

## Effect of Exercise Duration on Klotho and GluN2B Gene Expressions in Hippocampus of Wistar Rats with Moderate Intensity Exercise

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### Abstract

Exercise could delay the degeneration process by increasing Klotho, which is an endogen antioxidant. Klotho protects against oxidative damages, regulates hippocampal synaptic plasticity, and enhances GluN2B expression. This study aimed to analyze the effect of 14-day and 8-week moderate-intensity exercise on Klotho and GluN2B mRNA expressions in rat hippocampus. This study was an animal experimental study using Wistar rats that was performed from June to September 2019 at the Central Laboratory, Universitas Padjadjaran, Indonesia. Rats were divided into 4 groups: control (14-day control and 8-week control) and exercise group (14-day exercise and 8-week exercise). The exercise groups ran at a moderate intensity (20m/min) for 30min/day, 5x/week. Hippocampus was then subjected to klotho and GluN2B gene expression analysis. The exercise groups showed a higher Klotho expression, both in 14 days ( $0.844 \pm 0.124$  vs.  $1.057 \pm 0.013$ ) and 8 weeks ( $0.897 \pm 0.072$  vs.  $1.380 \pm 0.168$ ) although statistically insignificant ( $p=0.24$ ). No difference in GluN2B gene expression was observed between control and exercise groups on 14 days ( $1 \pm 0.09$  vs.  $1.22 \pm 0.09$ ) and 8 weeks ( $1.24 \pm 0.03$  vs.  $1.38 \pm 0.05$ ;  $p=0.11$ ). In conclusion, 14-day and 8-week moderate-intensity exercise did not alter Klotho and GluN2B expressions in rat hippocampus.

**Key words:** Duration, Exercise, GluN2B, hippocampus, Klotho

## Pengaruh Durasi Olahraga terhadap Perubahan Ekspresi Gen *Klotho* dan *GluN2B* di Hipokampus Tikus Galur Wistar yang Mendapatkan Perlakuan Lari Intensitas Sedang

### Abstrak

Olahraga dapat menghambat proses degenerasi saraf dengan meningkatkan salah satu antioksidan endogen, yaitu Klotho. Klotho dapat melindungi sel neuron dari kerusakan oksidatif dan meningkatkan sinaptik plasticity pada hipokampus. Penelitian ini bertujuan untuk melihat pengaruh olahraga lari pada hewan coba pada 14 hari dan 8 minggu terhadap perubahan ekspresi gen *Klotho* and *GluN2B* di hipokampus. Penelitian ini merupakan penelitian eksperimental dilaksanakan pada bulan Juni hingga September 2019 di Laboratorium Sentral Universitas Padjadjaran. Penelitian ini menggunakan tikus jantan galur wistar. Tikus dibagi menjadi 4 kelompok perlakuan dan kontrol. Olahraga dilakukan dengan *treadmill* tikus, kecepatan 20m/min, 30 menit perhari selama 5 kali perminggu. Kemudian, mRNA otak digunakan untuk PCR dengan menggunakan primer *klotho* dan *GluN2B*. Hasil penelitian menunjukkan bahwa tidak terdapat perbedaan yang bermakna ( $p=0.24$ ) pada ekspresi gen Klotho pada 14 hari ( $0.844 \pm 0.124$  vs  $1.057 \pm 0.013$ ) dan 8 minggu ( $0.897 \pm 0.072$  vs  $1.380 \pm 0.168$ ) antara kelompok kontrol dan kelompok lari. Ekspresi gen dari *GluN2B* juga tidak menunjukkan perbedaan yang signifikan ( $p=0.11$ ) pada 14 hari ( $1 \pm 0.09$  vs  $1.22 \pm 0.09$ ) dan 8 minggu ( $1.24 \pm 0.03$  vs  $1.38 \pm 0.05$ ). Simpulan, bahwa 8 minggu olahraga tidak meningkatkan ekspresi *Klotho* dan *GluN2B* yang signifikan.

**Kata kunci:** Durasi, olahraga, *GluN2B*, hipokampus, *Klotho*

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## Introduction

The elderly population is increasing along with technological advances in the health sector. Nevertheless, an increase in life expectancy can increase the prevalence of degenerative diseases. One of the degenerative diseases that increases in prevalence is dementia. A global study on dementia showed that the number of dementia patients worldwide has reached around 43.8 million in 2016. Increase in life expectancy without a good quality of life could increase the global burden.<sup>1</sup> Therefore, interventions are needed to prevent dementia.

The risk factors for dementia are divided into unmodifiable and modifiable factors. Unmodifiable factors of dementia are age, family history, and genetics, such as the presence of apolipoprotein E allele. Meanwhile, the modifiable factors dementia are current smoking, social engagement, year education, and lack of physical activity.<sup>2</sup>

Exercise has a potential effect as a neuroprotector. Aerobic exercise like running for 8 weeks prevents neuronal degeneration by reducing lipid peroxidation and preventing ROS production.<sup>3,4</sup> Another study in a rat model of Alzheimer's disease showed that physical exercise prevents memory deterioration in a rat model through the improvement of endogenous antioxidants.<sup>3</sup>

*Klotho* is known as an anti-aging gene in humans.<sup>4</sup> In the brain, *Klotho* has been proven to be a neuroprotective agent and regulator of synaptic plasticity.<sup>4,5</sup> Studies show that *klotho* interacts with Insulin Growth Factor-1 (IGF-1) to regulate  $\beta$  amyloid by increasing its clearance via choroid plexus.<sup>6</sup> It plays a role as a neuroprotective agent against neuronal oxidative damage induced by  $\beta$  amyloid and glutamate by regulating thioredoxin/peroxiredoxin (Trx/Prx) system.<sup>4</sup> Study shows that *klotho* could enhance GluN2B expression, an N-methyl-D-aspartate (NMDAR) subunit that plays an important role in learning and memory.<sup>7</sup> Exercise could enhance *klotho* expression.<sup>8</sup> Short duration of interval training has been proved to increase *klotho* expression in heart tissue of rat model of cardiac ischemia. On the other hand, moderate-intensity exercise upregulated the NMDRs subunit in the hippocampal area while high-intensity exercise suppressed NMDRs expression.<sup>9</sup>

Several studies have demonstrated a correlation between *Klotho* and GluN2B. A study from Dena B. Dubal *et al.* showed an increase of GluN2B in postsynaptic densities and enhanced

NMDAR long-term potentiation as a consequence of *klotho* elevation in human amyloid precursor protein (hAPP) mice.<sup>10</sup> Long term potentiation is one of the synaptic plasticity forms which plays an important role in learning and memory.<sup>11</sup>

However, no study has compared the effect of exercise duration on *Klotho* and GluN2B expressions in the hippocampus. This study was conducted to compare the effects of 14 days and 8 weeks of exercise on hippocampal *klotho* and GluN2B in Wistar rats.

## Methods

This was an experimental study on 36 male Wistar rats obtained from Biofarma Laboratory, Bandung, Indonesia at the age 10 weeks with a body weight of 201-250 grams. Rats were kept at room temperature with 12-hours light and dark cycle with food and water ad libitum. The animals were habituated on a treadmill for 2 weeks before treatment. The animal experiment was held at the Animal Laboratory of Physiology Division, Faculty of Medicine, Universitas Padjadjaran, and the Central Laboratory, Universitas Padjadjaran from June to September 2019. All experimental protocols had been approved by the Research Ethics Committee of Universitas Padjadjaran no 1237/UN6.KEP/EC/2019.

IDEA's treadmill machine for rats with five lanes was used. All animals were habituated on the treadmill machine for 2 weeks until achieving the desired speed. Rats ran on an individual lane for assigned speed (Control: 0 m/min, 14 days and 8 weeks groups: 20 m/min). Moderate intensity exercise was based on lactate threshold as described in a previous study.<sup>9,12</sup> Lactate threshold was reached on 20 m/min treadmill exercise. Physical exercise duration was 30 min/day, 5 times a week for each group. Exercise periods were 14 days and 8 weeks of physical exercise treatment.<sup>9</sup>

After the physical exercise treatment, all rat groups were terminated under anesthesia. Rats were decapitated, the vital organ was isolated, and the brain was kept under cold temperature. Hippocampus was isolated from the cerebrum, kept in Eppendorf tubes, and stored in -80°C until RNA isolation.

RNA was isolated from hippocampal tissue using TRIzol as a reagent (Thermo Fisher Scientific, Uppsala, Sweden). The sample was subjected to semi-quantitative PCR with One-Step RT PCR Kit (Bioline, London, UK) using the Sensoquest Labcycler Thermocycler

**Table Primer For PCR**

Gene	Primer Sequence	Tm	Product
Klotho <sup>12</sup>	F: CTCTGAAAGCCTACGTGTTGG	56°C	275 bp
	R: TAGAAACGAGATGAAGGCCAG		
GluN2B <sup>13</sup>	F: GGCATTGCTATCCAAAAGGA	61°C	157 bp
	R: GCTGCCCCCAACATATAGAA		
GAPDH	F: GTTACCAGGGCTGCCTTCTC	61°C	177 bp
	R: GATGGTGATGGGTTTCCCGT		

Tm: Primer melting temperature, F: forward primer, R: reverse primer

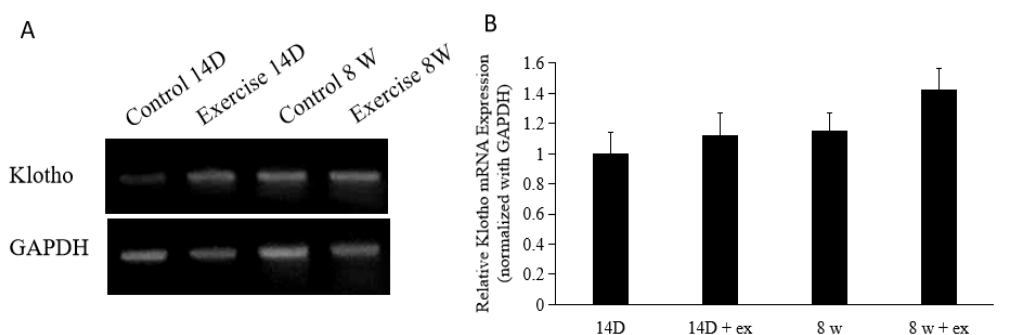
(Genecraft, Koln, Germany). Primer sequences used in this study are presented in Table 1. Gel electrophoresis was performed after PCR and the gel band of each gene was visualized using Bluepad Dual Transilluminator BioDocAnalyze while the band thickness was quantified using ImageJ software (NIH, USA).

All statistical analyses were performed using IBM SPSS version 20.0 software. Data were served as means  $\pm$  standard error of means (Mean  $\pm$  SEM). Data analysis was started with the Shapiro-Wilk test to check normality and

Levene's test for homogeneity. This was then followed by the analysis on statistical difference among groups using One Way ANOVA and Least Significant Difference Test. In this study, a p-Value of  $< 0.05$  was considered statistically significant.

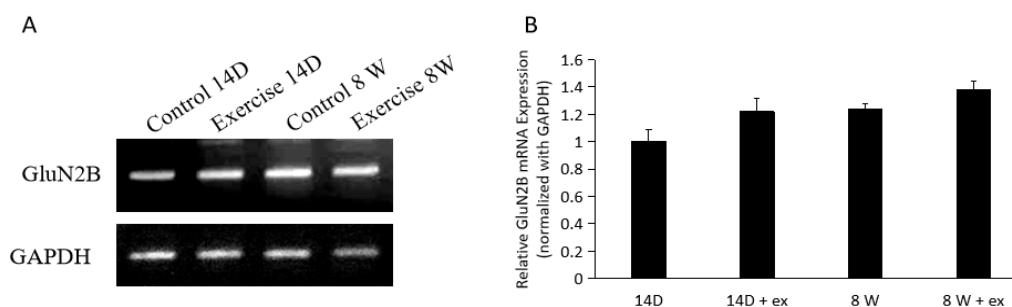
## Result

To assess the effect of moderate-intensity exercise with two different durations on internal antioxidant gene expression, the Klotho gene



**Figure 1 Klotho mRNA Band Expression**

(A) Relative expression of klotho gene, normalized by GAPDH; (B) Data represent mean  $\pm$  SEM



**Figure 2 GluN2B Relative Gene Expression at 14 Days and 8 Weeks Exercise**

(A) GluN2B expression was normalized with GAPDH; (B) Value is expressed as Mean  $\pm$  SEM

expression was measured on day 14 and week 8 of the treatment period (Figure 1). Klotho mRNA bands are presented in Figure 1A. It was demonstrated that 14 days ( $0.844 \pm 0.124$  vs  $1.057 \pm 0.013$ ) and 8 weeks ( $0.897 \pm 0.072$  vs  $1.380 \pm 0.168$ ;  $p = 0.24$ ) of exercise increased the Klotho level although not statistically significant (Figure 1B).

To investigate whether the Klotho increment affects the synaptic plasticity in different duration of exercise, the mRNA level of NMDAR subunit GluN2B in hippocampus was measured (Figure 2A). No change was observed in GluN2B expression among groups in 14 days of physical exercise duration ( $1 \pm 0.09$  vs  $1.22 \pm 0.09$ ) and the prolonged duration of exercise until 8 weeks did not alter GluN2B gene expression compared to the control group ( $1.24 \pm 0.03$  vs  $1.38 \pm 0.05$ ), with  $p=0.11$  (Figure 2B).

## Discussion

Many previous studies have shown that exercise gives many benefits for humans. Exercise induces physiology responses by affecting homeostasis in the body. Exercise, especially exhaustive exercise, could damage tissue by producing excessive reactive oxygen species (ROS).<sup>10</sup> Nevertheless, moderate-to-high exercise could increase antioxidant results, resulting in elevation of ROS concentration which enhances physiological functioning.<sup>14</sup>

Regular exercise has multiple anti-aging effects on the body. Klotho is known as an anti-aging gene, which is highly expressed in the brain and kidneys.<sup>3</sup> Several studies show that klotho increases as a result of exercise. A study from Ji et al. showed that aerobic exercise could significantly increase klotho mRNA and protein in both rat's brain and kidney.<sup>8</sup> During exercise, especially with moderate-to-high intensity, skeletal muscle generates Reactive Oxygen Species (ROS). Accumulation of ROS leads to tissue damage due to DNA and protein alteration.<sup>15</sup> In a cell culture experiment, Klotho has the ability to prevent oxidation and dissociation.<sup>16</sup> ROS disturbs neuronal signaling especially dopaminergic neurons through activating ROS-sensitive apoptosis signal-regulating kinase -1 (ASK1)/p38. Klotho overexpression modulates the ASK1/p38 pathway, thus protects the dopamine neuron from ROS induce apoptosis.<sup>17</sup>

Aerobic exercise training for 48 weeks on rat model leads to higher klotho expression in brain and kidney.<sup>18</sup> A previous study discovered

that intermittent high-intensity exercise for 5 days induces klotho expressions in plasma.<sup>19</sup> The results of this present study showed that no significant difference in Klotho expression between the 14-day and 8-week groups. Although insignificant, a higher Klotho expression level was found in the exercise group. Prolonged exercise duration (8 weeks) showed a higher Klotho expression compared to the 14 days exercise. Discrepancies identified in this study are possibly caused by stress oxidative level as the regulator of Klotho expression. Different intensity exercises and different duration produced various stress oxidative levels. It suggests training intensity combined with duration plays an important role in regulating klotho expressions.

Several studies have demonstrated a correlation between *Klotho* and *GluN2B*. A study from Dena B. Dubal et al. showed an increase of GluN2B in postsynaptic densities and enhanced NMDAR long-term potentiation as a consequence of *Klotho* elevation in human amyloid precursor protein (hAPP) mice.<sup>9</sup> Two weeks of treadmill exercise upregulates the NMDARs receptor in mice model.<sup>20</sup> In contrast, No difference in GluN2B gene expression was observed in this present study between 14 days and 8 weeks of exercise. These results correlate with Klotho expression in the hippocampus. It indicates that GluN2B expression is partly modified by Klotho expression. However, other forms of NMDAR receptor were not measured in this study; thus, further studies are needed to elucidate the correlation of exercise with other NMDAR receptors.

Certain limitations in this study should be considered. First, Klotho expression is regulated by stress oxidative level but this study did not observe oxidative stress level, ROS, or another endogenous antioxidant levels in relationship to Klotho expression. Second, only moderate intensity was used in this study that further studies are needed to reveal the effect of different intensity and duration of exercise on endogen antioxidant and synaptic plasticity.

In conclusion, 14 days and 8 weeks of moderate-intensity exercise does not change Klotho and GluN2B gene expression in the hippocampus of Wistar rats.

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