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The Effect of Combination Ovariectomy and D-galactose Administration on Alzheimer's Animal Model

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Abstract:

Background and purpose: Animal model helps researchers to evaluate new treatment plan for human and understand pathological mechanism involved in a development of disease. The use of rats as an animal model for Alzheimer's research has become a favorite among researchers. Rats are capable in mimicking Alzheimer disease due to their intelligence and quick adaptation to nature. At present there are several methods that can be used to induce Alzheimer's animals, but each method has advantages and disadvantages. We need to learn other methods that can provide many advantages and few disadvantages. The Amyloid-beta 42 (A β -42) and Reactive Oxygen Species (ROS) are thought to play an important role in the pathology of Alzheimer's disease. This study aims to investigate whether ovariectomy and D-galactose can be an effective method for inducing Alzheimer's animal models. This was an experimental study with control group design. Twelve female Sprague Dawley rats were involved and grouped into two groups. Control group who did not undergo ovariectomy and the experiment group underwent ovariectomy and was given intraperitoneal D-galactose 500 mg/kg.bw. Amyloid beta-42 plasma and Y-Maze test were conducted after 6 weeks. The results showed that the experiment group has lower mean Y-Maze score ($42,79 \pm 6,97$) compared with control group ($74,27 \pm 4,01$) and the Amyloid-beta 42 plasma was higher in the control group. In conclusion ovariectomy and D-galactose are proven to induce cognitive decline and higher plasma Amyloid-beta 42 in the Alzheimer's animal model.

Keywords: Alzheimer's, D-galactose, Ovariectomy, Sprague Dawley, Y-Maze

Introduction:

Higher life expectancy worldwide leads to higher elderly population. High population of elderly brings various health issues such as neurodegenerative disease, Alzheimer's ¹. There is no medical therapy available to cure Alzheimer. The current therapy works on slowing down Alzheimer's progressivity and its risk factors. The pathology of Alzheimer's is not yet fully understood so thus more study needs to be done to understand this disease more thoroughly ². Animal model helps researchers to evaluate new treatment plan for human and understand pathological mechanism involved in a development of disease. Due to limited knowledge regarding the cause of Alzheimer, every available model shows limitation so thus its application must be considered carefully.

At present, no animal model is able to mimic Alzheimer's disease in a natural way, therefore the majority of studies are carried out using phenotype simulations in animal models, or using transgenic animals as the most advanced techniques ³.

Although transgenic animals show many advantages in research, they still show some weaknesses. Besides there may be a high mortality rate and might show deleterious effects, transgenic animals are also very expensive and will further burden research funding. In some studies that do not provide transgenic animals, rats are the animal of choice in Alzheimer study. Due to their intelligence and quick adaptation, rat are capable of mimicking Alzheimer disease in human. Rats have memory and cognitive abilities that can be measured using

behavioral tests which are very important features in choosing an Alzheimer's animal model³. Previous study showed rat capable in future decision making based on their current knowledge. This ability is called metacognitive which previously considered one of primate unique feature^{4,5}.

At present there are several methods that can be used to induce Alzheimer's animals, but each method has advantages and disadvantages, so we need to learn other methods that can provide many advantages and few disadvantages. Increased Amyloid-beta 42 (A β -42) production and Reactive Oxygen Species (ROS) are widely believed to be the main causes in Alzheimer's pathology^{6,7}. D-galactose is a reducing sugar that is easy to react with amino acids in peptides, to form Advanced Glycation End Products (AGEs). Chronic administration of D-galactose at low doses has shown to induce changes that mimic natural aging processes in animals, includes cognitive decline, oxidative stress, and metabolic disorders. Premature aging in animals which induced by chronic exposure to D-galactose is commonly used as a model for studying the neurodegenerative disease⁸.

This study aims to investigate whether ovariectomy and D-galactose can be an effective method for inducing Alzheimer's animal models. The combination of ovariectomy and D-galactose as a ROS stimulator is thought to support each other to induce Alzheimer's by disrupting the amyloid-beta cascade pathway and damaging the mitochondria by increasing ROS. This method is based on principle that estrogen is endogen A β regulator. Ovariectomy diminishes the negative feedback to Luteal Hormone (LH), this process leads to increased LH level that supports A β production through amyloidogenic pathway⁹.

In normal condition, estrogen in the brain plays role in maintaining synapses plasticity and hippocampal neuronal growth that are involved in periodical memory formation¹⁰. Estrogen also preserves the nerve from beta amyloid toxicity, lowers brain inflammation, and prevents hyperphosphorylation of tau protein¹¹. Estrogen regulation on A β has not been fully understood, but the mechanism might involve indirect action of estradiol aromatization pathway, and gonadotropin and gonad hypothalamus-hypophyses pathway. In cell culture experiment, estradiol directly reduce the A β oligomer level and indirectly increases A β clearance^{12,13}.

There are two estrogen receptors in the brain, alpha and beta. Each of these receptors has a different role. Alpha receptor acts as a stimulator meanwhile beta receptor acts as an inhibitor. A

study shows that estrogen level in the plasma is decreased significantly after ovariectomy and correlated with risk factor of age-related Alzheimer disease. Decreased estrogen level after ovariectomy causes lower neural cell viability and leads to cell death (apoptosis). Furthermore, ovariectomy also decreases alpha receptor in hippocampus that leads to excessive tau protein expression. Based on a previous animal study, estrogen has a protective effect in the rats neuronal hippocampal that underwent A β toxicity and this protective effect is related with increased mitochondria respiration function. This phenomenon shows estrogen-induced neuronal induction mechanism in degenerative process is estrogen activation function of cellular mechanism and the end result is improvement of mitochondria viability. Therefore mitochondria is an ideal targeted therapy in neurodegenerative disease^{14,15}.

Materials and Methods:

Chemicals and reagent kits

D-galactose were purchased from PUDAK Scientific and Rats A β -42 ELISA Kit was purchased from Elabscience.

Animal grouping and treatment

The study design was approved by the Ethics Committee of Faculty of Medicine Andalas University, Indonesia. This was an experimental study with 12 female Sprague Dawley rats (12 weeks old, 150-200 gram) and kept in SysLab Laboratory, Bogor. The rats were fed with standard pelleted and distilled water *ad libitum*. The room temperature was maintained at 24°-25°C with constant dark-light cycle. They were divided into two groups consisted of 6 rats. The control group, which did not undergo ovariectomy and the experimental group which undergo ovariectomy and given intraperitoneal D-galactose 500 mg/kg.bwt.

Ovariectomized and D-galactose Group

Intra-muscular anesthesia was performed on rats using ketamine (50 mg / KgBW) with a 1 cc syringe. The anesthesia will take about 45 minutes. Shave the lateral sides of the rats then disinfect with 70% alcohol and iodine solution. Make a 2 cm incision following the spine at a distance of 1.5 cm. Find the ovaries and tie them with the catgut chromic. Then, sew the muscles back with catgut chromic followed by the skin with silk chromic using a simple interrupted technique. Observed wound recovery every day. 7 days post-ovariectomy, start administration of D-galactose 500mg/kg.bwt intraperitoneally daily for 6 weeks.

Behavioural experiments and blood collection

After 6 weeks cognitive function was assessed using Y-Maze and blood sample was

collected for A β -42 test. Y-Maze test is defined as spontaneous switch percentage if the score is greater than 50%, which indicated normal cognitive function. Spontaneous switch percentage score was tested with T test to investigate mean difference between groups. Y-Maze Spontaneous alternation test is a behavior test to assess rats instinct in exploring new environment. Rats tend to explore the new labyrinth arm compared with going back to the previous arm. Various brain structures including hippocampus, septum, base of forebrain, and prefrontal cortex are involved in this process¹⁶. Up to this day, Y-Maze is often used as a method to investigate learning ability and behavior evaluation among animal model so this is a good method in reflecting animal memory ability¹⁷.

Y-Maze consists of three arms, rats tend to enter the different arm as their natural instinct for searching food. Counting the mistakes in entering the arm may reflect spatial memory and learning ability in animal model. In other word, Y-maze test is practical to investigate memory function and capable to assess cognitive impairment in rats^{17,18}. The method of inducing cognitive impairment by ovariectomy has a principle that estrogen is an endogenous regulator of A β ⁹. This study involved female rats that underwent ovariectomy when they reached 3 months old because at this age rats are prone to hormonal change. The previous study, using 3 months old rats showed that serum estrogen level was higher compared with 6 months old female rats. High dose D-galactose administration aims to trigger metabolic disturbance that leads to excessive ROS production. ROS and mitochondria function are two contributing variables. High levels of ROS in the mitochondria cause cell damage and thus contribute to the pathological process in the brains of rats undergoing ovariectomy which can be assessed from A β -42 levels^{18,19}.

At the end, the rats were sacrificed for blood collections. Blood is collected by cardiac puncture to investigate plasm A β -42.

Statistical analysis

Data obtained were presented as mean \pm SEM. The statistical significance was determined using T-Test Comparisons producing a p value $<$ 0.05 were considered significant. The analysis was performed using SPSS software (version 16).

Results:

Subjects including 12 female rats aged 12 weeks with body weights ranging from 150-200 grams. The weighing of rats aimed to ensure that the rats body weight meets the inclusion criteria. The analysis of Y-maze was conducted at the Syslab Bogor and ELISA quantitative measurement

for plasma A β -42 was perform at the Biomedical Laboratory of Andalas University and all research was conducted between October 2018 – June 2019. To find out the differences in rat weight, parametric analysis was performed using the T-test. The analysis showed that the p value was 0.27 ($p >$ 0.05), which means that there was no significant difference in sample weight between groups and all the rats were included into the inclusion criteria.

The percentage of spontaneous alternation was expressed as mean \pm standard deviation. It was calculated by counting the number of arms sequentially visited by the rats. The results showed that the experimental group has a lower percentage of spontaneous alternation compared to the control group Fig.1. The control group had an average percentage of spontaneous alternation 74,27 \pm 4,01 which represents a normal cognitive function while the experimental group showed the percentage of spontaneous alternation 42,79 \pm 6,97 which represents impaired cognitive function, with p value 0,01. According to the Stanford Behavioral and Functional Neuroscience Laboratory, the percentage of spontaneous alternation of Y-Maze Test values of more than 50% is considered normal.

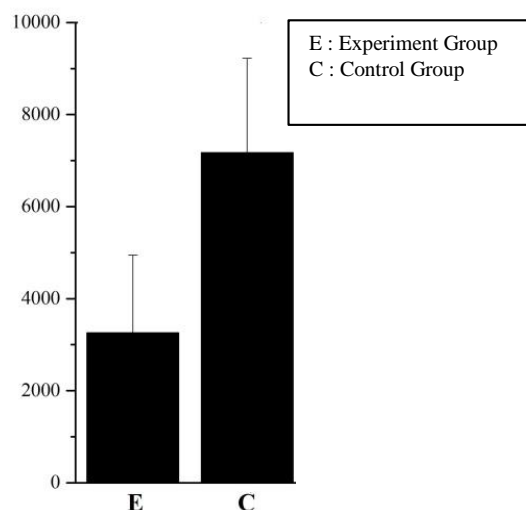


Figure 1. The result of A β -42 levels

Plasma A β -42 level was tested statistically with ANOVA. The control group showed a significantly different in A β -42 levels compared to rats in the positive control group. The average level of A β -42 in the control group was 7313,332 while in the experiment group 2643,658 (p value 0,001)

Discussion:

Cognitive function assessment based on the principle that rats have a natural instinct to explore new objects. Y-Maze is used to study the ability of rodents in environmental recognition and memory functions so that this method is effective in reflecting the ability of animals to function their memory. Y-Maze consists of three arms, rats will

tend to enter different arms as a reflection of their instinct to survive. Calculation of the number of arms entered by Maze and errors entering the arms reflects the ability of spatial memory and learning ability in animal model. In other words, the Y-Maze experiment is practical enough to be used to study the evaluation of memory function so this method is very well used in research to evaluate cognitive impairment in rats model¹⁷.

Induction of cognitive impairment with ovariectomy uses the principle that estrogen is an endogenous regulator of A β . Ovariectomy causes a loss of negative estrogen feedback on lutein hormone (LH), causing an increase in LH levels that support the production of A β through amyloidogenic pathways. Hormone Replacement Therapy (HRT) can decelerate Alzheimer's by increasing A β clearance, a component of amyloid plaque (10). Another study on estradiol showed that estradiol can increase the level of dissolved A β (sAPP α) which is a non-amyloidogenic product of APP metabolism. Low estrogen level after ovariectomy can affect estrogen receptors, α and β receptors in the hypothalamus, resulting in decreased A β clearance. This study used 3-month-old pre-puberty female rats that were very sensitive to the loss of ovarian hormones and were considered the most capable to show the characteristics of hormonal deficiency^{20,21,22}.

In previous studies, female rats that underwent ovariectomy at 3 months of age showed a higher serum estrogen level compared to 6 months old female rats. The administration of high-dose D-galactose aims to trigger metabolic disorders that cause excessive ROS production (18). D-galactose is a physiological nutrient and reducing sugars that can react freely with amino acids in protein to form Advanced Glycation End (AGEs) products. High intake of D-galactose can trigger aging by producing ROS through D-galactose metabolism. Another study conducted in rats given D-galactose 100 mg/KgBW intravenously for 6-10 weeks were reported to show progressive decreases in learning abilities and memory function and an increase of free radical production in brain²³. Reactive oxygen species (ROS) and mitochondrial function stimulate each other. High level of ROS in mitochondria can cause damage because mitochondrial DNA is sensitive and double chain so that hydrogen ions are easy to move. This process is called lipid peroxidation. Brain is the easiest organ to experience oxidative processes because it consists of fat tissue^{24,25}.

Damaged mitochondria cannot maintain cell energy requirements. Decreased brain metabolism rate often occurs before the appearance

of clinical symptoms, causing decrease in ATP level needed for normal energy homeostasis. Reduced energy metabolism leads to increased expression of β -secretase and cytoskeletal changes, including the appearance of epitopes that trigger NFT. This causes an increase in ROS production which induces interference with oxidative phosphorylation which results in a decrease of ATP level needed for normal energy homeostasis. As a result, more and more neurons will die. Clinically, this will be seen in a decline in cognitive function¹⁴. This study showed a significant difference between the control group and the experimental group. This can occur due to increased A β and mitochondrial disorders caused by increased ROS.

Conclusions and Recommendations:

Animal induction in Alzheimer's models with ovariectomy and D-galactose has been shown to cause cognitive impairment in experimental model. Further research is needed to understand the mechanism in influencing cognitive function. However, for studies with limited time and funding, induction of Alzheimer's model rats with ovariectomy and D-Galactose is worth considering.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for republication attached with the manuscript.
- The author has signed an animal welfare statement.
- Authors sign on ethical consideration's approval
- The study was approved by the Ethical Review Committee of the Medical Faculty, Andalas University (No.530/KEP/FK/2018).

Authors' contributions statement:

F. F., Y. S., R. S., and N. I. L. contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript

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تأثير الجمع بين استئصال المبيض و تناول د-جالاكتوز على النموذج الحيواني لمرض الزهايمر

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الخلاصة:

استحداث الامراض وعلاجها مختبريا (باستخدام الحيوانات المختبرية) يعطي معلومات قيمة حول ميكانيكية حدوث الامراض وتطورها. ومن اهم هذه الامراض مرض الزهايمر حيث توجد طرق عديدة لاحداث المرض في الجرذان ولكل طريقة محاسن وعيوب والتحدي الاكبر هو التوصل الى طريقة لاحداث المرض ذات محاسن عالية وعيوب قليلة. يُعتقد أن أميلويد بيتا 42 (Aβ-42) وأنواع المركبات الغير مستقرة والتي تحتوي على الاوكسجين في تركيبها (ROS) تلعب دور مهم في امراضية الزهايمر. تهدف هذه الدراسة الى احداث مرض الزهايمر لدى اناث الجرذان من خلال استئصال المبيض وحقن أميلويد بيتا 42 (Aβ-42) وهل تعتبر هذه الطريقة هي الطريقة الافضل. تعتبر هذه الدراسة دراسة مختبرية مع تصميم المجموعة الضابطة. تم تضمين 12 من اناث الجرذان وتقسيمهم الى مجموعتين. المجموعة الضابطة التي لم تخضع الى استئصال المبيض والمجموعة الثانية التي خضعت الى عملية استئصال المبيض وحقنت مع بيتا كالكتوز 500 ملغم لكل كم. تم اجراء فحصي Amyloid beta-42 و Y-Maze بعد ستة اشهر من استئصال المبيض. اظهرت هذه الدراسة أن المجموعة الثانية لديها متوسط درجات فحص Y-Maze أقل (6,97 ± 42,79) مقارنة بالمجموعة الضابطة (4,01 ± 74,27) وأن قيم Amyloid-beta 42 كانت أعلى في المجموعة الضابطة. لقد ثبت أن استئصال المبيض و الحقن باستخدام D-galactose يؤديان إلى التدهور وارتفاع مستوى أميلويد بيتا 42 في البلازما في النموذج الحيواني لمرض الزهايمر.

الكلمات المفتاحية: مرض الزهايمر، د-جالاكتوز، استئصال المبيض، جرد المختبر نوع (Sprague Dawley) ، فحص Y-Maze