and respectively 0.001 (p < 0.05), it means that Centella asiatica concentrations of 1.25 µg / ml and 2.5 µg / ml are unable to maintain locomotor activity in zebrafish larvae up to the age of 8 dpf. In the Centella asiatica group at the concentration of 5 µg / ml also increased locomotor activity at age 6 dpf and decreased at age 8 dpf, but this was not significantly different from the p value of 0.071 (p > 0.05), which has meaning that the Centella asiatica concentration of 5 µg / ml can increase locomotor activity in zebrafish larvae up to the age of 8 dpf and the closest to the negative control group when compared with Centella asiatica concentrations of 1.25 µg / ml and 2.5 µg / ml. This result is in line with research Khotimah et al. (2015) which states that Centella asiatica extracts concentrations of 2.5 µg / ml, 5 µg / ml and 10 µg / ml significantly increase motility and dopamine levels in zebrafish induced by rotenone. In the study of Marks et al., 2005 the level of activity in zebrafish maintained in hypoxia was lower than that of zebrafish maintained in normoxia. The study also measured a lower level of aggression in zebrafish maintained in hypoxic conditions compared to zebrafish maintained in normoxia, where this results in behavioural restriction activities resulting in decreased accumulation of lactate. At advanced hypoxia levels, a fundamental change in the availability of glycogen (glucose) decreases or the activity of lactate dehydrogenase increases which can limit the ability to swim in zebrafish. Zebrafish with lower activity levels are expected to last longer in chronic hypoxic conditions because it avoids the harmful effects of persistent lactate metabolism.

In this study, several limitations were obtained, including the calculation of locomotor activity used in this study was still manual by recording the swimming activity of zebrafish larvae using a camera handphone for 5 minutes. In addition,
when observing the body length and locomotor activity of zebrafish larvae, they must be moved from the hypoxic tube to the plate to facilitate observation, so that for ± 3 hours of observation the CCH model zebrafish larvae are exposed to normoxic conditions. This condition means that the CCH method in this case, is still not perfect. It is not known yet for sure whether *Centella asiatica* can inhibit mitochondrial dysfunction, reduce damage to neuron cells by decreasing Iba-1 and caspase 3, and be able to increase the length of the zebrafish larvae through increased growth hormone and growth factors.

**CONCLUSION**

Chronic constant hypoxia (CCH) can inhibit body length and reduce locomotor activity in zebrafish larvae ages 4, 6, and 8 dpf significantly. A significant (moderate) relationship was found between the *Centella asiatica* ethanol extract concentration of 1.25 μg / ml, 2.5 μg / ml, 5 μg / ml with increased body length and locomotor activity in zebrafish larvae of CCH models.

**CONFLICT OF INTEREST**

There is no competing interest regarding the manuscript

**FUNDING**

None

**ETHICS CONSIDERATION**

Ethics approval has been obtained by the Ethics Committee, Faculty of Medicine, Universitas Brawijaya, Saiful Anwar General Hospital, Malang, Indonesia prior to the study being conducted.

**AUTHOR CONTRIBUTION**

All authors contributed to the process of this research.

**REFERENCES**

