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Effects of treadmill exercise training on cerebellar estrogen and estrogen receptors, serum estrogen, and motor coordination performance of ovariectomized rats

Saidah Rauf [1, 2], Sri Kadarsih Soejono [1], Ginus Partadiredja [1*]

[1] Department of Physiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2] Masohi Nursing Study Program, Health Polytechnic Maluku, Ministry of Health of the Republic of Indonesia

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ABSTRACT

Objective(s): The present study aims at examining the motor coordination performance, serum and cerebellar estrogen, as well as ERβ levels, of ovariectomized rats (as menopausal model) following regular exercise.

Materials and Methods: Ten female Sprague Dawley rats aged 12 weeks old were randomly divided into two groups; all of which underwent ovariectomy. The first group was treated with regular exercise of moderate intensity, in which the rats were trained to run on a treadmill for 60 min per day for 12 weeks. The second group served as control. Rotarod test was carried out before and after exercise treatment. All rats were euthanized thereafter, and blood and cerebellums of the rats were collected. The serum and cerebellar estrogen as well as cerebellar ERβ levels were measured using ELISA assays.

Results: The number of falls in the rotarod task of the exercise group was significantly lower than that of control group. The cerebellar estrogen level of the exercise group was significantly higher than that of control group. Accordingly, there was a significantly negative correlation between the number of falls and cerebellar estrogen level in the exercise group.

Conclusion: The present study shows that a lengthy period of regular exercise improves the cerebellar estrogen level and motor coordination performance in ovariectomized rats.

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Introduction

Menopausal period in women is characterized with a considerable decrease of estrogen hormonal level (1-3). The decrease of estrogen hormone (hypoestrogenicity) interrupts the normal functioning of female organs, including the brain (2, 3). Low level of estrogen in the brain results in the decrease of cognitive, affective, nociceptive, movement, and motor coordination functions (4). These alterations of movement and motor coordination functions, which are pivotal functions of cerebellum (5), indicate that cerebellum appears to be a target organ of estrogen. It seems that the function of cerebellum is strongly influenced by hormonal status (6).

In rodents, menopausal-induced reduction of estrogen level can be appropriately mimicked by bilateral ovariectomy procedure. Adult mice underwent ovariectomy have been shown to suffer from sensorimotor (including equilibrium capacity) and exploratory behavior deficits comparable to their old counterparts. These behavioral impairments were apparently associated with the decrease of plasma estradiol levels (7). Previous studies have also shown a decrease in total protein synthesis (8), an increase in estrogen receptor β-mRNA containing Purkinje cells (9), but no change in estrogen receptor α (ERα) (10) in the cerebellum of ovariectomized rats.

The physiological effects of estrogens in the brain are accomplished through genomic pathway (1, 11) which are mediated by two estrogen receptors, i.e. ER_{α} and estrogen receptor β (ER_{β}) (12). Both ER_{α} and ER_{β} are widely distributed in cerebellum (6, 13, 14). However, the physiological effects of estrogen in cerebellum of adults are mainly mediated by ER_{β} (13).

Several investigations discovered that physical exercise accelerates motor coordination improvement in both humans and animals (15-17). Physical exercise was also observed to be associated with increased extragonadal aromatization (18) and neurotrophin synthesis (19-21), all of which affect estrogen receptors

**Corresponding author:* Ginus Partadiredja. Department of Physiology, Faculty of Medicine, Universitas Gadjah Mada Yogyakarta 55281, Indonesia. Tel: +62 274-6492-492; email: gpartadiredja@ugm.ac.id

expression (18, 22-24). It remains unclear, however, whether physical exercise affects motor coordination performance, cerebellar and serum estradiol levels, as well as ER_β in ovariectomized adult rats; and how these variables correlate to each other. The present study aims at investigating such effects and correlations.

Materials and Methods

Animals

Ten female Sprague Dawley rats aged 12 weeks old which were initially weighing 170-200 g, were used in this study. Sprague Dawley strain was chosen as experimental animals since this strain is considered to be suitable for studies on hormonal changes (25). The rats were obtained from Animal House of Gadjah Mada University. They were housed in cages under 12 hr of naturally light-dark cycle. Food and water were given *ad libitum*. They were allowed to acclimatize for one week. The experimental protocol and animal handling was approved by the Ethics Committee of Faculty of Medicine, Gadjah Mada University (approval number KE/FK/194/EC).

The rats were randomly assigned into two groups, i.e. exercise group (n=5), and control group (n=5). After one week of acclimatization, both ovaries of all rats of both groups were removed via a 2–3 cm ventral midline incision on the abdomen under anesthesia (ketamine HCl 40 mg/ kg body weight; PT Guardian Pharmatama, Jakarta, Indonesia). Seven days after ovariectomy, exercise training started.

Exercise training protocol

The exercise protocol referred to that adopted by Hao *et al* (18) with slight modifications. Briefly, the protocol consisted of two periods, i.e. adaptation period and exercise period. The rats of exercise group were adapted to the exercise protocol and treadmill apparatus (Gama Tread version 2010, Faculty of Medicine, Gadjah Mada University) in a training room for one week. During the adaptation period, the running speed, the treadmill slope, and the duration of exercise were increased gradually. The speed was increased from 10 m/min up to 18 m/min; the slope was increased from 0° up to 5°; while the duration was increased from 15 min up to 60 min. Subsequently, during the exercise period the rats were trained to keep running constantly on the treadmill at a speed of 18 m/min and at a slope of 5° for a total duration of 60 min per day. This regimen of exercise was designed to be of moderate intensity and was calculated to obtain VO_2 max of approximately 56%, based on the regimen developed by Bupha-Intr *et al* (26). The exercise was performed five times per week (every Mondays, Tuesdays, Wednesdays, Fridays, and Saturdays) for 12 weeks with two days of rest period in each week (every Thursdays and Sundays). The control group was only moved to the training room at the same time when the exercise group performed exercise.

Rotarod task

The motor coordination of rats was assessed on a rotarod apparatus (The Ugo Basile model 7700, Veresi, Italy). The protocol of rotarod test was based on those described in previous studies (15, 27, 28) with slight modifications. The tests were carried out in two series, namely seven days after ovariectomy and on the last day of exercise. Each series consisted of three trials, which were performed at the intervals of 60 min (15). The duration of each trial was 3 min (27, 28).

In order to habituate to the apparatus, prior to the tests, each rat was left for 1 min on the running surface of the stationary rotarod. The rat was then removed from the rotarod and the rotarod was turned on to rotate at a speed of 16 rounds per min. The rat was returned to the surface of the rotarod. It had to walk forward in order to maintain its position on the running surface of the rotarod during the three minutes trial. The number of falls of the rats was recorded for further statistical analyses. The number of falls was defined as the average of the total number of falls of the rats during the three trials of each series.

Serum and tissue collection

The rats were euthanized approximately 24 hr after the last exercise training. Prior to euthanasia, 2 ml of blood was collected from retro-orbital sinus of each rat under anesthesia (ketamine HCl 40 mg/ kg body weight; PT Guardian Pharmatama, Jakarta, Indonesia) and it was allowed to clot for 2 hr at room temperature. The blood was subsequently centrifuged at 1800 ×g for 10 min at a temperature of 4 ˚C (29). Serum was separated from the blood and stored at -20 ˚C freezer prior to estrogen level measurements.

Immediately after blood collection, the cerebellums of the rats were removed from their skulls and subdivided into left and right parts. The extracted left cerebellums were homogenized in TEGM (10 mM Tris-HCl, 5 mM EDTA, 10% glycerol, and 2.3 mM MgCl; pH 6.8). Every 50 mg of the cerebellums was dissolved in 1 ml TEGM. The homogenates were subsequently incubated for 18 hr in 4 ˚C refrigerator. The homogenates were then centrifuged at 1000 ×g for 20 min, and the supernatants were used for the determination of ER_β concentration. The concentration was determined using rat estrogen receptor 2 (ER beta) ELISA kit (Cusabio Biotech Co., Ltd., PR China) in a Biorad microplate reader (Benchmark, Japan) operated at a wavelength of 450 nm.

The right cerebellums were homogenized in phosphate buffered saline (PBS). Every 100 mg of the cerebellums was dissolved in 1 ml PBS. The homogenates were incubated for 12 hr in -20 ˚C freezer.

Tabel 1. The body and cerebellar weights in control and trained ovariectomized rats

Weights	Exercise group (n=5)	Control grup (n=5)	*P*
Body weights (g)			
Before exercise	164 [a]	170 [a]	0.829 [c]
After exercise	221 [a]	226 [a]	0.834 [c]
Cerebellar weights (mg)	325.02 ± 9.48 [b]	325.02 ± 9.48 [b]	0.287 [d]

[a] Values are expressed as Medians; [b] Values are expressed as Means±SEM; [c] Mann-Whitney test (between groups); [d] Unpaired t-test test (between groups)

The homogenates were then centrifuged at 5000 ×g for 5 min. The supernatants of these homogenates were used for the examination of estradiol concentration. The concentration was determined using rat estradiol ELISA kit (DRG instruments GmBH, Germany) in a Biorad microplate reader (Benchmark, Japan) operated at a wavelength of 450 nm.

Statistical analysis

The data of body weights of rats were analyzed using Mann-Whitney test, since these data did not pass the normality test. On the other hand, the data of cerebellar weights were analyzed using unpaired t-test, since these data passed the normality and variance tests. The mean differences of the number of falls before and after treatment (within groups) for both exercise and control groups were analyzed using paired t-test. The differences of the number of falls between groups were analyzed using unpaired t-test. In addition, unpaired t-test was used to measure the mean differences of serum and cerebellar estrogen as well as cerebellar ER_β levels between groups. Spearman correlation test was used to assess the correlation between the number of falls and the levels of cerebellar and serum estrogen as well as ER_β levels.

The statistical analyses were performed using SPSS version 19 software. All data were presented in means±standard error of mean (SEM) and significance levels were set at $P<0.05$.

Results

Body and cerebellar weights

Table 1 presents data on the body and cerebellar weights of all rats. There was no significant difference of body weights between groups both before and after exercise. There was also no significant difference in cerebellar weights between exercise and control groups.

Rotarod test

Table 2 presents data on the number of falls in motor coordination task before and after exercise in both control and exercise groups. There was no significant difference in the number of falls between groups before exercise. On the other hand, the number of falls of exercise groups was significantly lower than that of control group ($P<0.05$) following exercise. In the exercise group, the number of falls was significantly lower after exercise than that before exercise. In contrast, in the control group, the number of falls after treatment was significantly higher than that before treatment.

Levels of estrogen and cerebellar ER_β

Table 3 shows data on the serum and cerebellar estrogen as well as cerebellar ER_β levels in both control and exercise groups. The levels of cerebellar estrogen were significantly higher in the exercise group than in the control group. On the other hand, there were no significant differences in the serum estrogen and cerebellar ER_β levels between exercise and control groups.

Correlation

Spearman correlation test showed that there was a significantly negative correlation between the number of falls and cerebellar estrogen levels (r= (-) 0.650; P= 0.042). However, no correlation between the number of falls and cerebellar ER_β levels (r= (+) 0.49; P= 0.893) as well as between the number of falls and serum estrogen level (r= (+) 0.36; P= 0.920) was found.

Discussion

The present study found that a regularly and lengthy period of physical exercise prevented ovariectomy-induced motor coordination performance deficits in rats. Exercise also prevented the decline of cerebellar estrogen levels due to ovariectomy. In addition, motor coordination performance correlated significantly with cerebellar estrogen levels.

We cannot compare our study with others since literature search in the biomedical research database to date does not reveal any study of the effects of ovariectomy on the behavior of rats similar to ours.

Table 2. Effect of exercise training on the number of falls in motor coordination test before and after treatment in the exercise and control ovariectomized rats

	Exercise group (n = 5)	Control group (n = 5)	*P* [a]
Before exercise	23.60 ± 3.74	24.20 ± 10.78	0.959
After exercise	10.60 ± 1.47	89.20 ± 11.84	0.002
P [b]	0.006	0.040	

Values are expressed as Means±SEM; [a]*P*-values of unpaired t-test (between groups); [b]*P*-values of paired t-test (within groups)

Table 3. Effect of exercise training on the levels of serum and cerebellar estrogen and cerebellar ER_β in the exercise and control ovariectomized rats

	Exercise group (n=5)	Control group (n=5)	*P*[a]
Serum estrogen levels (pg/ml)	26.06 ± 4.06	24.54 ± 8.66	0.876
Cerebellar estrogen levels (pg/100 mg tissue)	31.64 ± 3.54	19.66 ± 2.70	0.027
Cerebellar ER_β levels (ng/50 mg tissue)	0.033 ± 0.008	0.026 ± 0.004	0.418

Values are expressed as Means±SEM; [a]*P*-values of unpaired t-test (between groups)

The only study on rodents which may be comparable to ours is that of Baeza and colleagues (7). Our study corroborates this study in which ovariectomy brought about the decrease of sensorimotor abilities such as reflex, balance, muscle strength, and motor coordination in adult mice. Such deficits of motor coordination performance might be reversed by physical exercise as was shown in previous studies using rats model of cerebellar injury and ataxia (15, 30).

Physical exercise, a type of physical activity assumed in a planned and structured movement to maintain or improve physical fitness (31), may modify motor coordination function by affecting neuronal plasticity of cerebellum (32), a key part of brain participating in regulating motor coordination (5). It is plausible that the effects of physical exercise on cerebellum are mediated through estrogen, as the present study demonstrated that cerebellar estrogen levels, but not serum estrogen levels, had a significantly negative correlation with motor coordination performance. Estrogen may influence cerebellum (4) by preventing neuronal deaths, inducing the formation of synapses and increasing information transmission through nerve impulses (33, 34) as well as maintaining the structure (1, 35) and function of cerebellum (34).

At the time of shortage of estrogen in the body, such as following ovariectomy or menopausal period, serum estrogen level may decline. However, estrogen level in tissues, including in the cerebellum, might be maintained through extragonadal production in order to ensure the sufficient availability of estrogen for normal functioning of steroid-dependent organs. It has been demonstrated that cerebellum is an organ that can produce steroid hormones *de novo* (36). Estrogen biosynthesis in the cerebellum might be enabled by the presence of P_{450} aromatase enzyme (29). It has been put forth that P_{450} aromatase enzyme is responsible for converting testosterone into estradiol, and androstenedione into estron (37-39). Exercise possibly leads to an increase of synthesis of this enzyme. Previous studies in our laboratory have indicated that regular exercise might give rise to an increase of CYP_{19} aromatase expressions in adrenal cortex and adipose tissues of ovariectomized rats (unpublished results). CYP_{19} aromatase is thought to be implicated in the production of P_{450} aromatase enzyme (40). The precise mechanism of how exercise increases cerebellar estrogen levels by extragonadal aromatization remains unclear, but it may involve IL-6. Physical exercise increases skeletal muscle's production of lL-6 that are subsequently released in large quantities into the circulation (41). It has also been reported that lL-6 was released in human brain following a 60 min-physical exercise (42). IL-6 in circulation triggers extragonadal steroidogenesis by increasing aromatase activity in the adrenal cortex, bone, and fat tissue (43). In addition, it is considered to be involved in the regulation of neurosteroid synthesis in the brain (44).

Estrogen normally mediates its functions via estrogen receptors (ER) (12, 34, 37). Direct genomic pathway which involves the interaction between ER and its ligand (estrogen) is the common mechanism of estrogen action on brain tissue (1, 35). In adolescent cerebellum, estrogen acts mainly on ER_β rather than ER_α (13, 45). Furthermore, ER_βs are more widely distributed in various types of cells of the cerebellar cortex (14, 46), whilst ER_αs seem to be more confined to Purkinje cells only (13). Hence, it had been anticipated that, consistent with the increase of cerebellar estrogen levels, the cerebellar ER_β levels also increased accordingly. However, this was not the case of our study. The fact that ER_β levels remained constant following regular exercise raises questions of whether estrogen might actually have exerted its effects through ER_αs instead of ER_βs. Alternatively, estrogen might have stimulated other yet unknown pathways.

Previous studies have shown that the expression of ER in neurons at the central nervous system is regulated by insulin-like growth factor-I (IGF-I) (47) and depends on estrogen concentration (47, 48). In a circumstance when estrogen in tissue is lacking, IGF-1 increases the transcriptional activity of ER_α. Otherwise, if the tissue estrogen level is sufficient, IGF-1 suppresses the transcriptional activity of ER_α through phosphatidylinositol 3-kinase (PI3K) pathway. Meanwhile, ER_β is a target gene of the transcriptional activity of ER_α (49). This implies that the expression of ER_β may actually reflect to that of ER_α. In the present study, the higher estrogen level in the exercise group as compared to control group may suppress the transcriptional activity of both ERs.

Conclusion

The present study found that regular physical exercise prevents ovariectomized-induced deterioration of motor coordination and cerebellar estrogen levels of rats. It is likely that the beneficial effects of exercise are exerted through estrogen. Nevertheless the precise mechanisms by which exercise and estrogen

alter cerebellar functioning require further investigations.

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Conflict of interests

The authors declare that there is no conflict of interest.

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