

Aerobic exercise combined with environmental enrichment improves spatial memory and enhances neuroigin 1 expression: an animal study

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ABSTRACT

BACKGROUND Numerous studies have revealed that aerobic exercise (AE) or environmental enrichment (EE) exert positive effects on the molecular, cellular, and structural changes responsible for functional plasticity. Therefore, this study was aimed to investigate whether the combination of AE and EE yields a greater effect on spatial memory and increases the expression of the adhesion molecule neuroigin 1 compared with either of the therapies alone.

METHODS Twenty 6-month-old male Wistar rats were randomly divided into four groups: (1) the control group (C), (2) the AE group, (3) the EE group, and (4) the AE-EE group. Escape latency in a water-E-maze (WEM) task was used as a parameter to assess spatial memory function. Neuroigin 1 protein expression was examined via the immunohistochemistry (IHC) technique followed by IHC optical density (OD) score analysis of the CA1 hippocampal region.

RESULTS Data acquired from the WEM task show that escape latency progressively decreased over time in all groups. However, animals in the AE-EE group required less time to complete the task compared with those in the control, AE, and EE groups. IHC OD scores revealed that the combination of AE and EE caused the highest expression of neuroigin 1 in the CA1 hippocampal region among the therapies studied. Statistical analysis indicated significant differences in OD score between the AE-EE and control, AE, and EE groups ($p \leq 0.001$).

CONCLUSIONS The combination of AE and EE rats increased neuroigin 1 expression and improved the spatial memory of male Wistar rats compared with application of either therapy alone.

KEYWORDS aerobic exercise, neuroigin 1, neuroplasticity, spatial memory

Memory decline is associated with a decrease in neuroplasticity starting from early adulthood.¹ Neuroplasticity is regulated and affected by various external stimuli, resulting in modification of the molecular, cellular, and structural changes responsible for functional plasticity. Emerging evidence suggests that an external stimulus, namely, environmental enrichment (EE), influences the development of the nervous system. Alterations in EE animals include increased levels of neurotrophic

factors, enhanced hippocampal dendritic structural plasticity, and stimulated synaptic plasticity and neurogenesis, all of which contribute to spatial memory performance.²⁻⁴ Improvements in memory functions are also induced by aerobic exercise (AE) through modification of gene expression, enhanced synaptic protein expression and postsynaptic structures, and increased synaptic transmission.⁵ Structural changes in the CA1 hippocampal region have been observed to lead to improvements in

spatial memory function in rats subjected to low-intensity treadmill training.⁶ Recent studies have shown that synaptic plasticity is regulated by interactions between presynaptic and postsynaptic neurons, including cell-adhesion molecules, scaffolding proteins, and the synaptic vesicle machinery. Neuroligin 1, a postsynaptic cell-adhesion molecule that binds to the presynaptic neurexin and postsynaptic density 95 protein (PSD-95), plays an important role in signal transduction and synaptic formation and maturation, which are essential for cognitive function.^{4,5,7} Thus, the aim of this study was to investigate whether the combination of AE and EE provides a greater effect on spatial memory than either of the therapies alone.

METHODS

The study design and methods was approved by the Ethics Committee of the Faculty of Medicine Universitas Indonesia (No. 849/UN2.F1/ETIK/2016). This study was conducted from August 2016 until February 2017. Animal care and surgical procedures were performed at standardized place in *Balai Penelitian dan Pengembangan Kesehatan RI* (Jakarta), protein analysis was done at the Immunopathology Laboratory, Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia, and tissue analysis and imaging were conducted at the Pharmacokinetic Laboratory, Department of Pharmacology and Therapeutic, Faculty of Medicine, Universitas Indonesia.

Subjects

Twenty 6-month-old male Wistar rats were randomly assigned to one of four groups, namely: (1) the control group (n = 5), (2) the AE group (rats submitted to AE; n = 5), (3) the EE group: (rats housed in an EE cage; n = 5), and (4) the AE-EE group (combination of AE and EE; n = 5). The animals were housed at 25 ± 1 °C with a 12 hours/12 hours light/dark cycle and unlimited access to food and water throughout the experiment.

Exercise protocol

Light-intensity AE was provided in this experiment by using an animal treadmill. Prior to the experiment, subjects underwent a familiarization period, which consisted of running on the treadmill for 10 min for five

consecutive days at a rate of 10 m/min. The exercise protocol was given 5 days a week for 6 weeks (Monday to Friday). In each session, the animals were allowed to warm up for 5 min by running at speed of 10 m/min; the training speed and duration were set to 20 m/min and 30 min, respectively.^{8,9}

Environmental enrichment (EE) cage

EE refers to housing conditions under which laboratory animals experience higher levels of sensory, motor, social, and cognitive stimuli compared with standard laboratory housing conditions.⁴ EE cage adapted from the Marlau™ cage protocol with slight modifications were used in this study. The size of the cage is: length = 800 mm × width = 600 mm × height = 510 mm and consisted of two floors. The ground floor was divided into a feeding area (G1) and a play area (G2) connected by a one-way door from G1 to G2. The play area was equipped with water bottles, a white rectangular house, and three running wheels in pink, blue, and yellow. G2 was connected to the upper floor, in which the maze was placed, through a climbing ladder. The second floor was also connected to G1 (the other side of the cage) through a sliding tunnel.¹⁰

Housing protocol

Prior to the study, subjects in the EE and AE-EE groups were kept in an enriched environment for 1 week for familiarization with the environment (without changing the maze configuration). After the familiarization period, the animals were kept in their respective housing throughout the experiment. Standardization of novelty and cognitive stimulation were ensured by changing the maze configuration thrice a week on Monday, Wednesday, and Friday using six mazes (series A–F), each consisting of two different configurations.¹⁰ Rats in the control group were kept in isolation cages, while rats in the AE group were kept in regular rat cages.

Water-E-maze (WEM) task

For this study, the WEM apparatus which evaluates spatial learning and memory were used. The WEM device is an E-shaped glass pool filled with water. It consists of a U-shaped main trench and three trenches branching perpendicularly to the main trench, two edge trenches (ti), and the middle trench (ta). The length of the main trench was 1.25 m, the

middle trench measured 35 cm, and the edge trenches measured 25 cm each. The height and width of each trench were 60 and 25 cm, respectively. The M point in the middle trench was the starting point of the learning and memory test and a ladder was laid in one of the edge trenches as a goal (G) for the animal on their efforts to escape from the water. Escape latency (in seconds) was recorded in this study.⁸

During the training phase, the rats were exposed to the WEM apparatus 5 days per week. Each rat randomly explored the device for no longer than 180 sec. If the rats did not find the ladder, they were gently guided to it and allowed to remain on it for 10 sec to help them become familiar with the WEM environment. For the spatial learning test, the rats were subjected to three trials without an interval and allowed 120 sec for each trial to reach the ladder.^{8,9} The test was performed on all groups at weeks 0, 2, 4, and 6.

Immunostaining

Paraffin sections (4 μm) of the hippocampus of all subjects were subjected to immunohistochemistry (IHC) to identify expression levels of neuroigin 1. Hippocampal sections were incubated with anti-neuroigin 1 antibody (Santa Cruz Biotechnology, Inc.) at 1:750 dilution. The negative control was treated as described above except that the antibody against anti-neuroigin 1 antibody was omitted. Cell Purkinje which is located in rat cerebellum is used as positive control. Horseradish peroxidase-based staining systems (anti-mouse, rat, rabbit, and guinea pig) from Biogear were used as secondary antibodies.

Imaging

Images were captured as five large fields of view by a binocular light microscope (Leica DM500) equipped with a built-in camera (Leica ICC50 HD) at 400 magnification in various target areas of the CA1 hippocampal region. Protein expression was analyzed via an open-source plugin called Immunohistochemistry Profiler, which is compatible with ImageJ software, for IHC analysis using color deconvolution and computerized pixel profiling to enable assignment of an automated score to each image.

IHC optical density (OD) score

Although IHC Profiler counts a pixel intensity and computes its score automatically, the final score is

$$\text{IHC Optical Density Score} = \frac{\begin{matrix} \text{Percentage contribution of high positive} \times 4 + \\ \text{Percentage contribution of positive} \times 3 + \\ \text{Percentage contribution of low positive} \times 2 + \\ \text{Percentage contribution of negative} \times 1 \end{matrix}}{100}$$

shown in a semiquantitative manner (high positive, positive, low positive, or negative). The following algebraic formula is used to calculate the IHC OD score (from 1 to 4) of the IHC images.¹¹

Statistical analysis

Statistical analysis were performed using SPSS software, version 22 (IBM), and graphs were drawn using GraphPad Prism. Average escape latency data within a group between week 0 and weeks 2, 4, and 6 were analyzed using paired t-test, while IHC OD data between groups were analyzed using the Kruskal–Wallis test followed by Mann–Whitney post-hoc analysis. In this study, $p < 0.05$ was considered statistically significant.

RESULTS

Water E-maze (WEM) task

The results of the memory spatial test using the WEM device are presented in Figure 1. Figure 1 illustrates a reduction in escape latency in the control group, although paired t-test revealed no significant difference in escape latencies between week 0 (18.5 [3.9]) and weeks 2 (16.6 [4.4]; $p = 0.578$), 4 (13.3 [8.2]; $p = 0.233$), and 6 (15.7 [11.4]; $p = 0.368$). Moreover, the data show that the escape latency of rats in the control group increased at week 6 compared with that at week 4 (Figure 1). Paired t-test showed significant differences in escape latency in the aerobic group between weeks 0 (18.9 [4.0]) and 4 (10.9 [4.0]; $p = 0.049$) and between weeks 0 and 6 (7.1 [4.1]; $p = 0.003$). No significant difference in escape latency was observed in the EE group between week 0 (17.5 [10.2]) and weeks 2 (9.2 [5.6]; $p = 0.159$) and 4 (8.9 [5.1]; $p = 0.082$); however, a significant difference in escape latency between weeks 0 and 6 (5.9 [1.4]; $p = 0.020$) was found. Statistical analysis of the AE-EE group results showed significant differences in escape latencies between weeks 0 (19.8 [11.3]) and 4 (7.1 [3.1]; $p = 0.041$) and between weeks 0 and 6 (4.6 [1.6]; $p < 0.001$). Altogether, these data suggest that the escape latency progressively decreases with time in all groups, indicating spatial

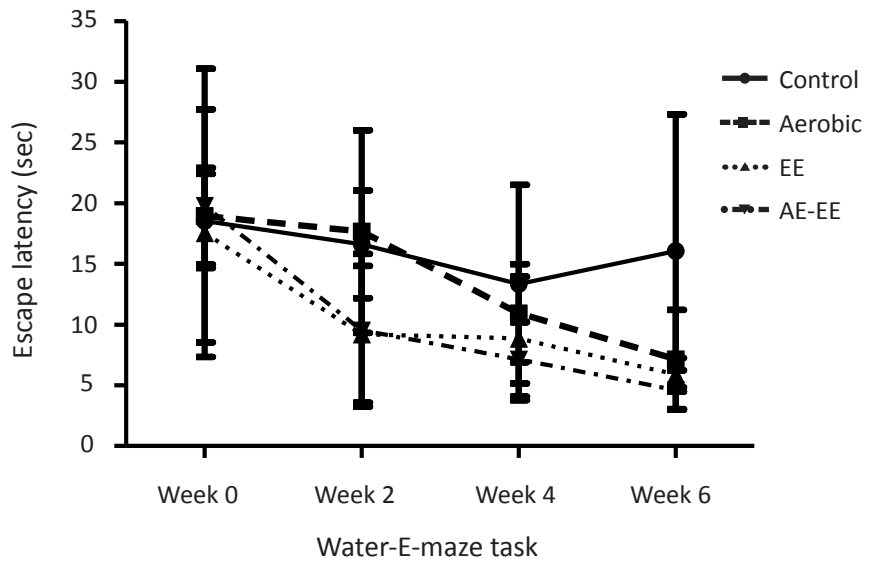


Figure 1. Escape latency during the Water E-waze (WEM) task. Each point represents the block mean (SD) of the WEM task. Lower numbers of escape latency indicate better performance. Among the groups studied, the AE-EE group (n = 5) exhibited the best performance throughout the experiment. AE=aerobic exercise; EE=environmental enrichment

learning. However, animals in the AE-EE group required less time to complete the WEM task compared with those in the control, AE, and EE groups.

Neuroigin 1 protein expression

To identify the expression of neuroigin 1, IHC staining of this protein was carried out on pyramidal cells of the CA1 hippocampal area. As shown in Figure 2, the AE-EE group revealed denser IHC expression than the other groups. The IHC OD scores of rats in the AE-EE group (1.6 [0.09]) were higher than those of

rats in the control group (1.3 [0.11]), the aerobic group (1.4 [0.15]), and the EE group (1.4 [0.24]), as revealed in Figure 3.

DISCUSSION

Stored information related to the environment and spatial orientation was needed by the rats to find the ladder in the WEM device. The decrease in average escape latency in the WEM task over-time showed learning and spatial memory formation in the animals. Deterioration in the spatial memory test

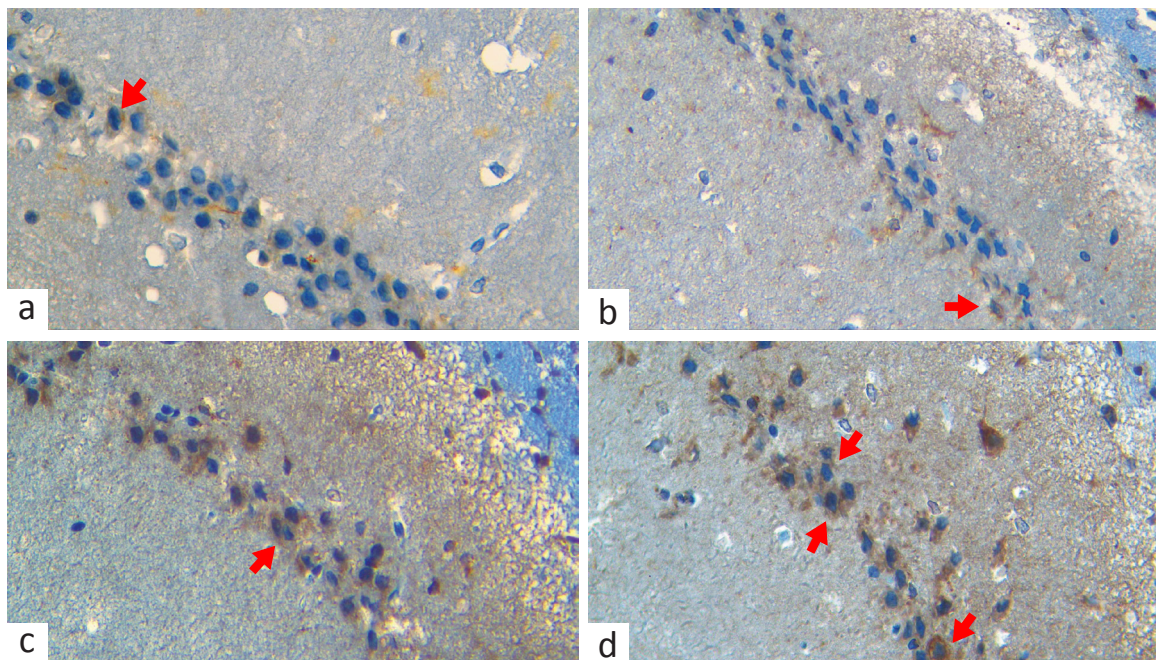


Figure 2. Expression of neuroigin 1 (red arrow) in the CA1 hippocampal region after 6 weeks of treatment: (a) control group; (b) AE group; (c) EE group; and (d) combination of AE-EE group. AE=aerobic exercise; EE=environmental enrichment

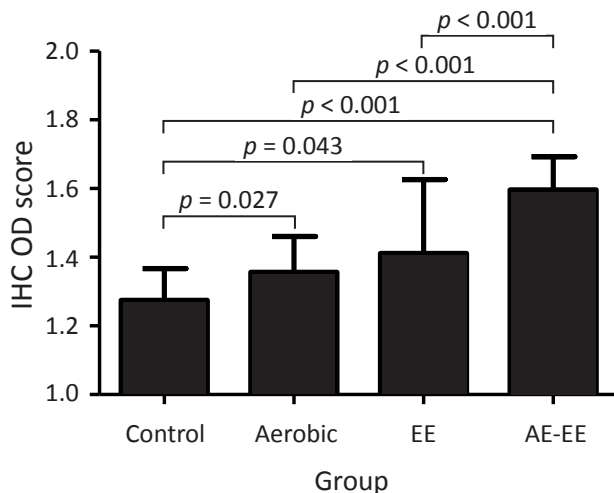


Figure 3. Neurologin 1 protein immunostaining determined from the mean of IHC OD scores in the CA1 hippocampal region. Significant differences were determined by the Kruskal–Wallis test followed by the Mann–Whitney post-hoc test. IHC=immunohistochemistry; OD=optical density; EE=environmental enrichment; AE=aerobic exercise

of rats in the control group was assumed to be due to the placement of rats in the isolation cage because social isolation affects cognition and disrupts spatial memory.^{12,13}

Using the WEM protocol, the rats from the AE-EE group showed reduced escape latency over time and performed better than the other groups in completing the spatial memory task. These results indicate that the combination of AE and EE has a great impact on spatial memory in experimental animals. Various studies employing the Morris water maze, the Y-maze, and the radial arm maze have demonstrated the role of AE in facilitating structural and hippocampus-dependent spatial memory.^{3,6,9} Similar results obtained from previous studies exposing rodents to an enriched environment with a variety of novel, cognitively challenging, and multimodal activities suggest that EE exerts protective effects against age-related cognitive decline and improves hippocampus-dependent learning and memory.⁴

Both AE and EE have been reported to increase several pre- and post-synaptic proteins involved in the formation of synapses in the rat hippocampus. Synaptic formation is induced by interactions between presynaptic and postsynaptic neurons, including cell-adhesion molecules, scaffolding proteins, and proteins associated with the synaptic vesicle machinery, such as synaptophysin, PSD-95, neuroligin 1, and β -neurexin.^{5,14,15} Physical

exercise using a treadmill is believed to facilitate synaptogenesis through increased regulation of neuroligin 1 in the hippocampus.⁵ Neuroligin 1 interacts with its presynaptic partner neurexin to promote synapse development, structural stability, and plasticity.^{16,17} Its intracellular tail binds to PSD-95, a protein found in the postsynaptic sites of excitatory synapses. The interaction of PSD-95 and neuroligin 1 has been proposed to accelerate the maturation of synapses and strengthen its connections.^{17,18} Studies also show that AE increases cell proliferation, neuron differentiation, neurogenesis, and cell survival, leading to improvements in synaptic plasticity.^{3,6,9}

The effect of EE on neuroligin 1 expression is unknown. Indeed, the results of this study may initiate future studies on the role of neuroligin 1 in functional excitatory synaptic formation.^{16,19–21} At the cellular level, application of EE increases dendritic branches, synaptogenesis, and neurogenesis in the hippocampus.²² Changes to this morphology are supported by changes in gene expression, especially those related to synapse function and neuroplasticity.⁴ These facts may represent a mechanism underlying the beneficial effect of EE on neuroligin 1, which enhances brain function by improving learning, memory, and cognition. Taken together, AE and EE appear to increase neuroplasticity differently. AE increased both cell proliferation and neurogenesis, while EE appeared to increase neurogenesis only without affecting cell proliferation.²³

In conclusion, the combination of aerobic exercise combined with environmental enrichment improves spatial memory and enhances neuroligin 1 expression in 6-month-old male Wistar rats better than AE or EE alone.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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