NEURO PROGENITOR CELLS (NPCS) AND PPARγ EXPRESSION IN THE BRAIN OF PROTEIN-DEFICIENT RATS AFTER ADMINISTRATION OF PASAK BUMI (Eurycoma longifolia Jack) EXTRACT

Didik Dwi Sanyoto¹, Triawanti², Dimas Ikhsan Airlangga³

¹Division of Anatomy, Biomedical Department, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin
²Department of Biochemistry and Biomolecular, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin
³Student at Faculty of Medicine, Universitas Brawijaya, Malang

Correspondence Author: didikdwisanyoto@ulm.ac.id

Abstract:
Malnutrition due to protein deficiency disrupts the neurogenesis process that affects cell development and brain function. One of the efforts to overcome the matter is to use the pasak Bumi (Eurycoma longifolia Jack) which has compounds believed to increase the process of neurogenesis. The aim of study to analyze the effect of pasak bumi extract on the expression of NPCs (Tuj-1) and PPARγ cell in the brains of malnourished rats. Groups of experimental animals that were were divided into 5 groups, namely (P1) malnourished rats + placebo (aquades); (P2) malnourished rats + pasak bumi extract (EPB) 7.5 mg/kg BW; (P3) malnourished rats + EPB 15 mg/kg BW; (P4) malnourished rats + EPB 22.5 mg/kg BW; (P5) malnourished rats + EPB 30 mg/kg BW. As a control, normal rats were used which were given a placebo. Parameters examined include Tuj-1 and PPARγ using immunohistochemistry procedure. The results showed that the P3 group (15 mg/kg BW) had more cells expressing Tuj-1 than the other doses. There was a significant difference between the KP and the P3, P4, and P5 groups. There was no significant difference in the expression of PPARγ in all malnourished groups with EPB and KN intervention. Conclusion: extract of pasak bumi was able to improve the neurogenesis process in malnutrition but not PPARγ.

Keywords: Malnutrition, pasak bumi extract, NPCs, Tuj-1, PPARγ
Introduction

Neurogenesis is the process of forming neurons that can occur throughout life in certain brain regions. Neurogenesis begins with the presence of proliferative and multipotential Neural Stem Cells (NSC) and Neural Progenitor Cells (NPCs)\(^1,2\). These cells then fully differentiate into neuronal cells that mainly occur in several areas of the brain, including the subependymal zone in the lateral ventricles (V-SVZ) and the subgranular zone (SGZ) in the dentate gyrus inside the hippocampus which is associated with cognitive function and memory\(^3,4\). In addition to neurogenesis, NSCs can also develop into glial cells such as astrocytes and oligodendrocytes which function as support for neuronal cells, which is known as the process of gliogenesis\(^5\).

The process of neurogenesis occurs from the beginning of the second week of gestation and progresses rapidly until the first 1000 days of life\(^6,7\). The development of brain function in adulthood is determined in early childhood\(^8\). During this crucial period, the continuity of the neurogenesis process is susceptible to being influenced by external factors, one of which is nutrition\(^9\). A large body of epidemiological data indicates that nutritional deficiencies during in utero and/or childhood development result in long-lasting impaired attention and learning, decreased IQ scores, and decreased visuospatial working memory leading to low academic achievement\(^10,11,12\). Protein deficiency in early life can reduce enzyme activity, resulting in impaired protein synthesis and structure formation which affect the incorporation of lipids with cell membranes, including disrupted neuron cells. Incompletely formed neuron membranes can disrupt neuronal circuits which have an impact on morphological, neurochemical, and neurophysiological disorders of the brain\(^13,14\).

Prior study has shown that restriction of protein or protein-energy results in a smaller brain volume with reduced RNA and DNA content, fewer neurons, simpler dendritic and synaptic head architecture, also decreased concentrations of neurotransmitters and growth factors\(^8,15\). Observations on neurogenesis due to early protein malnutrition have shown decreased neurogenesis in neuronal cells in the CA1 region of the hippocampus\(^16\). Another study found that protein restriction early in life led to a decrease in neural progenitors in the hippocampus which decrease object recognition as adults\(^17\).

Malnutrition could be prevented and corrected through the provision of proper nutritional intake\(^18\). Several studies have shown that the process of neurogenesis can be enhanced through the administration of ginseng extract\(^19,20,21\). South Kalimantan has a biodiversity that has the potential to overcome malnutrition problems similar to ginseng, namely Pasak Bumi (\textit{Eurycoma longifolia} Jack). To date, Pasak Bumi is commonly used as an aphrodisiac, even though Pasak Bumi contains an active substance in the form of Quassinoid which is a flavonoid group. Results of a study on quassinoid in several plants have been shown to have neurorestorative effects that are associated with anti-oxidants and anti-inflammatory properties and activate several pathways responsible for neurogenesis\(^22,23\). A study by Sanyoto et al.\(^14\) showed that the administration of 15 mg/kg BW of Pasak Bumi extract to malnourished rats showed an increase in Brain-Derived Neurotrophic Factor (BDNF) levels which indicated a neurogenesis activity.
Increasing neurogenesis can also be characterized by NPC activity which is characterized by an increase in the number of Tuj-1 markers as well as the expression of the Peroxisome Proliferator-Activated Receptors (PPARγ) gene in the hippocampus. PPARγ is a subunit of PPAR which is a ligand-activated nuclear transcription factor. In addition to enhancing neurogenesis, PPARγ has various other biological functions, such as regulating lipid metabolism, increasing insulin sensitivity, modulating anti-tumor mechanisms, and inhibiting inflammation.

No studies have shown the relation of using Pasak Bumi extract on increasing the process of neurogenesis yet. This study aimed to analyze the effect of Pasak Bumi extract on the expression of Tuj-1 and PPAR cell counts in the brains of malnourished rats.

**Research Method**

This study had been received approval from the ethics committee of the Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia (Number 298/KEPK-FK UNLAM/EC/2019). This study was an experimental study with posttest-only with control group design. Rattus norvegicus as samples were female, 3 months old, and the number was 30 rats. Female Rattus norvegicus were mated by the male ones, and then they were separated. The rat pups that are born are selected male sex and then included in the group.

**Materials and Tools**

Pasak Bumi (*Eurycoma longifolia Jack*), low protein feed (AIN-76A), standard feed, aquadest, 70% ethanol, 90% ethanol, *Rattus norvegicus* rats, paraffin block from mouse tissue material, ether, PPARγ antibody, Tuj1 antibody, immunostaining kit mono staining, Rotary evaporator, digital analytical balance, measuring cup, blender, oral sonde, glass apparatus, cuvette, water bath, vortex, incubator, centrifuge, Olympus CX21 binocular microscope, Microtome Leica 2125 RM, hot plate, object-glass, Staining jar.

**Procedures**

*Making 70% ethanol extract of Pasak Bumi root*

The root of the Pasak Bumi is shaved and then dried without being exposed to direct sunlight. Then blended until it becomes a powder. 200 grams of Pasak Bumi root powder were soaked in 1.5 L of 70% ethanol at room temperature for 5 days and then filtered. Pasak Bumi root powder dregs were remacerated in 500 mL 70% ethanol at room temperature for 2 days and then filtered. The entire filtrate was collected and concentrated with a rotary evaporator at a temperature of 50°C to obtain a thick extract that still contains a small amount of solvent. Further evaporation using an oven at 40°C to obtain a thick extract.

*Preparation of experimental animals malnutrition*

Rat pups were made undernourished from the day they were born by feeding the rat’s mother that was breastfeeding with low protein feed (AIN-76A) for 4 weeks, after weaning the rat pups continued with low protein feed (AIN-76A) for 4 weeks. Rat pups received low-protein feed for 8 weeks (56 days). The rat was classified as undernourished if the serum protein level was below 4.7 g/dL. Rattus norvegicus's normal protein level is 4.7-5.2 g/dL. The malnourished rats were divided into 5 groups plus 1 group of normal rats. Each group consisted of 6 rats.

KN: Normal rat + placebo (aquadest)
P1 : Malnourished rats + placebo (aquades)
P2: malnourished rats + Pasak Bumi extract (EPB) dose of 7.5 mg/kgBW rats
P3: malnourished rats + Pasak Bumi extract (EPB) dose of 15 mg/kgBW rats
P4: malnourished rats + Pasak Bumi extract (EPB) dose 22.5 mg/kgBW rats
P5: malnourished rats + Pasak Bumi extract (EPB) dose of 30 mg/kgBW rats

Giving 70% ethanol extract of Pasak Bumi

After the rats were malnourished, they were given standard feed and 70% ethanol extract of Pasak Bumi according to the dose of each group. The normal control group (KN) and P1 were given standard feed and aquades as a placebo. Giving a solution of Pasak Bumi extract and placebo was carried out using a probe every morning 1 time a day for 5 weeks.

Examination of the expression of Tuj-1 and PPAR

The experimental animals were terminated using anesthesia after 5 weeks of treatment with Pasak Bumi extract. The blood specimen was taken from the heart while the skull was also dissected for brain extraction. The fixed brain tissue was carried out using formalin buffer solution for the paraffin block preparation. The brain preparations were stained with immunohistochemistry using the monoclonal antibody Tuj-1 and PPAR. It was viewed under a microscope and counted the number of cells expressing Tuj-1 and PPAR.

Data Analysis

The data were tested for normality and homogeneity. If normal, it is continued with ANOVA test analysis with 95% confidence level and Tuckey HSD further test. If it is not normal, then a non-parametric Kruskal Wallis test is carried out followed by Mann Whitney with a 95% confidence degree.

Results

In this study, the expression of Tuj-1 and PPARγ in the brains of the control group and malnourished rats was examined after the intervention. The results of immunohistochemical staining and the number of cells expressing Tuj 1 and PPARγ in each group are presented in Figure 1-4.

In Figure 2, it is shown that the malnourished group given a placebo (P1) had fewer neurons expressing Tuj-1 than the normal group. Meanwhile, the malnourished group who was given the 70% ethanol extract of Pasak Bumi with various doses showed that the group that was given a dose of 15 mg/kg BW had more cells expressing Tuj-1 than the other doses. Based on the ANOVA statistical test with a 95% confidence level, it was proven that there were significant differences between the treatment groups. After further testing, the results showed that there were significant differences between the KN group and the entire treatment group; between P2 and P3; between P3 and P5.

Figure 1. Results of immunohistochemical staining of Tuj-1 rat brain after administration of Pasak Bumi (Eurycoma longifolia Jack.) extract (KN= normal + placebo; P1= malnutrition + placebo; P2= malnutrition + 70% ethanol extract of Pasak Bumi (EPB) 7.5 mg/kg body weight; P3 = malnutrition + EPB 15 mg/kg body weight; P4 = malnutrition + EPB 22.5 mg/kg body weight; P5 = malnutrition + EPB 30 mg/kg body weight)
Figure 2. Average number of cells expressing Tuj-1 after administration of Pasak Bumi extract (Eurycoma longifolia Jack.) (KN= normal + placebo; P1= malnutrition + placebo; P2= malnutrition + 70% ethanol extract Pasak Bumi (EPB) 7.5 mg/kg body weight; P3 = malnutrition + EPB 15 mg/kg body weight; P4 = malnutrition + EPB 22.5 mg/kg body weight; P5 = malnutrition + 30 mg/kg body weight EPB; p = 0.000, the same letter indicates no significant difference)

Figure 3. Results of PPARγ immunohistochemical staining of rat brain after administration of Pasak Bumi (Eurycoma longifolia Jack.) extract (KN= normal + placebo; P1= malnutrition + placebo; P2= malnutrition + 70% ethanol extract of Pasak Bumi (EPB) 7.5 mg/kg body weight; P3 = malnutrition + EPB 15 mg/kg body weight; P4 = malnutrition + EPB 22.5 mg/kg body weight; P5 = malnutrition + EPB 30 mg/kg body weight)

It can be seen in Figure 4. that the normal group (KN) showed the highest PPARγ expression compared to the malnourished group. Meanwhile, the expression of PPARγ in the malnourished group with the EPB intervention at a dose of 15 mg/kg BW (P3), 22.5 mg/kg BW (P4), and 30 mg/kgBW (P5) showed almost the same amount and was higher than the group given a placebo. and a dose of 7.5 mg/kgBW. After the Kruskal Wallis statistical test was carried out because the data was not normal, it was obtained that there was no significant difference between the treatment groups (p = 0.249).

Discussion

Protein restriction in early life leads to a decrease in neural progenitors in the hippocampus. This causes a decrease in object recognition as adults which affects spatial memory formation\textsuperscript{17}. The study by Wang and Xu\textsuperscript{28} found that the rats that experienced protein malnutrition in the womb had a lighter brain weight than the control, the total protein level in the hippocampus was significantly lower, the hippocampus BDNF
content was lower, the MWM test showed the ability to learn and memory is disturbed.

One parameter to measure neurogenesis is Tuj-1, which is a marker to identify Neuron Progenitor Cells (NPCs), neuron cells at the beginning of their development. This can be seen from the increase in neurogenesis (neuronal differentiation) which is characterized by an increase in the number of Tuj-1 positive cells. Tuj-1 is an antibody that can detect the presence of beta 3 tubulins. Tubulin is the main building block of microtubulin which is the cytoskeleton of cell structures and plays a role in maintenance, mitosis, meiosis, and intracellular transport. Tuj-1 is tubulin that is specifically involved during neuronal differentiation. Immunohistochemical staining with Tuj-1 can detect cell bodies, dendrites, and axons of immature neurons (Neuron Progenitor Cells/NPC).

In this study, it was shown that the normal group had the number of NPCs characterized by the number of cells expressing the highest Tuj-1 among all groups and there was a significant difference between the KN group and all treatment groups. This shows that in cases of malnutrition, there is a disturbance in the neurogenesis process. Protein malnutrition in the long term reduces the ability of cells to proliferate. Effect on impaired cell differentiation, including size, complexity, synaptogenesis, and dendritic arborization, and a decrease in the number of hippocampal neurons. Granule cells in the hippocampal dentate gyrus are most susceptible to the effects of nutrient restriction, especially in the CA1 layer. The process of migration of neurons from the site of neurogenesis to certain areas is driven mainly by immunoglobulins and chemokines. The lack of these two materials could have an impact on the migration process itself.

In the treatment group, it was seen that the administration of 15 mg/kg BW of EPB resulted in the highest Tuj-1 expression compared to other groups but was not able to increase the neurogenesis process close to the normal group. Pasak Bumi roots contain various flavonoid compounds that not only act as antioxidants but also as anti-inflammatory. A study by Triawanti et al. reported that the administration of 70% ethanol extract of Pasak Bumi roots could improve oxidative stress in the brains of undernourished rats. This was indicated by a decrease in the levels of H2O2 and MDA and an increase in the activity of the superoxide dismutase enzyme. A study by Sanyoto et al. proved that 70% ethanol extract of Pasak Bumi root at a dose of 15 mg/kg BW can increase brain BDNF secretion which indicates a neurogenesis process. Cichon et al. reported that various flavonoid compounds can support neuroplasticity, neuroprotection, neurogenesis, and also low toxicity. The role of flavonoids in synaptogenesis and neuronal proliferation, among others, is through the CREB/BDNF/TrkB-PI3K/Akt signaling pathway.

The results showed that the normal group had the highest PPARγ expression among all groups, but there was no significant difference between all groups. PPARγ as a transcription factor can affect the expression of synaptic proteins that trigger an increase in synaptic plasticity. PPARγ is the key to the message response for translational processes due to nutritional disturbance stimuli, in the form of changes in gene expression, especially genes involved in lipid metabolism. PPARγ has a prominent role in neuroprotection and triggers an increase in cognitive performance, one of which is improving the spatial memory of experimental animals. Several studies have shown that cognitive performance can be enhanced through PPARγ nuclear receptors.

One possibility why PPAR expression in all of these groups was not significant is that there is a compensatory mechanism from
astrocytes in anticipating neuronal damage due to protein malnutrition. This is thought to be due to astrocytes in the protein malnutrition group experiencing reactive astrocytes so that the number of astrocytes increases. PPARγ is highly expressed on human primary astrocytes and is located intracellularly by immunofluorescent cytochemistry. These results indicate that PPARγ is a transcription factor in astrocytes (Chattopadhyay et al., 2000). The study of Cunha et al. proved that there was an increase in PPARγ expression in Mesenchymal Cells (MSC) of experimental animals that experienced protein malnutrition significantly when compared to controls. This suggests that protein malnutrition can alter the balance of adipogenesis. The process of differentiation and maturation of its mesenchymal stem cells is controlled by specific signal transduction and certain transcription factors including PPARγ.

Conclusions

It can be concluded that the process of neurogenesis can decrease in conditions of protein deficiency as indicated by a low number of NPCs. Administration of 70% ethanol extract of Pasak Bumi roots at a dose of 15 mg/kg BW can increase neurogenesis but cannot approach normal conditions. PPARγ expression in the normal group was not significantly different from the group that had protein deficiency and who was given 70% ethanol extract of Pasak Bumi roots.

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