Effect of red snapper fish intake on pyramidal cells in hypothyroidic rat brain model

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Abstract. Congenital hypothyroidism in infants results from partial or complete loss of thyroid glands, resulting in thyroid hormone deficiency. The untreated low thyroid hormone levels induce impairment in the maturation of the developing brain by regulating neurogenesis. Proper treatment in the specific onset timing and duration have shown to increase the chances of brain recovery. In this study, we examined the effect of daily intake of red snapper fish to improve the profile of brain cerebrum on hypothyroidic rat model, particularly on the number and distribution of pyramidal cells. Hypothyroid pups were produced by propylthiouracil induced female rats during pregnancy from E14 until P21. The rats were randomly divided into four groups, which include negative (congenital hypothyroidism) and positive control (thyroxine therapy) as well as red snapper-enriched diet at dosage 25% and 50%. After four weeks treatments, the rats were dissected and brain sections were made using paraffin embedding and hematoxylin-eosin staining to observe pyramidal cell numbers and distribution in cell layer III of the cerebral cortex. The result showed that the consumption of 50% red snapper diet increased the number of pyramidal cells in the cerebral cortex, parallel to the elevated thyroid hormone levels which was nearly equal to control.

Keywords: red snapper fish, hypothyroidism, pyramidal cells

1. Introduction
Hypothyroidism is a condition in which the thyroid gland is unable to produce sufficient amounts of thyroid hormone. The disease is often characterized by the abnormal size of the thyroid gland due to the impairment in the synthesis and secretion of thyroid hormones by the epithelial cells surrounding the follicles. In addition, pituitary gland dysfunction has been shown to also induce hypothyroidism. When this condition occurs from birth, it is called congenital hypothyroidism, which is responsible for several conditions, such as short stature or cretinism, decreased metabolic processes, and slow growth rate of the brain nerve cells resulting in intellectual development disorders ranging from moderate Intelligence Quotient (IQ) disorders to severe mental retardation [1][2]. The prevalence of congenital hypothyroidism in developing countries ranging from 1:2,000 to 1:3,000 births is higher compared to developed countries [3]. Hence, this condition requires much attention.

Thyroid hormones, including Thyroid Stimulating Hormone (TSH) and Free Thyroxine (FT4) are crucial for several physiological processes and development of the nervous system. During brain
development, these hormones stimulate and coordinate processes such as neuron proliferation, migration, growth of axons and dendrites, synapse formation, and myelination [4]. Disruption of these processes lead to abnormalities in neural networks, mental retardation, and other neurological disabilities such as impaired motor skills and visual processing, initiative loss, slow response, memory impairment, and drowsiness. With proper treatments and normalization of the euthyroid state, these neuropsychiatric symptoms tend to improve, although complete recovery is not certain [5].

Treatments of congenital hypothyroidism mainly rely on natural hormone replacement therapy using drug levothyroxine (L-T4). Neuronal changes in the cerebral cortex could be reversed to normalcy with adequate substitution therapy using LT-4 [6]. However, LT-4 induce common side effects such as diarrhea, muscle weakness, and headache while long-term usage potentially leads to cardiovascular disorders [7]. Therefore, there is a need to provide alternative treatments (e.g., diet), capable of improving the health status of hypothyroidism patients, with minimum potential side effect.

For the past 25 years, iodine, a trace element essential for thyroid hormone production has been incorporated into the diet in many countries in form of iodized salt. This method is considered a more sustainable way of improving health problems resulting from iodine deficiency [8][9]. Sea foods contain iodine, hence, serve as a good source for this nutrient [10]. Red snapper (Lutjanus sp.) is one of the most important fish commodities widely found in the Indonesian sea. It is highly consumed by the locals due to the delicious taste, availability, affordability, and high nutrition. Furthermore, the snapper fish family contains high amount of iodine (108.955 μg/100 g) and polyunsaturated fatty acids (PUFAs), which support brain function [11][12][13]. In hypothyroidism, we hypothesized that consumption of red snapper may play a positive role in the brain development since childhood, due to its iodine and fatty acids content, however, there are no previous reports in support of this hypothesis. Therefore, this study aims to determine the effect of red snapper intake on the brain histological structure in congenital hypothyroidic rats, particularly on the number and distribution of pyramidal cells in the cerebral cortex.

2. Material and methods

2.1 Animal care

The experiments were performed in line with the guidelines and regulations of animal care facilities of Integrated Laboratory Units, Sebelas Maret University, Indonesia. Ethical clearance was issued by the Health Research Ethics Committee, Faculty of Medicine, Sebelas Maret University with authorization number 477/UN27.06//KEPK/EC/2019. The Wild-type Sprague-Dawle (SD) rats were purchased from the Faculty of Pharmacy, Setia Budi University and were provided with food and drink ad libitum under controlled temperature, light, and humidity. Six females mated with two males, and the pups were used for the experiments after confirming the hypothyroidism status.

2.2 Inducing hypothyroidism in pregnant rats

The pregnant rats received 0.025 grams of Propylthiouracil (PTU) daily for three weeks, beginning from pregnancy week 3 to lactation day 15. PTU was dissolved in 100 ml distilled water and was mixed with 25-30 ml Carboxy Methyl Cellulose (CMC) to increase solubility. PTU was given ad libitum with water [14].

2.3 Measurement of serum TSH and FT4

Blood TSH and FT4 were measured at three weeks of age to confirm that the pups were under the physiological status of hypothyroidism. Thereafter, blood samples were collected from the orbital sinus using capillary blood corrector tubes, allowed to clot on ice, and then centrifuged at 2000g for 10 minutes. FT4 and TSH levels were measured using ELISA kit (CUSABIO) in line with the manufacturer instruction. This experiment was conducted at the Research and Development Center for Iodine Deficiency Disorders (Balai Penelitian dan Pengembangan Gangguan Akibat Kekurangan Yodium), Magelang, Indonesia.
2.4 Diets and proximate analyses
Red snapper fishes (*Lutjanus* sp.), weighing approximately 200 grams each, were obtained from a local supermarket. The fish were cleaned, scaled, and steamed for 15 minutes until the flesh was tender. Thereafter, a portion was mixed with basal diet broiler-2 (BR-2) according to the treatment dose. The rats were regularly fed twice daily with each supplement amounting to 10% of total body weight. Each day, the amount of leftover food was calculated.

Proximate analyses were performed at the Integrated Research and Testing Laboratory, Gadjah Mada University, Indonesia to determine nutritional contents, including protein, total fat, carbohydrate, water, ash content, and fiber using standard methods.

2.5 Oral administration of levothyroxine
The levothyroxine sodium was dissolved in aquadest and stored at -20°C. This solution was administered intragastrically once per day at final concentration of 0.005 μg/g.

2.6 Treatment groups
Three weeks old pups confirmed with hypothyroidism were divided into four groups of four individuals each, i.e., (1) a group receiving 25% red snappers fish diet, (2) a group receiving 50% red snappers fish diet, (3) a negative control group, and (4) a positive group control receiving levothyroxine. All treatments were given during the last four weeks. All treatments were performed during the last four weeks to 3-7 weeks old rats.

2.7 Histological analysis of brain pyramidal cells
The seven weeks old pups treated with congenital hypothyroid were euthanized anesthetically using chloroform. Thereafter, each pup was dissected using a surgical instrument to extract the brain, fixed in 10% neutral buffered formalin for 2 days and embedded in paraffin. Brain sections (5 μm) were mounted on glass slides and counterstained with hematoxylin and eosin. All the slides were examined using digital images taken with Nikon ECLIPSE Ti. The average number of pyramid cells was calculated from five fields of view for each section.

3. Result and Discussion
The pregnant female rats administered with PTU delivered offspring confirmed for hypothyroidism, as indicated by the pups’ TSH and FT4 blood serum level (Table 1). These pups were randomly assigned to 4 groups and received treatment for 8 weeks according to their group. After the experiment, the rats were dissected for brain tissue observation. The results showed that the hypothyroid rats had the lowest number of pyramidal cells (negative control, Fig. 1). However, there were no significant differences in the cells’ distributions among groups. In contrast, the groups treated with daily red snapper supplements showed a higher number of pyramidal cells as the number increased by 2.5 folds in the 50% dose-group compared to the control.

**Tabel 1. The pup’s blood serum thyroid hormone concentration after 30 days of PTU treatment**

| Hormone                        | Concentration          | Reference   |
|-------------------------------|------------------------|-------------|
| Thyroid stimulating hormone (TSH) | 20.59 ± 12.38 μIU/mL   | <19.8 μIU/mL         |
| Free T4 (FT4)                 | 1.07 ± 0.23 pmol/L     | >1.29 pmol/L atau + 13.7 pmol/L |

Neurogenesis defect in the hypothyroidic model rat has been widely reported. Thyroid hormone deficiency disrupts dendritic expansions in pyramidal cells of the cerebrum and visual cortex [15]. Furthermore, it also inhibits the dendritic branching of hippocampal pyramidal cells [16]. The brain is an important target of the thyroid hormone hence, its maturation is regulated by the hormone. Due to the close relationship between the brain cognitive capacity and synaptic connections formed by the dendritic branching of each neuron, many congenital hypothyroid patients were reported to have low intelligence and mental retardation [1].

The fewer pyramidal cells in the hypothyroidic rats may also associated with the mechanisms of the apoptotic pathway. Thyroid hormone upregulates the expression of bcl2, an anti-apoptotic gene to
protect the viability of post mitotic granular cells [17]. In addition, hypothyroidism also induces morphological changes in the mitochondria of brain cells. This changes result in the translocation of apoptogenic molecules from the membrane to the cytoplasm during cerebellar development [18].

Figure 1. Histological brain section of the four treatment groups (i.e., control, Levothyroxine treatment as well as 25% and 50% red snapper-enriched diet). (A) Pyramidal cells in the layer III of cerebrum cortex. (B) Quantification of pyramidal cells shown in A. n=4

Pyramidal cells constitute a larger portion of all neurons in the cerebral cortex, thereby, serving crucial function in the cognitive process. The increase in the number of these cells in the red snapper-enriched diets (Figure 1) is potentially caused by the corroborative action of thyroid hormone and other beneficial nutrients present in the fish. Also, it was observed that TSH and FT4 serum level in the red snapper-enriched diet group significantly improved to a normal condition (unpublished data). The high iodine content in the fish potentially contribute to this recovery. Red snapper contains a high amount of iodine (108,955 µg/100g), which is essential for production of thyroxine (T4) and triiodothyronine [11,19]. The utilization of iodized salt in developed country such as US was sufficient for human daily dietary requirements. This is indicated in the country’s low number of individuals with iodine deficiency-related problems [20].

Table 2. Proximate composition of red snapper-enriched feed

| No | Nutrient       | BR-2 (%) | 25% fish+ BR-2 (%) | 50% fish+ BR-2 (%) |
|----|----------------|----------|-------------------|--------------------|
| 1  | Protein        | 11.90    | 13.43             | 12.54              |
| 2  | Total fat      | 0.11     | 0.13              | 0.10               |
| 3  | Carbohydrate   | 71.72    | 55.82             | 40.89              |
| 4  | Water          | 11.34    | 26.67             | 43.45              |
| 5  | Ash            | 4.93     | 3.95              | 3.02               |
| 6  | Fibers         | 21.38    | 27.88             | 11.16              |

Besides its iodine content, other nutrients in the red snapper fish may also contribute to the increased number of pyramidal cells. For example, the crude protein in the 50% enriched diet (0.6-1.5%) was higher compared to basal diet BR-2 (Table 2). Also, the water content increased to almost four times, when the basal diet was combined with 50% fish diet thereby, adding a moister texture into the feed. Despite the different feed textures and (possibly) taste, all treatment groups finished the daily food supplement. Nevertheless, the result from proximate analyses did not specify details on the amino acids or fats constituents in each sample. Previous studies showed that red snapper is one of the marine fish rich in Omega-3 fatty acids in form of Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [21,22]. In an experiment conducted using iodine deficient-induced hypothyroidic rat model by Pal et al (2013), it was reported that regular intake of polyunsaturated fatty acids promotes function, morphology, and biochemical parameters of the cerebellum, despite constancy in the level of blood thyroid hormone. Meanwhile, when the fatty acid supplements were combined with potassium iodide,
the neurotrophic biomolecules were repaired and thyroid hormone levels returned to normal. Also, there was an increase in motor coordination, memory capacity, and learning ability. The combination of fatty acid and iodine also reduced apoptosis and oxidative stress in the cerebellum [23].

This study highlights the positive impact of red snapper fish intake on the brain neuronal profile of rat with hypothyroidism. Due to the realization of the fish-based diet health benefits, the use of red-snapper for hypothyroidism treatment is likely to increase. However, further studies are needed to investigate the detail mechanisms on how red snapper increases pyramidal cells.

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