

## Tomato (*Lycopersicon commune*) Juice and Physical Exercise Increase Number of Neurons and ER $\beta$ expression in Post-Ovariectomy Rats Brain

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**Background:** Estrogen deficiency condition can degrade the quality of life, decline in cognitive function will be more severe through age. Phytoestrogen compounds can be found in pegaga leaf extract, tomatoes, and papaya is an easy and inexpensive way to increase estrogen levels in post menopause women through extra gonadal estrogen induction. Therefore, the aims of this study were to examine the effect of tomato juice, physical exercise, and combination of these treatments on promoting neurons and ER $\beta$  expression in somatosensory cortex that contribute to cognitive function of post-ovariectomy rats. **Method:** Twenty-eight female healthy Wistar rats (*Rattus norvegicus*), 8-10 weeks old, from Laboratory of Biochemistry, Faculty of Medicine Airlangga University include in this experiment. The animals were housed in the animal-care facility with ad libitum food and water. The temperature was maintained at 18°C-24°C. The treatments were done 2 weeks after ovariectomy. Tomato were made in Laboratory of Pharmacognocny and Phytochemistry, Faculty of Pharmacy, Airlangga University, from inner part of the tomato fruits (mucous like substance) with freeze dry method (-40°C). **Results:** The weight of white rat *Rattus norvegicus* post ovariectomy in this study was between 133-170 gram with a mean weight  $154.32 \pm 9.72$  gram. Hematoxylin/eosin staining showed neuronal deficit in the control rats brain. In figure 1, the tomato group showed the largest of neurons number ( $145.43 \pm 17.728$ ), followed the combination group ( $140.57 \pm 22.449$ ), the exercise group ( $136.86 \pm 23.104$ ) and the smallest number in the control group ( $96.43 \pm 28.965$ ). Four weeks after treatments the number of neurons increased significant in the tomato group ( $p=0.001$ ), exercise group ( $p=0.004$ ) and combination group ( $p=0.002$ ) from the control group. This study showed no significant different between tomato and exercise group ( $p=0.500$ ), tomato and combination group ( $p=0.701$ ) and between exercise and combination group ( $p=0.769$ ). **Conclusions:** In conclusion, our data demonstrated that post ovariectomy rats showed deficit numbers of neurons and decreased ER $\beta$  in the somatosensory cortex. Treatment with physical exercise, tomato juice, and combination of these treatments increased the number of neurons and ER $\beta$  expression in the somatosensory cortex.

**Keywords:** Neurons, ER $\beta$  expression, Post-Ovariectomy, Rats.

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### INTRODUCTION

Women living almost about half of their lives with estrogen deficiency condition. One of the symptoms that can degrade the quality of life at the age of menopause is a decline in cognitive function and this symptom will be increased with age.<sup>1,2</sup> At least 10% of the population aged 65 years or more experience

cognitive impairment and increased by about 50% at age 85 years. Epidemiological studies have shown that women have a higher incidence of dementia, particularly Alzheimer's disease than men.<sup>3</sup> The ageing-related decrease of cell plasticity that is normally maintained by the continuous addition of new neurons brought about by neurogenesis and contribute to senescence-dependent impairments of brain function. In aging, a dysregulation of the HPA system leads to elevated levels of circulating corticosteroid levels that will inhibit precursor cell proliferation in the brain. While neurotropic factors and growth factors are decline with age, transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) might increase and in old mice inhibited the proliferation of early precursor cells.<sup>2,4</sup> Many studies reported that estrogen hormone replacement therapy reduces the risk of Alzheimer's and cognitive function in postmenopausal women and elderly.<sup>5</sup> In vivo studies reported the effects of estrogen on the areas in the brain such as cerebral cortex and hippocampus.<sup>6</sup> The hippocampus is involved in learning and memory processes, that hippocampal glutamatergic neuron express ER.<sup>7</sup> The neuroprotective effect of estrogen in physiologic doses is well establish, but the molecular and cellular mechanisms remain controversial.<sup>8</sup> Most studies show that the cerebral cortex is most strongly protected by estrogen, followed by the striatum and hippocampus region.<sup>9</sup> During menopause, the decline in estrogen may increase the risk of diseases and affect quality of life. Dietary soy phytoestrogens have been shown to improve memory function in postmenopausal women.<sup>5</sup> Due to the use of estrogen replacement therapy increases the risk of malignancy in the breast and endometrial, then phytoestrogens that have estrogenic effects as an alternative to estrogen replacement therapy.<sup>10</sup>

Phytoestrogens are a component of the plant with the structure and effects similar to mammalian  $17\beta$ -estradiol.<sup>10,11</sup> Epidemiological data describing how they could be used as a way the symptoms of menopause and associated diseases, but the benefits for brain function and behavior has not been known.<sup>12</sup> Estrogenic activity of phytoestrogens depend on binding affinity to the estrogen receptor, which determined the aromatic ring and the hydroxyl group at a specific place. The effects of phytoestrogens can directly by occupying ER, but also can indirectly through sex

hormone binding globulin (SHBG). Phytoestrogen signals on brain by activation of classical estrogen receptors (ERs), there are ER $\alpha$  and ER $\beta$ .<sup>10,11,13-16</sup> Estrogen exerts chronic or genomic effects and rapid signaling or non-genomic effects via the regulation of the activation of kinase signaling pathways.<sup>7,10,11,17,18</sup> In vivo study reported that estrogen also bind G-protein-coupled transmembrane-bound receptor, leading to effects on cell proliferation and survival in the adult brain.<sup>19,20</sup> In the human brain, ER $\beta$  appears to be the predominant receptor in areas the cerebral cortex, hippocampus, anterior olfactory nucleus, cerebellum, dorsal raphe, substantia nigra, midbrain and several brain stem nuclei. In the macaque monkey brain, ER $\beta$  is widely express in the adult hippocampus, that a key region regulating cognitive and emotion function.<sup>13,20</sup>

Phytoestrogen compounds can be found in pegaga leaf extract, tomatoes and papaya. Examination radioimmunoassay solid phase in the tomato fruit mucilage obtained phytoestrogen content of  $1037.0 \pm 37.7$  pg/g.<sup>22</sup> Tomato (*Lycopersicon commune*) included in the Solanaceae family are often found in parts of Indonesia. Tomato is a fruit daily consumed by many people, in addition to cheap, easy to take it. Physical exercise of moderate intensity (60-75% maximum heart rate) is an easy and inexpensive way to increase estrogen levels in post menopause women through extra gonadal estrogen induction. The combination of physical exercise and phytoestrogens (*Marsilea crenata* Presl) reported to yield an increase in estrogen levels higher than just physical exercise or consumption of phytoestrogen.<sup>23</sup> In vivo study reported that tomato juice increase bone density of menopause rats.<sup>24</sup> Has never been reported to influence the consumption of tomatoes as phytoestrogens on cognitive function and there was still little information of the effects physical exercise and combination of physical exercise and phytoestrogen on the cognitive function. Therefore, the aims of present study were to examine the effect of tomato juice, physical exercise and combination of these treatments on promoting neurons and ER $\beta$  expression in somatosensory cortex that contribute to cognitive function of post-ovariectomy rats. Because samples were taken from the brain, therefore this study using post-ovariectomy Wistar rats (*Rattus norvegicus*).

## MATERIALS AND METHODS

### Animals, Tomato Juice, and Tissue Preparation

Twenty-eight female healthy Wistar rats (*Rattus norvegicus*), 8-10 weeks old, from Laboratory of Biochemistry, Faculty of Medicine Airlangga University include in this experiment study. The animals were housed in the animal-care facility with ad libitum food and water. The temperature was maintained at 18°C-24°C. The treatments were done 2 weeks after ovariectomy. Tomato juice was done in Laboratory of Pharmacognocny and Phytochemistry, Faculty of Pharmacy, Airlangga University, was made from inner part tomato fruits (mucous like substance) with freeze dry method (-40°C). This process was continue with sublimation in the vacuum freeze dried chamber with pressure of 0.036 psi (0.0025 bar), and the temperature was increased until reached 38°C. The tomato juice dose was calculated from conversion human dose to animal dose<sup>25</sup>, whereas the average human dose of tomato fruits 400-600 gram/day.<sup>26</sup> Subjects were randomized divided into 4 groups, there are : the control group just got aqua 2cc / day per scoop, the administration of tomato juice group ( 220mg / kg / day dissolved in aqua 2cc per scoop), the physical exercise group ( swimming without load for 30 minutes, 3 times / week), and the group of combination of tomato juice administration and physical exercise. After four weeks treatments, animals were decapitated, and brains were removed and put into 4% paraformaldehyde for histology evaluation and immunohistochemistry analysis.

### Histology and Cell Counting

In this study, we used hematoxyllin/eosin (HE) staining to examine the histology of brain with light microscopy. Brains were postfixed in 4% paraformaldehyde overnight and processed routinely for paraffin embedding. Sagittal 4-µm serial section were made and directly mounted on gelatin-coated slides. The sections were deparaffinized in xylene and rehydrated, then stained with hematoxyllin/eosin (HE). Neuronal counting from the somatosensory cortex (postcentral gyrus) were determined histology by light microscopy, magnifying 1000 times.

### Immunohistochemistry

Paraffin sections were deparaffinized in xylene, and rehydrated. Sagittal sections (4-µm-thick) of the somatosensory cortex of postcentral gyrus were rinsed with PBS for 3 minutes, then incubated in citrate buffer, rinsed again with phosphate buffer saline (PBS) 2 times for 3 minutes. Process follow by section were incubated with H<sub>2</sub>O<sub>2</sub> 3% for 10 minutes, follow by washed in PBS 2 times for 3 minutes. Sections were then incubated in monoclonal rat anti-ERβ for 1 hours at room temperature, rinsed with PBS 2 times for 3 minutes, and then incubated with secondary Ab (biotinylated) for 30 minutes, follow by washed with PBS 2 times for 3 minutes. Sections were incubated with streptavidin-HRP for 30 minutes, rinsed with PBS for 3 minutes, and then incubated with 3,3'-diaminobenzidine chromogen for staining for 3-5 minutes. After this step, rinsed with PBS for 3 minutes, and with aquadestilata 2 times for 3 minutes' follow by incubated with Meyer's Hematoxylin for 10 minutes. Sections were then mounted on slides and cover-slipped with *Permount*. Calculation of ERβ expression was observed as a brown color in the nuclei of neurons, each slide on a microscope field of view with a magnification of 1000 times.

### Statistical Analysis

All data were presented as mean ± SD. Statistical significant differences were determined using one-way ANOVA and post hoc test to determine differences between groups. Correlation was determined with Pearson test. All statistical test carried out using SPSS 23 and statistical significance was set at p<0.05 for all analysis.

## RESULTS

### Histological Changes in The Post Ovariectomy Rats Brain

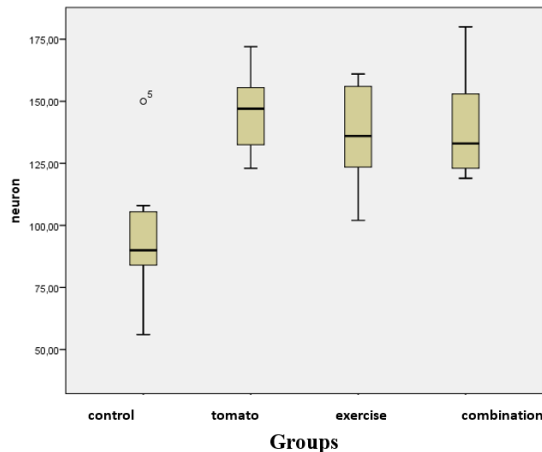
The weight of white rat *Rattus norvegicus* post ovariectomy in this study between 133-170 gram with a mean weight 154.32 ± 9.72 gram. Hematoxylin/eosin staining showed neuronal deficit in the control rats brain. In figure 1, the tomato group showed the largest of neurons number (145.43 ± 17.728), followed the combination group (140.57 ± 22.449), the exercise group (136.86 ± 23.104) and the smallest number in the control group (96.43± 28.965).

Four weeks after treatments the number of neurons increased significant in the tomato



group ( $p=0.001$ ), exercise group ( $p=0.004$ ) and combination group ( $p=0.002$ ) from the control group.

This study showed no significant different between tomato and exercise group ( $p=0.500$ ), tomato and combination group ( $p=0.701$ ) and between exercise and combination group ( $p=0.769$ )



**Figure 1.**

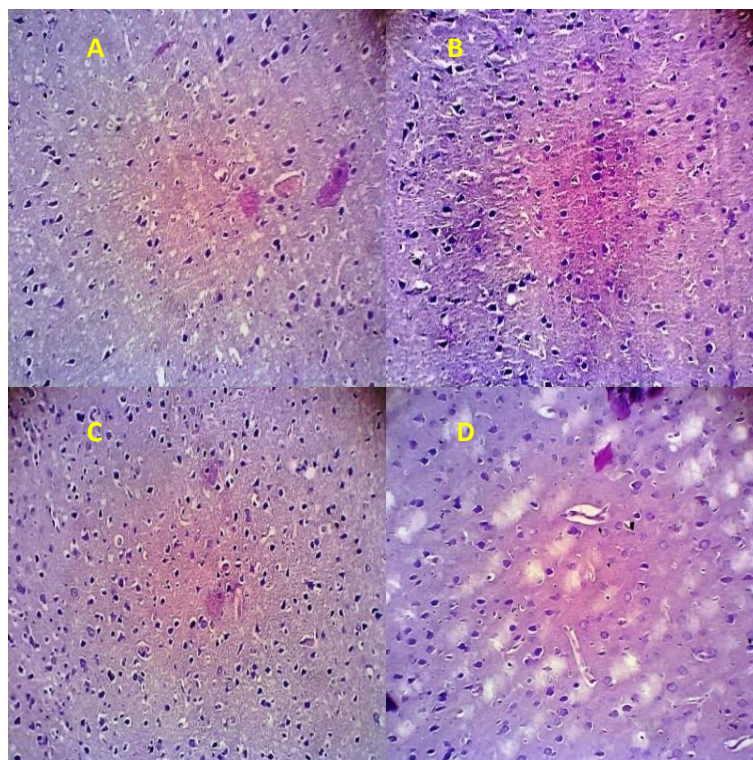
Sagittal section ( $4\mu\text{m}$ ) of the somatosensory cortex show histology comparison of post ovariectomy rats brain in the control group and

the treatment groups. Note the increased of mean number of neurons in the treatment groups. The tomato group, exercise group and combination group are not significant between groups.

### Immunoreactivity in The Post Ovariectomy Rats

The combination group showed the largest of ER $\beta$  expression ( $18.71 \pm 1.380$ ), followed by the tomato juice group ( $12.86 \pm 2.193$ ), the physical exercise group ( $8.14 \pm 1.345$ ) and the smallest expression in the control group ( $5.29 \pm 1.113$ ) (Figure 3).

In this study we obtained significant increased ER $\beta$  expression in tomato group ( $p=0.000$ ), exercise group ( $p=0.011$ ) and combination group ( $p=0.000$ ) after 4 weeks' treatments. LSD Post Hoc test showed a significant difference between tomato and exercise group ( $p=0.000$ ), tomato and combination group ( $p=0.000$ ), exercise and combination group ( $p=0.000$ ). Pearson test showed correlation between the number of neurons and ER $\beta$  expression ( $r=0.514$ ;  $p=0.005$ ).

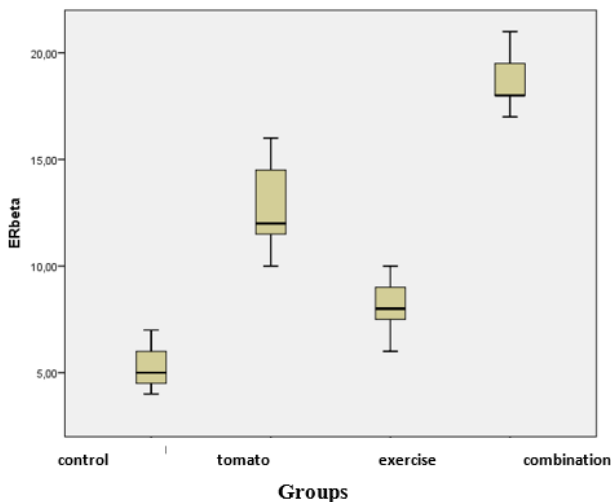


**Figure 2.**

Sagittal section ( $4\mu\text{m}$ ) of the somatosensory cortex post ovariectomy rats. The sections dyed with a hematoxylin/eosin, magnifying 400 times. Histology comparison showed neuronal deficit in the control post ovariectomy rats brain (A), compared with the treatment groups (B, C, D).

## DISCUSSION

In this study, we found significant neurons deficit and decreased ER $\beta$  expression on somatosensory cortex in post ovariectomy rats. Study on ER $\beta$  knock out mice (BERKO) showed morphological abnormalities in the brain, neuronal hypo cellularity with severe neuronal deficits in somatosensory cortex area.<sup>27</sup> In vivo studies reported that loss of estrogen caused may lead loss of synaptic connections in hippocampus or decline in basal forebrain cholinergic function or choline acetyl transferase activity.<sup>17</sup>

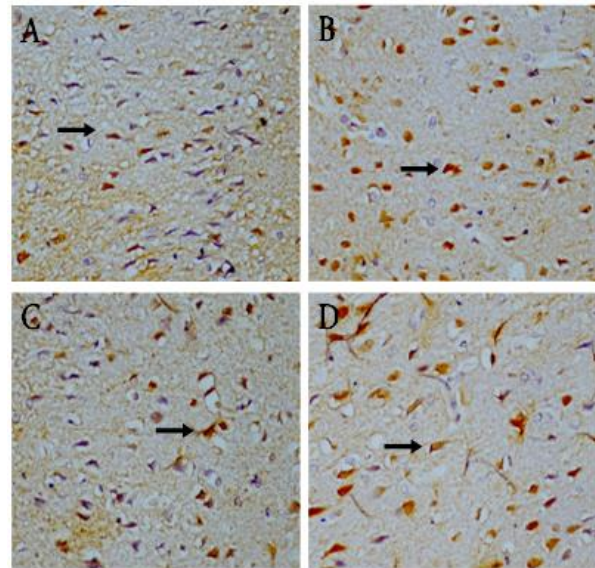


**Figure 3.**

Mean of ER $\beta$  expression in the sagittal section (4 $\mu$ m) of the somatosensory cortex show immunoreactivity comparison. Note the increased number of ER $\beta$  expression in the treatment groups. The combination group show the highest ER $\beta$  expression, followed by the tomato group and the exercise group.

Other studies reported that in very old rats and post ovariectomy rats demonstrated decreased levels of ER $\beta$  expression on the brain.<sup>15,16,28</sup> Study in ER $\beta$  KO revealed an abnormal neuronal migration and increase of apoptotic neuronal death. There was a severe neuronal deficit in the somatosensory cortex.<sup>29</sup> This suggest that ER $\beta$  is the functional ER within the region of the cortex and have an important role in the maintenance of number of neurons in rat's brain. In ovariectomized rats, dendritic spine density in ventromedial hypothalamic nucleus (VMN), CA1 region on hippocampus, and medial prefrontal cortex is decreased as compared to gonadal intact rats.<sup>30</sup> The nervous system is capable of plasticity and

that the dendritic spine is the major site of this activity.



**Figure 4.**

ER $\beta$  expression in sagittal sections (4 $\mu$ m-thick) of the somatosensory cortex sections of post ovariectomy rats. Estrogen receptor  $\beta$  was detected by immunohistochemistry, using the monoclonal ER $\beta$  antibody. The expression signal is indicated by the brown reaction of diaminobenzidine chromogen in the neurons ( $\rightarrow$ ). Note the increased of ER $\beta$  expression on the treatment groups (B, C, D) compared with the control group (A).

Statistical analysis showed that there was significantly increase the number of neurons after treatment in all groups ( $p < 0.05$ ), but the effect of tomato juice administration, physical exercise (swimming without resistance) and combination of these treatments showed the same effect ( $p > 0.05$ ) There was significant increase of ER $\beta$  expression after treatment in all groups ( $p < 0.05$ ) and each of the treatment was have the different effects ( $p < 0.05$ ).

The effects of tomato juice (phytoestrogen) on neurons and ER $\beta$  expression of somatosensory cortex in our study were consistent with previous studies. In vitro studies reported that when neuron cultures from the cortex and hippocampus were exposed to neurotoxic substances, phytoestrogen demonstrated neuroprotective antioxidant effects. For ovariectomized rats, phytoestrogens have associated with increased expression of BDNF that involved in neurogenesis and preservation of neurons.<sup>16</sup> In

vitro study with cultured of H19-7/IGF-IR neural cell line reported that genistein, daidze, and  $17\beta$ -estradiol elevated the expression of BDNF mRNA and the effects BDNF-Trk pathway have the important role in the regulation of neuronal cells proliferation.<sup>5</sup> Endogenous BDNF and Tsk signaling pathways mediate cortical progenitors' survival and neurogenesis. Brain-derived neurotrophic factor (BDNF) is a polypeptide, a neurotrophins produced by the neurons is crucial in neuronal development, survival and plasticity, an important role in memory formation. Phytoestrogen increased choline acetyltransferase and nerve growth factor messenger RNA in the frontal cortex and hippocampus in the female rats.<sup>10</sup> Neuron signals are known to regulate the effects of estradiol on astrocytes. Astrocytes also express ER $\beta$ , and these cells are involved in the neuroplastic and neuroprotective actions of the hormone.<sup>6,31,32</sup>

The physiological functions of astrocytes include control of hormonal release by neuron, neuroprotection and modulation of neuronal regeneration.<sup>9,27</sup> Astrocyte secrete neurotrophic factors BDNF that can induce cell proliferation.<sup>19</sup> In vivo studies reported that treatment of ovariectomized rats with estradiol  $17\beta$  induces certain hippocampal neurons to form new synaptic connections with other nerve cells.<sup>6</sup> The intracellular signaling pathways leading to neuroprotection involve PI3K, AKT and mitogen-activated protein kinase (MAPK).<sup>7,8,18,33</sup>

Estrogen is a powerful inducer of MAPK. In adult rats, estrogen increases serotonin receptor mRNA and IGF-1 mRNA expression in the brain to influence brain cells proliferation through stimulate precursor cell proliferation.<sup>2,19</sup> Estradiol as a negative regulators of cell death, has been shown to promote the expression of Bcl-2 in NT2 neurons and Bcl-XL in PC12 cells and cultured hippocampal neurons.<sup>33</sup> Many in vitro studies in several neuronal-culture model system reported that estradiol ( $E_2$ ) protects against toxicities caused by serum-deprivation,  $\beta$ -amyloid, excitotoxins and oxidative stress. In the animal models of cerebral ischemia, estradiol increased ER $\beta$  expression and has been shown to attenuate neuronal death through inhibition of nuclear factor- $\kappa$ B (NF- $\kappa$ B).<sup>8,27</sup>

In this study, physical exercise increased the number of neurons and ER $\beta$  expression.

Previous studies in animal models reported that physical exercise has an acute up-regulation effect on precursor cell proliferation and neurogenesis. It has been suggested mediated by vascular endothelial growth factor (VEGF) or insulin growth factor 1 (IGF-1). In vivo studies reported that IGF-1, FGF mRNA and BDNF mRNA were elevated in rodents by exercise.<sup>34</sup> Physical exercise induced hippocampal neurogenesis in adult and aged mice and running increases cell proliferation and neurogenesis in adult mouse dentate gyrus.<sup>2,35</sup> Individuals with traumatic brain injury (TBI), exercise-induced improvements in cognitive function after participation in vigorous aerobic exercise training.<sup>36</sup> Brain-derived neurotrophic factor (BDNF) activity may mediate these effects. This neurotrophic factor is a protein found in high concentration primarily in the hippocampus, cerebral cortex, hypothalamus and cerebellum and have a role in neurogenesis, dendritic growth, and long-term potentiation of neurons.<sup>37</sup>

The combination group showed the highest ER $\beta$  expression and increased the number of neurons. This suggest that phytoestrogen and IGF-1 have synergistic effects on ER $\beta$  expression in the promotion of neuronal survival and neuroprotection.<sup>18</sup> While the effects of each treatment on ER $\beta$  was significantly different, all of each treatment have been the same effects on number of neurons. There was correlation between the number of neurons and ER $\beta$  expression ( $p < 0.05$ ). This suggest the importance of the role of ER $\beta$  for the development and plasticity of neurons. Adult neurogenesis requires a specific molecular and cellular microenvironment such as neurogenic niche. Within this niche, gap junctions, paracrine effects of neurotransmitters, neurotrophic factors and growth factors control sequential steps in neurogenesis.<sup>2</sup> Each treatment in this study (tomato juice, exercise and combination) have the difference complex mechanism to influence this neurogenesis process that will need the future research. The neuroprotective effects of phytoestrogen and physical exercise might depend on its interaction with intracellular receptors and crosstalk with other intracellular signaling pathways.

## CONCLUSION

In conclusion, our data demonstrated that post ovariectomy rats showed deficit numbers



of neurons and decreased ER $\beta$  in the somatosensory cortex. Treatment with physical exercise, tomato juice and combination of these treatments increased the number of neurons and ER $\beta$  expression in the somatosensory cortex. There was correlation between the number of neuron cells and ER $\beta$  expression. This study providing information regarding the potential implication of phytoestrogen and physical exercise for the prevention and treatment against neurodegenerative diseases in estrogen deficiency condition. Further studies are needed to elucidate the mechanism of neuronal loss in post menopause and age-related neurodegeneration, the neuroprotective effects of phytoestrogen and physical exercise.

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