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Original Research Article

Expression of p16INK4a in the Brain of Animal Model of D-Galactose Induced Aging

Dwi R. Anggraini[1]*, Muhammad Ichwan[2], Yetty Machrina[3], Syafruddin Ilyas[4]

[1]*Department of Anatomy, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia*
[2]*Department of Pharmacology and Therapeutic, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia*
[3]*Department of Physiology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia*
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera, Medan, Indonesia

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ABSTRACT

Oxidative stress and mitochondrial dysfunction play a major role in aging. Chronic administration of D-galactose has been reported to cause a decline in cognitive and motor skills similar to the symptoms of aging, therefore, it is considered a model of accelerated aging. The p16INK4a as aging biomarkers have been investigated. The study evaluated and compared natural aging and D-galactose induced aging model in the hippocampus. Mice were divided into 5 groups (n = 5), i.e., K0: young control (3 months of age), K1: natural aging (24 months of age), P1, P2, and P3: treatment group (3 months of age, D-galactose was administered subcutaneously at the doses of 100 mg/kgbw, 200 mg/kgbw, and 400 mg/kgbw, respectively) for 8 weeks. Hippocampus tissues were collected. The p16INK4a expression of hippocampus was evaluated by RT-PCR and immunohistochemistry test. The study showed weight gain in K1 (natural aging) and P3 (D-gal 400mg/kgbw) was significant ($p = 0.010$ and $p = 0.019$, respectively). The expression of p16INK4a in P1 (D-gal 100 mg/kgbw) was similar to naturally aging group and the highest expression compared to the others ($p > 0.005$). Immunoexpression of p16INK4 in P1 showed the highest increased expression (10.6 ± 1.95) compared to the other treatment groups (2.2 ± 1.3 and 1.2 ± 1.6, respectively). D-galactose inducted at dose 100 mg/kgbw for 8 weeks is a suitable animal model for aging brain.

***Keywords*:** Animal model, D-galactose, Brain aging, p16INK4a.

Introduction

Aging is a complex natural phenomenon associated with a continuous decline in a number of pathophysiological processes, manifested by degenerative changes in structure and function of cells. Significant amount of data was found to prove that aging process has a strong correlation with the continuously declining antioxidant function, contrasting to the highly accumulating oxidant products and mitochondrial ROS (reactive oxygen species).[1-3]

Aging has become a global problem, as the aging population continues to increase. How to slow down the aging process is a major challenge in clinical and biological research. Recent study of aging at the molecular level has been comprehensively reviewed worldwide to understand the basic mechanisms of aging as well as the pathophysiological effects and behavior of aging.[4] Animal aging models are important for aging studies, because not all molecular mechanisms can be studied in humans and limitation of ethical problems. Some standard models have been studied including fish, birds, mice, and dogs.[5,6] The use of accelerated aging models are considered common due to the practical conditions of the research project, such as duration and budget.[7,8,9] D-galactose causes oxidative stress in various tissues by increasing the production of ROS and AGEs (Advanced glycation end products) which also occurs in normal aging. Research aging models with administration of D-galactose can moreover be utilized to study aging in the brain.

Corresponding author E mail: dwirita@usu.ac.id
Tel: +6281376119774

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This is an effective alternative to accelerate the aging process. There are three distinctive metabolic pathways that are specific for D-galactose in the body, the main pathway being the Leloir pathway.[10] The second pathway is the conversion of excess D-galactose to galacticol by galactose reductase. Failure in galacticol metabolism will increase accumulation in cells, affect osmotic pressure and damage the antioxidant defense system. The third, excessive levels of D-galactose are oxidized by galactose oxidase to reactive aldehydes and hydrogen peroxide.[11]

The p16INK4a which is a cell cycle inhibitor and one of the most potent indicators of cellular aging, has been investigated recently. Expression of p16INK4a has been shown to markedly increase with aging in rodent, baboon and human tissues tested, presumably as a result of accumulating DNA damage with increasing age.[12]

The marked accumulation of p16INK4a in various age tissues also supports the hypothesis that senescence may contribute to aging. Undetectable expression of p16INK4a mRNA in young cells, can result in senescent cell remaining within tissues,[12-15] and may be potentially activated by stress. According to the current hypothesis, the accumulation of aging cells in the organism with slow regenerative capacity, will result in organ failure and homeostasis and consequently tissue aging.[16] In addition, some extrinsic factors of lifestyle, such as smoking and sedentary or inactivity, and chronic diseases and their treatments, cause p16INK4a expression, thereby increasing cellular aging.[17]

Materials and Methods

Animal and treatment

A total of 25 male mice, weighing 25- 40 g were divided into 5 groups, namely K0, K1, P1, P2 and P3. K0: 3 months of age mice, given a standard diet, as young control group. K1: 2 years of age mice, given a standard diet, as naturally aging group. Treatment group (P1, P2 and P3) 3 months of age, given D-galactose subcutaneously at the doses of 100mg/kgbw, 200mg/kgbw and 400mg/kgbw/day

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respectively, for 8 weeks. They were kept under controlled conditions (room temperature, 22‑24˚C; relative humidity, 40‑60%) and acclimatized to the housing environment for 1 week prior to the experiment with free access to feed and distilled water. Body weight from each group were recorded every four weeks. The mice were sacrificed on the last day of treatment, hippocampus organs were directly gathered for bioassay or stored at ‑70˚C for later use. The study was conducted following the consent of the Ethics Committee of Universitas Sumatera Utara Number: 161/KEP/USU/2020.

Immunohitochemical staining
Immunostaining of p16INK4a of the hippocampal brain tissue was performed using 5-µm-thick with PBS containing 0.05 M EDTA followed by 4% formaldehyde fixed, paraffin-embedded tissue sections, which were deparrafinized using xylol. As a p16INK4a polyclonal antibody (Thermo Fischer, PA5-20379) was used and detected using Powervision (DAKO A/S, Denmark) and peroxidase-DAB visualization. Two independent pathologist performed evaluations of the immunostained samples. Every sample was given a score according to the intensity of the nuclear staining (1= weak staining; 2 = moderate staining and 3=strong staining) and the extent of stained cells (1=1-10%; 2=11-50%; 3=51-80% and 4=81-100%). The final immunoreactive score was determined by multiplying the intensity and extend of positivity scores of stained cell, with the 1-12 scores and graded with low =1- 4; medium = 5-8 and high= 9-12.[18]

Gene expression analysis using RT-PCR
Total RNA was separated from hippocampal tissue using RiboZol RNA extraction reagent (# N580, AMRESCO), according to the protocol of the kit used. The purity and concentration of isolated RNA were determined using a 260/280 nm ratio read using a MicroTrake Take microvolume and a BioTek PowerWave XS microplate reader (BioTek Instruments Inc., Winooski, Vt., USA). Complement DNA (cDNA) was reverse transcribed using the iScript cDNA Synthesis Kit (# 1708891, Bio-Rad Laboratory). The samples used were duplicates by using CFX Connect Real-Time System (Bio-Rad Laboratory) and SYBR Green Super Mix (# 1708880, iQ SYBR Green Supermix, Bio-Rad Laboratory). Primer for p16INK4a F:GTGTGCATGACGTGCGG R: CACCTGAATCGGGGTACGAC. (Elkins Park, PA, USA). The RT-PCR stages used are as follows: 3 minutes at 95°C, 10 seconds at 95°C and 45 seconds at 58°C (step 2 repeated 49 times)10 seconds at 95°C, 2 seconds at 65°C. The value of C (q) was determined automatically by CFX Manager ™ v.3.1 (Bio-Rad Laboratory). The quantification of transcripts to internal housekeeping control genes GAPDH was determined with Livak methods using $2^{-\Delta\Delta Ct}$ formula.[19]

Statistical analysis
The results were presented as mean ± standard error of the mean and analyzed by the SPSS using one-way ANOVA analysis and continued with Post Hoc Test. Statistical significance was considered at $p < 0.05$.

Results and Discussion

D-galactose accelerated aging process can be recorded in animal models of aging. Animal models of D-galactose induced aging have similarities with natural aging animals in levels of oxidative stress, free radical injury, non-enzymatic glycation. The results were in line with the study of Sebayang, and Susantiningsih *et al.* showing that the occurrence of weight gain is related to age. Giving D-galactose continuously results in accumulation of galactose in the tissue.[22,23]

Expression of p16INK4a in the induced aging model.
The expression of gene p16 was analyzed by comparing the cycling threshold (CT) target gene with CT housekeeping gene as ΔCT. To get the level of expression of the target gene relative to control, fold change was sought using the Livak formula. Relative gene expression was analyzed using reference genes (GADPH) based on the Livak formula and analyzed qualitatively.
The p16INK4a expression in natural aging group increased compared to young control. This is consistent with the hypothesis that p16INK4a expression increases with aging.[15] P1 group (D-galactose 100 mg/kgbw) showed the highest expression compared to other treatment groups and was similar to the expression in naturally aging (Figure.1).

Immunoexpression of p16INK4a in the induced aging model
Immunohistochemistry staining results are revealed in Table 2. The results showed high expression of protein in the K1 (12.0 ± 0). This is in accordance with the literature that the increasing age of a person shows a high expression of p16.[19] The treatment group P1 (100 mg/kgbw/day) showed the highest increased expression (10.6 ± 1.95) compared to the other treatment groups (2.2 ± 1.3 and 1.2 ± 1.6, respectively). The burden of aging and aging-associated diseases poses crucial medical and financial problems to the growing aging population. Determining underlying cellular pathogenesis of aging progression is a needed anti-aging strategy. In D-galactose-induced aging, oxygen stress and neuroinflammation are the underlying causes of brain senescence, leading to accumulated biomarker of senescence in tissue including Saβ-galactosidase, p16INK4a and p21.[25] The expression of p16 is notably increased with aging in most rodent and human tissues. Accumulation of p16 contributes to aging by negatively regulating the cell cycle.[26] Previous study showed that injected D-galactose increased the expression of oxidative stress and decreased the expression of antioxidants.[27,28] The dose of D-galactose used varies between 50 - 400 mg/kgbw/day with a duration of 6-8 weeks.[27-29] D-galactose provides an aging effect similar to natural aging. D-galactose (150 mg/kgbw) injected subcutaneously for 6 weeks, caused the effects of aging, cognitive impairment and neurodegenerative diseases such as dementia.[25,31] The study showed brain aging with D-galactose induced at dose 100 mg/kgbw for 8 weeks. The accelerated induced aging model might be promising tool for human brain aging model in mice.

Table 1: Body weight of mice

Groups	Body weight (mean ± SD)	p-value
K0	34.68 ± 2.61	
K1	40.64 ± 0.70	0.010*
P1	31.42 ± 2.36	0.710
P2	35.58 ± 1.83	0.546
P3	38.66 ± 1.56	0.019*

*: p-value = Significant Statistical test results using independent t-test showed that there was a significant difference of K1 and P3 among K0 group ($p < 0.05$). The weight gain in the treatment group which is D-galactose induced corresponds to an increase in the dose given. This is because lipid turnover in adipose tissue is reduced during the aging process leading to weight gain.[21] The body weight (g) of the mice is shown in the Table 1.

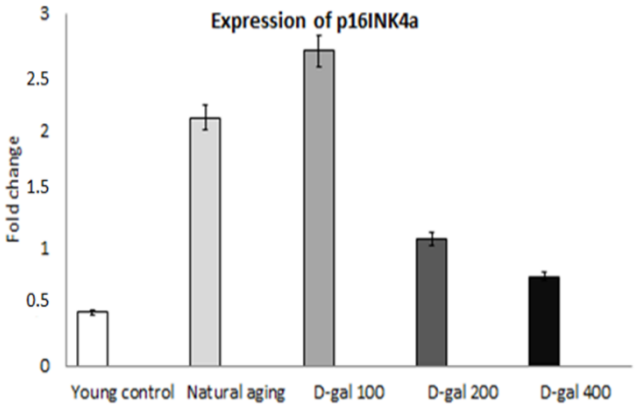

Figure 1: The p16INK4a expression of hippocampus.
The results of the data for all treatments were tested for normality using the Shapiro Willk test method. It was found that the data were normally distributed so that it could be multivariate analysis using the ANOVA test. It was found that the p-value was not significant, $p > 0.05$ ($p = 0.119$).

Table 2: Scoring of p16INK4a expression of hippocampus tissue

Groups	p16INK4a expression (mean ± SD)	Scoring
K0	6.6 ± 4.5	moderate
K1	12.0 ± 0	high
P1	10.6 ± 1.95	high
P2	2.2 ± 1.3	low
P3	1.2 ± 1.6	low

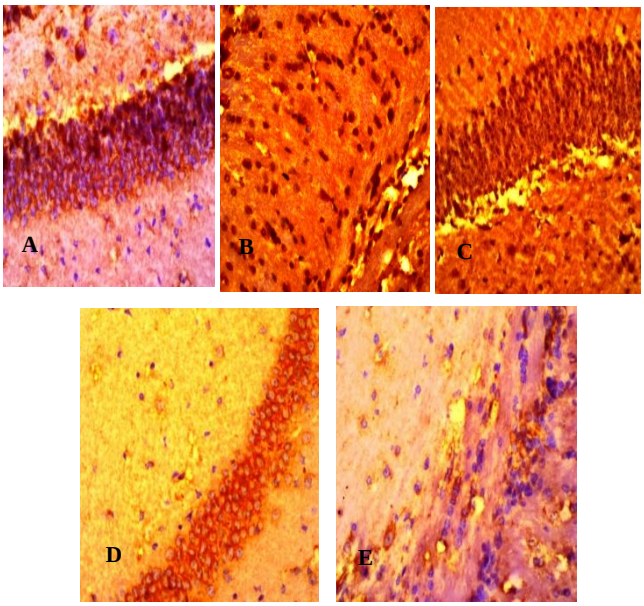

Figure 2: Immunohistochemistry analysis of p16INK4a expression of CA1 hippocampus A. K0, moderately positive nuclear staining; B.K1, highly positive nuclear staining intensity; C. P1, highly positive nuclear staining intensity; D and E. low (weakly) positive nuclear staining in P2 and P3 group, respectively. Photomicrographs were taken in high-powered, 400x**.**

Conclusion

In summary, the study provided appropriate dose of D-galactose for experimental animal models of brain aging in mice at dose of 100 mg/kgbw/day for 8 weeks.

Conflict of Interest

The authors declare no conflict of interest.

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