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RESEARCH ARTICLE

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PCNA Gene Expression as A Marker of Alzheimer's Disease in The Brain of Long-Tailed Macaques (*Macaca fascicularis*)

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Abstract
Early diagnosis of Alzheimer's is still difficult to do, thus it is important to carry out further research to find biomarkers that can be used to detect early Alzheimer's disease. Proliferating Cell Nuclear Antigen (*PCNA*) is known as a proliferation marker, which has the potential to detect neurogenesis in the brain. This study aimed to identify *PCNA* gene expression in the brain as a marker of Alzheimer's disease in *Macaca fascicularis*. *Macaca fascicularis* was used in this study because of their similarities with humans in terms of their behavioral complexity, as well as high cognitive abilities and the formation of a pathological characteristic of Alzheimer's disease in the brain. This study used the brains of 7 monkeys in the hippocampus and cortical regions. Monkeys have previously been divided into old and adult age groups. The detection of *PCNA* gene expression was done using the RT-qPCR method. The results showed the gene expression tended to be higher in the adult group and the hippocampus region, although based on statistical analysis showed no significant differences.
Key words: Alzheimer's disease, *Macaca fascicularis*, Neurogenesis, *PCNA*, RT-qPCR

1. Introduction

Alzheimer's disease is the most common cause of dementia. Dementia is a general term to describe a progressive brain disease syndrome. This syndrome consists of a decrease in cognitive abilities, orientation skills, language skills, a decrease in the ability to control emotions, social behavior and motivation (Nisa and Lisiswanti 2016). According to the World Health Organization (WHO), by 2023, it is estimated that more than 55 million people in the world will suffer from dementia, with 60-70% suffering from Alzheimer's. This value is estimated to increase by 10 million every year. This rapid increase in dementia cases is in line with the increase in people's life expectancy. The increase in cases occurs especially in people aged 70 to 80 years (Reitz *et al*. 2011).

Despite the increasing importance and impact of dementia on society, dementia is currently still a low priority health problem in Indonesia. Access to dementia diagnosis and treatment is also hampered by the common belief in society that dementia is a normal part of aging and a lack of knowledge about the existence of treatments for dementia symptoms (Prince *et al*. 2016). The increasing number of cases of dementia, especially Alzheimer's disease, is becoming a problem in society in various countries around the world, so a deeper understanding of this disease is needed. Research continues to be conducted to find out more information about Alzheimer's disease and understand the appropriate treatment for this disease.

The long-tailed monkey (*Macaca fascicularis*) is a type of non-human primate (NHP) that is often used as an animal model in Alzheimer's disease research. This is due to its long life span, behavioral complexity, and high cognitive abilities (Darusman *et al*. 2013). Based on research by Bartus and Dean (2009), it shows that there are similarities in age-related mild cognitive impairment in non-human primates and humans with Alzheimer's. In addition, it is known that there are similarities in the formation of Amyloid-beta (Aß) plaques, which are a pathological characteristic of Alzheimer's disease in the brains of

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old long-tailed monkeys and humans with Alzheimer's disease (Oikawa *et al*. 2010).

Understanding protein interactions in a disease can explain the molecular basis of the disease, which is expected to provide information regarding methods for preventing, diagnosing and treating the disease. This is because in carrying out its functions, proteins often interact with other proteins and other molecules (such as DNA and RNA), which can mediate metabolism and cellular processes in the body (Gonzalez and Kann 2012). This understanding of protein interactions can also be applied in research on Alzheimer's disease to find out more in-depth information regarding the pathogenesis of this disease.

PCNA (Proliferating Cell Nuclear Antigen) is a protein that plays a role in DNA replication and is known as a cell proliferation marker. Based on Tobin *et al.* (2019), *PCNA* in the brain is known to be involved in the proliferation of Neural Progenitor Cells (NPCs), which are progenitor cells to produce new neurons in the process of neurogenesis. So, further research needs to be carried out regarding the potential of *PCNA* as a cell proliferation marker to detect a decrease in the process of adult neurogenesis (adult neurogenesis) to detect early Alzheimer's.

2. Materials and Methods

2.1. Sample Collection

The samples used in this study were RNA isolate samples extracted from brain tissue in the cortex and hippocampus regions of seven long-tailed macaques (*Macaca fascicularis*) which were divided into two groups, namely the old monkey group (n = 3; IA3446, C6023, T3885) aged ≥ 15 years and a group of adult monkeys (n = 4; T3879, IA3512, T3916, IA33790) aged 10-12 years. All individual monkeys used came from Primate Research Center (PRC-IPB).

2.2. RNA Extraction and cDNA Preparation

The extracted RNA was diluted using Nuclease Free Water (NFW). cDNA isolation was carried out by reverse transcription using the Sensifast™ cDNA Synthesis Kit (Bioline, Meiridian, Bioscence, USA) with a Miniamp Thermalcycler RT-PCR machine. The reagents used consisted of 10 µL RNA as sample, 1 µL RT-enzyme, 5 µL Nuclease Free Water, and 4 µL 5x trans amp buffer for each sample.

2.3. RT-qPCR amplification

The amplification process was carried out using the Bio-Rad CFX Opus System qPCR machine. Each reaction uses reagents consisting of 2 µL cDNA as template, 6 µL Nuclease Free Water (NFW), 10 µL Sensifast Sybr mix and 1 µL each of forward and reverse primers for the *PCNA* or beta-actin (ACTB) gene as reference gene, resulting in 20 µL of reagent (Table 1). The predenaturation temperature used was 95°C for 2 minutes, then denaturation was carried out at 95°C for 10 seconds, annealing was carried out at 55°C for 30 seconds. This process was carried out 44 times.

2.4. Data analysis

Data analysis was carried out using Microsoft

Table 1. Primers used in the RT-qPCR procedure

Gene Symbol	Gene Name	Primer Forward (F)/Reverse (R)
ACTB	*Beta-actin*	*F:* ACAGAGCCTCGCCTTTGC R: CACGATGGAGGGGAAGAC
PCNA	*Proliferating Cell Nuclear Antigen*	F: TGATGAGGTCCTTGAGTG R: GAGTGGTCGTTGTCTTTC

Note: ACTB (beta-actin used as reference gene)

Excel and SPSS 27 software. The data obtained from qPCR was in the form of Cycle Threshold (Ct), which was then processed to obtain the ΔCt value, namely the difference between Ct *PCNA* and Ct ACTB. The ΔCt value is then processed using the 2-ΔCt formula to obtain the Relative Quantification (RQ) value. The RQ value is then processed to obtain the *PCNA* gene expression value in the form of fold change. The fold change value is defined as the ratio between 2 quantities, obtained by comparing the RQ value between the two age groups (older group compared with the adult group) and between the two regions tested (cortex region compared with the hippocampus region). Data normality was tested using the Shapiro-Wilk test, and data homogeneity was tested using the Levene test, the data was tested using the independent t-test to see differences between age groups and between regions.

3. Results

3.1 Comparison of *PCNA* Gene Expression Based on Age Groups in *Macaca fascicularis*

The results of data processing and statistical tests showed that the data were normally distributed and homogeneous, and showed that there was no significant difference between *PCNA* gene expression in the adult and old *Macaca fascicularis* groups with a P-value of 0.155 (>0.05) (Table 2).

3.2. Comparison of *PCNA* Gene Expression Based on Brain Region in *Macaca fascicularis*

Neuropathological changes that are signs of Alzheimer's disease are the presence of senile plaques or amyloid-beta deposits and the presence of neurofibrillary tangles. These changes mainly start from the hippocampus and entorhinal cortex before finally spreading to other parts, such as the

Table 2. *PCNA* gene expression by age group

Age Group	Number of Samples	Average RQ ± SD	P-value
Adult	6	0.108 ± 0.043	0.505
Aged	6	0.091 ± 0.042	

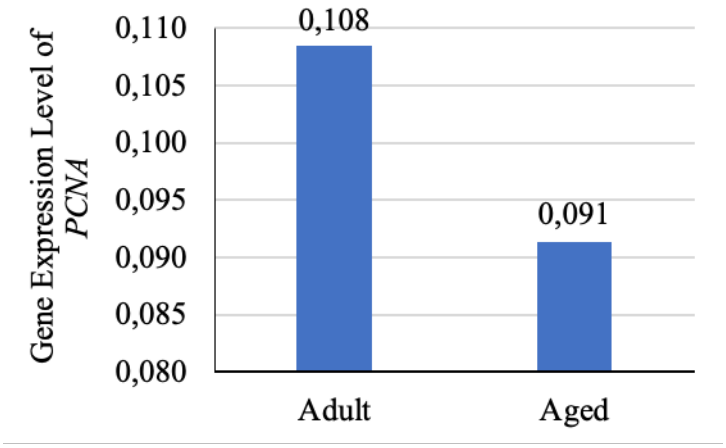

Figure 1. *PCNA* gene expression by age group in *Macaca fascicularis* brain

temporal, parietal and frontal brain (Jahn 2013). The results of the study showed that there was *PCNA* gene expression in the two regions studied, namely the hippocampus and cortex regions of the brain of long-tailed monkeys. The data processing results showed that the data were normally distributed and homogeneous, and showed that there was no significant difference between *PCNA* gene expression in the two regions with a P-value of 0.820 (>0.05). Apart from that, the data obtained has a large standard deviation (Table 3).

The calculation results show that the *PCNA* gene expression in the cortex region is 0.94 fold change from the *PCNA* gene expression in the hippocampus region, which means that the *PCNA* gene expression value in the cortex region is 94% of the *PCNA* gene expression value in the hippocampus region, or there is a decrease of around 6%.

3.3. Comparison of *PCNA* Gene Expression in *Macaca fascicularis* Based on Age Group and Brain Region

PCNA gene expression levels were also compared by age group in the hippocampus and cortex brain regions. Comparisons were made to see differences in *PCNA* gene expression levels in age groups in different regions. Data processing and statistical tests in both regions showed that the data were normally distributed and homogeneous, and showed that there were no significant differences in *PCNA* gene expression between the two age groups in each region, with a P-value for the hippocampus region of 0.063 and the cortex region of 0.673 (Table 4).

Table 3. *PCNA* gene expression based on brain region

Brain Regions	Number of Samples	Average RQ ± SD	P-value
Hippocampus	6	0.103 ± 0.038	0.820
Cortex	6	0.097 ± 0.049	

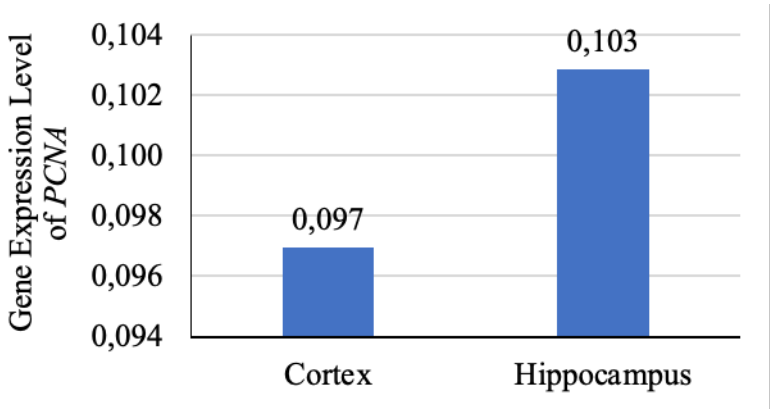

Figure 2. *PCNA* gene expression based on region in the brain of *Macaca fascicularis*

Table 4. *PCNA* gene expression based on brain region and age group

Brain Regions	Age Group	Number of Samples	Average RQ ± SD	P-value
Hippocampus	Adult	3	0.130 ± 0.028	0.063
	Aged	3	0.076 ± 0.023	
Cortex	Adult	3	0.087 ± 0.050	0.673
	Aged	3	0.107 ± 0.057	

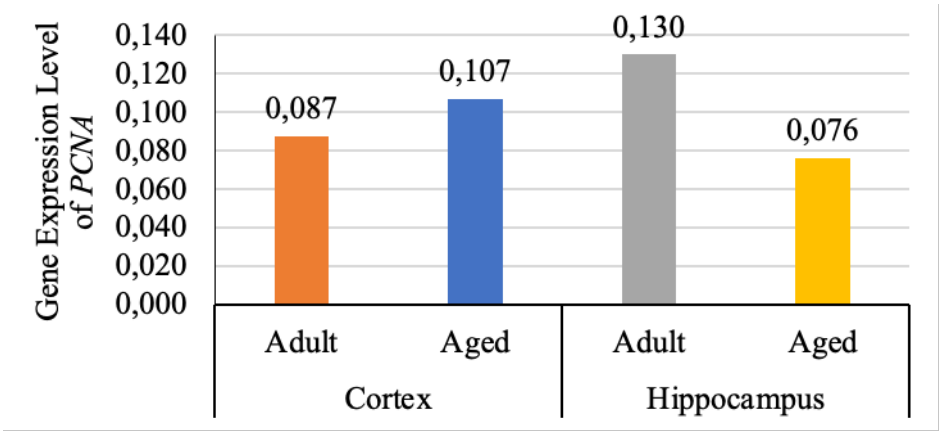

Figure 3. *PCNA* gene expression by age group in *Macaca fascicularis* in different brain regions

The results of data processing show that the highest *PCNA* gene expression is in the hippocampus region of the adult age group with an average RQ value of 0.130, while *PCNA* gene expression in the hippocampus region of the elderly age group is the lowest with an average RQ value of 0.076 (Figure 3).

4. Discussion

PCNA gene expression (figure 1) based on age groups in the brain of *Macaca fascicularis*, it is known that *PCNA* gene expression tends to be higher in the adult group compared to the old group. The calculation results show that the *PCNA* gene expression in the old group is 0.84 fold change from the *PCNA* gene expression in the adult group. These results may indicate decreased *PCNA* gene expression with increasing age in long-tailed monkeys.

This could indicate differences in the proliferation response in the brains of aging monkeys. The higher value of *PCNA* gene expression in adult animals could indicate that the neurogenesis process in the brain is more active in adult animals. Neurogenesis is the process of forming functional and mature nerve cells, produced by Neural Stem Cells (NSCs) in the brain (Abdissa *et al*. 2020). Neurogenesis was previously thought to occur only during the embryonic and perinatal stages in mammals. However, it has now been proven that there is a process of adult neurogenesis in rodents, non-human primates (NHP), and humans (Tobin *et al*. 2019). Although this neurogenesis process still occurs in adult animals or humans, based on several other studies, it can be seen that this process occurs in lower numbers compared to young animals/humans (Moreno-Jimenez *et al*. 2019). The formation of new neurons is thought to provide a neural substrate to accommodate new experiences, resistance to stress and anxiety, and is thought to prevent neurodegeneration (Kumar *et al*. 2019).

Disturbances in the process of adult neurogenesis are a common characteristic or sign in various neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) (Winner and Winkler 2015). This disturbance in the neurogenesis process shows that apart from the loss/damage of existing neurons, in brains with degenerative diseases the endogenous capacity of the brain of adult people/ animals for cell renewal is also compromised or lost. Research by Rodriguez *et* al. (2008) using mice, detected a decrease in proliferation as part of neurogenesis which was directly correlated with the presence of amyloid-beta plaques. This is also in accordance with the statement by Gage and Mu (2011), that changes in the early stages of Alzheimer's development, such as amyloid-beta deposition and

inflammation can interfere with the maturation of new neurons and inhibit hippocampal neurogenesis. These results are also in accordance with research by Tobin *et al*. (2019), where a decrease in neuroblasts was detected using DCX+*PCNA*+ as a marker in humans with mild cognitive decline. This shows that disturbances in the process of adult neurogenesis can be an early sign of Alzheimer's disease.

PCNA gene expression based on regions in the *Macaca fascicularis* brain shows that *PCNA* gene expression in the hippocampus region is higher compared to the cortex region. The hippocampus is a part of the brain that is vital in the process of learning, memory, and spatial navigation. Apart from that, the hippocampus also has a role in behavior, as well as regulation of hypothalamic function (Anand and Dhikav 2012). The cortex is a part of the brain that is composed of complex associations of neurons that cover the outermost part of the brain. The cortex is responsible for complex brain phenomena such as perception, thinking ability, language ability, episodic memory, and voluntary movement (Molnar et al. 2019). Damage or changes in these two regions can cause damage to the function of each region, which allows symptoms of Alzheimer›s disease to appear.

Adult neurogenesis is currently known to occur primarily in the dentate gyrus (DG) of the hippocampus and subventricular zone (SVZ), with the majority of cells proliferating in the SVZ migrating and settling in the olfactory bulb (OB). However, until now, controversy still exists regarding whether adult neurogenesis also occurs in other brain structures such as the cortex, hypothalamus, striatum, amygdala, and other parts (Jurkowski *et al*. 2020). *PCNA* has a role in the proliferation of Neural Progenitor Cells (NPCs) in the brain. The results in this study show that in the cortex region, *PCNA* gene expression values are not significantly different from *PCNA* gene expression in the hippocampus region, which may indicate that cell proliferation from the neurogenesis process occurs in the two brain regions studied.

Different results were obtained in the cortex region. The expression level of the *PCNA* gene in the cortex region had a higher value in the elderly group, with an average RQ value of 0.107, and 0.087 in the adult age group (Figure 3). The calculation results show that the *PCNA* gene expression value in the elderly group in the cortex region is 1.23 fold change from the *PCNA* gene expression in the adult group in the cortex region. This means that the *PCNA* gene expression value in the elderly group in the cortex region is an increase of 23% from the *PCNA* gene expression value in the adult group in the cortex region. This indicates that there is an increase in *PCNA* gene expression in the cortical region with increasing age.

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