



Effect of Combined Electroacupuncture and Selegiline Treatment in Alzheimer's Disease: An Animal Model

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The complexity of pathological mechanisms in Alzheimer's disease (AD) poses significant challenges to the development of corresponding drugs. Symptom-specific pharmacological interventions and alternative treatments provide promising treatment possibilities. Therefore, we considered a combination of selegiline (SEL) and electroacupuncture (EA). We used an animal model with AD to investigate the effect of a combination of these treatments on cognitive function. 5XFAD mice received a week of SEL treatment and 2 weeks of EA. Novel object recognition and Y-maze tests were subsequently performed to assess their cognitive functions. To determine the molecular action of the combination treatment, Western blots, A β_{1-42} enzyme-linked immunosorbent assays (ELISA), and micro-positron-emission tomography were also performed to assess pathological markers and processes. The results were assessed based on the difference between untreated transgenic, SEL-treated, and SEL- and EA-treated groups of mice. Mice in the combined treatment group demonstrated significantly better cognitive functions, and lesser neuroinflammation than the comparative groups. In addition, mice treated with a combination of SEL and EA did not demonstrate a direct modulation of insoluble A β but demonstrated greater glucose metabolism. Our findings demonstrated that SEL combined with EA treatment was associated with better cognitive functioning due to inhibition of neuroinflammation and increased glucose metabolism relative to the comparative groups in a mouse model with AD.

Keywords: Alzheimer's disease, cognitive dysfunction, electroacupuncture, selegiline, 5XFAD

INTRODUCTION

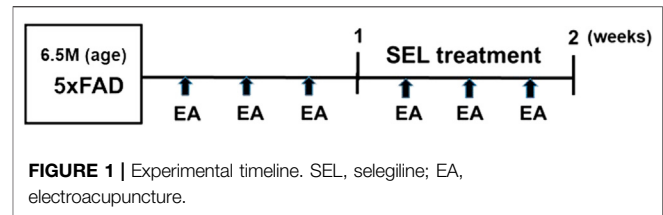
Alzheimer's disease (AD), which is characterized by age-related neurodegenerative processes that lead to loss of cognitive and memory functions, is associated with neurofibrillary tangles formed by hyperphosphorylated tau and β -amyloid (A β) deposition, and neuroinflammation of the cerebral cortex and hippocampus (Choi et al., 2018; Oyama et al., 1993). The neuroinflammation and amyloid plaques observed in AD are found to be associated with activated astrocytes and microglial cells. The latter is associated with proinflammatory factors, and tumor necrosis factor-alpha or interleukin 6 cytokines in serum and brain tissue (Fillit et al., 1991; Strauss et al., 1992). Activated microglia are also found to co-localize with A β plaques in the brains of patients with AD (Sastre et al., 2006; Calsolaro and Edison, 2016), and their inflammatory functions are found to be triggered by A β

precursor protein (β -APP) (Barger and Harmon, 1997). In addition, autophagic dysfunction causes memory impairment due to $A\beta$ accumulation in 5XFAD mice (Álvarez-Arellano et al., 2018). The same study demonstrated inflammasome activation caused by $A\beta$ plaque accumulation due to autophagy impairment and microglial phagocytic activity reduction. There has also been some evidence regarding the involvement of APOE protein in neuroinflammation and glucose metabolism during AD pathogenesis (Jagust and Landau, 2012; Liu et al., 2013).

Despite the identification of these major pathological markers, no approved drugs targeting $A\beta$ or hyperphosphorylated tau have been established, due to the complexity of their mechanisms of action. In contrast, symptom-specific pharmacological treatments involving dopaminergic drugs have been better researched and used AD treatment. This is premised on the fact that patients with AD demonstrate dysregulations in the dopaminergic system, including the reduction of dopamine and its receptors (Gibb et al., 1989; Storga et al., 1996; Burns et al., 2005), and the progressive dopaminergic neuron loss contributes to memory impairments and synaptic plasticity (Nobili et al., 2017). To this effect, Selegiline (SEL; L-deprenyl), a monoamine oxidase (MAO) inhibitor used for Parkinson's disease, has been trialed for the treatment of memory dysfunction in AD. While it has been found to improve synaptic plasticity and learning and memory by inhibiting GABA production from reactive astrocytes in AD mice (Jo et al., 2014), there is no evidence supporting its long-term efficacy for cognitive improvement (Piccinin et al., 1990; Mangoni et al., 1991; Sano et al., 1997).

In addition, research has demonstrated that non-drug therapy is helpful in maintaining and even improving cognition among patients with AD (DeVreese et al., 1996; Kukull and Ganguli, 2000; Dysken et al., 2014). In this regard, complementary and alternative medicine therapies such as electroacupuncture (EA) have provided a promising avenue for the treatment of neuronal diseases (No authors listed 1998; Li et al., 2014), with improvements in cognitive function noted among patients with AD (Jia et al., 2017; Shao et al., 2019) and reductions in neuroinflammation and oxidative processes observed in animal studies (Yang et al., 2016; Cai et al., 2019). Specifically, cognitive improvements following acupuncture were found to be associated with increased synaptic transmission, activation of $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7nAChR$) and the cholinergic anti-inflammatory pathway, and reduction of oxidative damage in the hippocampus, in AD models (Zhang et al., 2010; Liu et al., 2011; Yang et al., 2011; Han et al., 2018). In addition, memory improvements have been shown to correspond with decreased $A\beta$ plaques in the hippocampus of human amyloid precursor protein and presenilin-1 positive ($APP^+/PS1^+$) double transgenic mice that demonstrated memory improvements following EA (Yang et al., 2019).

Despite these advances, the combined effects of EA and psychopharmacology have not been studied. Therefore, we examined whether SEL combined with EA treatment improved cognitive function, in a 5XFAD animal model study. We also



evaluated if the combined treatment resulted in lesser neuroinflammatory indicators, increased anti-oxidative effects, and lesser incidence of autophagic markers compared to comparison groups.

MATERIALS AND METHODS

Procedure

The entire study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Oriental Medicine, and KIOM animal care guidelines were followed. This study involved 29 of female 5XFAD mice that were randomly allocated to three groups, namely 5XFAD transgenic mice (Tg), 5XFAD transgenic mice treated with SEL (Tg + SEL), and 5XFAD transgenic mice treated with a combination of SEL and EA (Tg + SEL + EA). The treatment period commenced when the mice were 6.5 months old. The experimental timeline is shown in **Figure 1**. The mice in all groups underwent the novel object recognition (NOR) and Y-maze tests following treatment, to evaluate their effect on cognitive function. The NOR probe trial and the Y-maze test were conducted the next day after the last administration of the EA and SEL treatments, based on the protocol followed in a previous study (Yang et al., 2016). All instruments used during these tests were cleaned with 70% ethanol between trials to reduce artifacts in the experiment after each test. Following cognitive testing, three to four prefrontal cortices and hippocampi were each randomly selected from each group to evaluate the treatment effects on neuroinflammation. Thus, the expression levels of microglia, astrocyte, and neuroinflammatory protein COX2 were examined in these brain regions using Western blots. Next, we investigated the anti-oxidative effect of SEL combined with EA treatment. Finally, microPET imaging was used to investigate glucose metabolic activity by SEL combined with EA treatment across groups.

Animals

5XFAD mice overexpressing APP transgenes with Swedish (K670N, M671L), Florida (I716V), and London (V717I) mutations, and PS1 mutations M146L and L286V were obtained from the Jackson Laboratory (Bar Harbor, ME, United States) maintained in the KIOM. 5XFAD males and B6SJ females were mated and transgenic offspring were selected by genotyping with polymerase chain reaction analysis (Oakley et al., 2006; Jeon et al., 2019). All animals were maintained in a specific pathogen-free environment with a temperature of $21 \pm 3^{\circ}\text{C}$ and humidity of $50 \pm 10\%$ with a

12-h light/dark cycle (light on 07:00–19:00 h). Access to water and food was ad libitum.

Selegiline and Electroacupuncture Treatment

The treatment protocol included 10 mg/kg SEL that was administered orally daily for a week and EA stimulation (1 mA, 2 Hz) at the Taegye (KI3) acupoint provided for 15 min, 3 times a week for 2 weeks (Figure 1). The KI3 acupoint is located on the medial aspect of the foot, between the medial malleolus and the Achilles tendon (Lu et al., 2017). Acupuncture needles (diameter 0.18 mm, length 8 mm) were purchased from Dongbang Medical (Boryeong, Korea). The EA treatment was performed according to the procedure described by Cai et al. (2019).

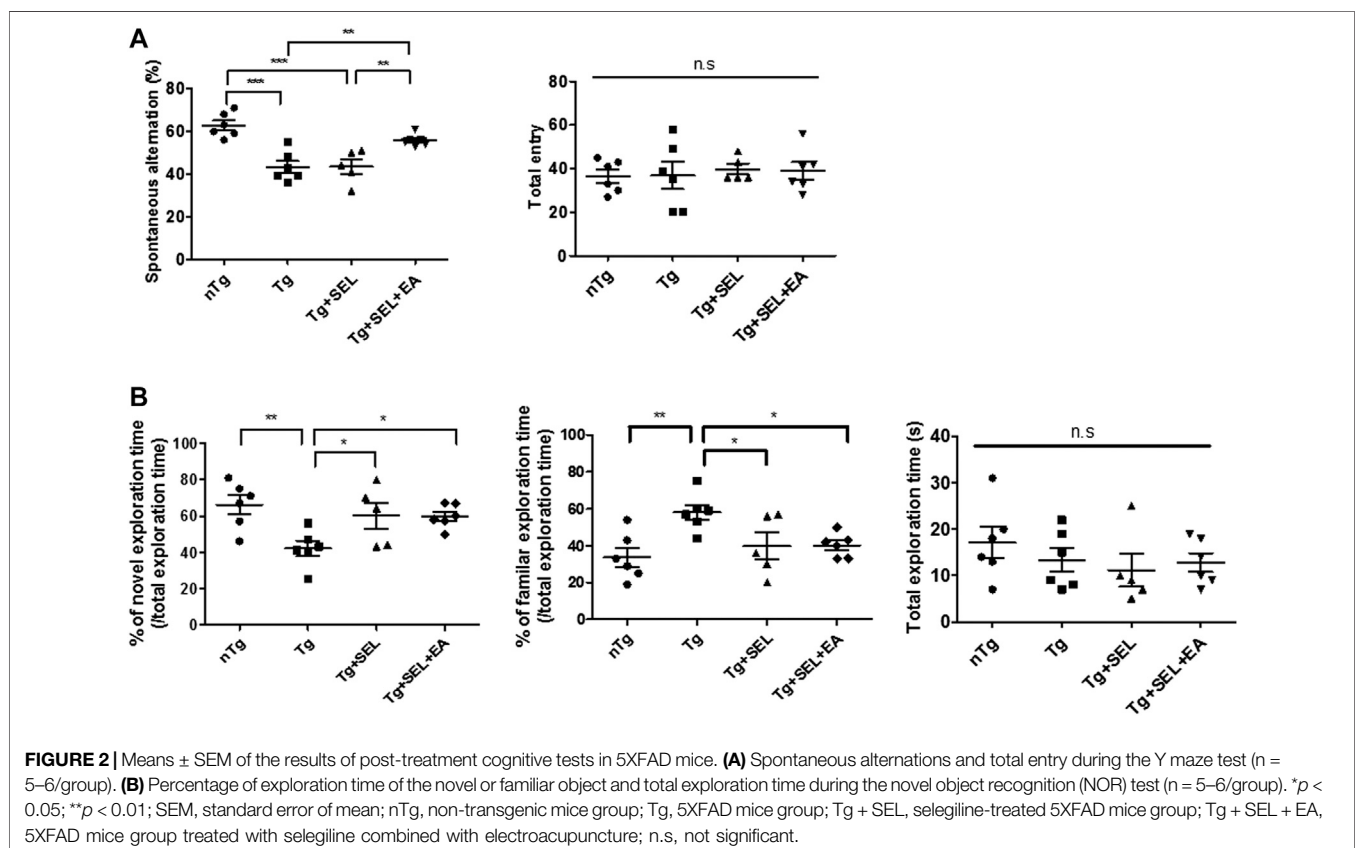
Novel Object Recognition Test

The NOR test was performed as previously described (Yang et al., 2016). NOR test was performed over 3 days, with the habituation stage taking place in the first day. This was followed by the training and probe stages, which were performed sequentially for 1 day each. Habituation of the mice to the arena took place for 10 min/day in a black polyvinyl plastic test box (40 × 40 × 40 cm) without any objects. During the training stage, the mice were put in the same box and exposed to two identical objects located at the edges of the box diagonally, and the mice were permitted to freely

explore the objects for 5 min. All goods were cleaned using a paper towels and 70% ethanol to remove odors between each test. In the probe test 24 h after the training, the mice were put in the same apparatus with one original object and the other exchanged for a new one with a different shape but similar texture. The mice were allowed to explore for 5 min. Objects were randomized between trials. The exploration time was recorded using a video camera-based Ethovision system (Noldus, Netherlands), and the percentage of exploration time of the novel object or familiar object was calculated using the following equation: $(T_{\text{novel}})/(T_{\text{novel}} + T_{\text{familiar}}) \times 100$, $(T_{\text{familiar}})/(T_{\text{novel}} + T_{\text{familiar}}) \times 100$. T_{novel} indicates the time spent exploring the novel object, and T_{familiar} represents the time spent exploring the familiar object.”

Y-Maze Test

The Y-maze apparatus was 40 cm long, 3 cm wide, and 15-cm high, with arms positioned at 120° angles from each other. All animals were placed in the maze’s center and were allowed to explore freely. The number of arm entries and triads were recorded for 8 min, and the calculation used to determine the percentage of alternation, as shown below, was based on that reported by Cai et al. (2019). A spontaneous alternation was defined as entry into three different arms consecutively (i.e., ABC, CAB, or BCA, but not ABA, BCB, etc.). The apparatus arms were cleaned using 70% ethanol and dried after each test. The percentage of spontaneous alternation was calculated as



$[(\text{number of alternations}/\text{total arm entries} - 2)] \times 100\%$. The number of total arm entries served as an indicator of locomotor activity.

Western Blots

The selected prefrontal cortical and hippocampal samples were homogenized using a radio immunoprecipitation assay buffer (Biosesang, Gyeonggi-do, Korea) containing phosphatase and proteinase inhibitors (Thermo Fisher Scientific, Waltham, MA, United States). The protein concentration was quantified using the Bicinchoninic Acid Assay Kit (Pierce, Rockford, IL, United States) and separated by 4–12% Bis-Tris Plus gels (Thermo Fisher Scientific). The equivalent 20 μg protein was loaded on Bolt 4–12% Bis-Tris Plus gels (Thermo Fisher Scientific). The membranes were blocked with 5% skimmed milk at room temperature and incubated with primary antibodies overnight at 4°C. Primary antibodies included 1:1,000 cluster of differentiation 11B (CD11B), heme oxygenase (HO)-1, and COX2 (Abcam, Cambridge, MA, United States); B cell lymphoma-2-associated X protein (Bax), transferrin (Santa Cruz Biotechnology, Dallas, TX, United States); APP, apolipoprotein E (APOE; Merck Millipore, Burlington, MA, United States); and 1:5,000 glial fibrillary acidic protein (GFAP; Agilent Technologies, Santa Clara, CA, United States). Further, 1:1,000 tubulin (Abcam, Cambridge, MA, United States) was used as a loading control for all immunoblots. The membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, United States). The protein bands were visualized with enhanced chemiluminescence and imaged using a ChemiDoc Imaging System device (Bio-Rad, Hercules, CA, United States). Quantification of immunoblots were normalized to tubulin and calculated using ImageJ (version 1.46j; National Institutes of Health, Bethesda, MD, United States).

A β_{1-42} Enzyme-Linked Immunosorbent Assays

Insoluble A β_{1-42} was elicited from all mice from each group and measured by a beta-amyloid 1-42 quantitative ELISA kit according to the manufacturer's instructions (Anaspec Inc., Fremont, CA, United States). Prefrontal cortex homogenates were briefly diluted to a 1:10,000 ratio in a sample dilution buffer and evaluated according to the manufacturer's protocol. The amount of insoluble A β_{1-42} was calculated using A β (1-42) protein standards.

MicroPET Scanning

MicroPET imaging was performed at the Osong Medical Innovation Foundation (Osong-eup, Chung Buk, South Korea) according to the protocol used by Cai et al. (2019). An animal positron-emission tomography (PET) scanner (Nano PET/CT, Mediso, Hungary) was used for imaging of mice under inhalation anesthesia (1.5–2.0% of isoflurane in 1.0–1.5 L/min of oxygen). For the quantitative analysis, regions of interest (ROIs) were analyzed in the frontal cortex regions using PMOD 3.8 software (PMOD Technologies LLC., Zurich, Switzerland). Subsequently,

the regional uptake was analyzed as mean standardized uptake value (SUV) using the following formula for the quantitative comparison of ^{18}F -FDG uptake.

$$\text{SUV} = \frac{\text{tissue radioactivity concentration}}{\text{injected activity}/\text{body weight}}$$

Statistical Analysis

The elicited data were analyzed using one-way analyses of variance (ANOVA) followed by the Newman-Keuls post hoc test for multiple comparisons or the Kruskal-Wallis test. This was followed by the Dunn's test, performed using Prism v.5.0 (GraphPad, La Jolla, CA, United States). The statistical significance levels were set at $p < 0.05$.

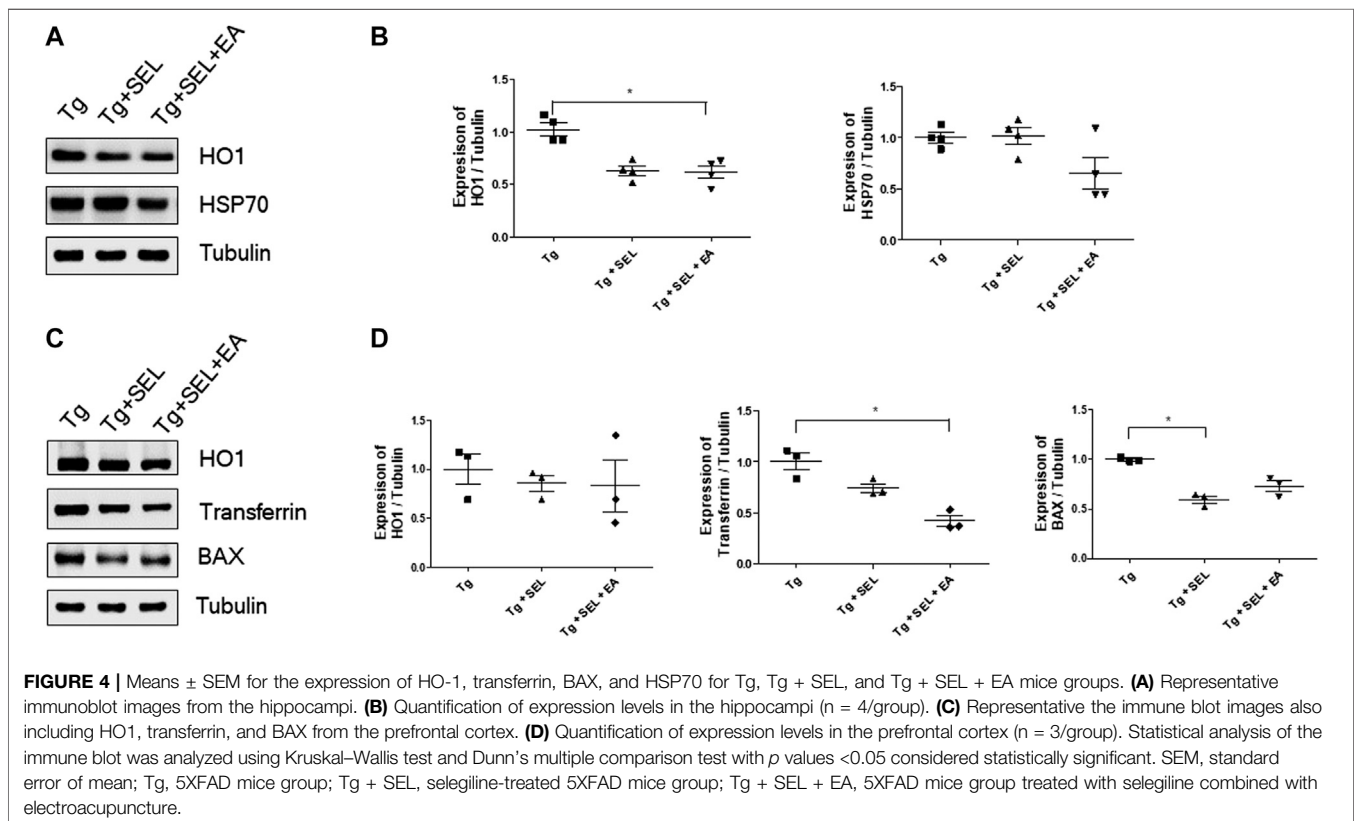
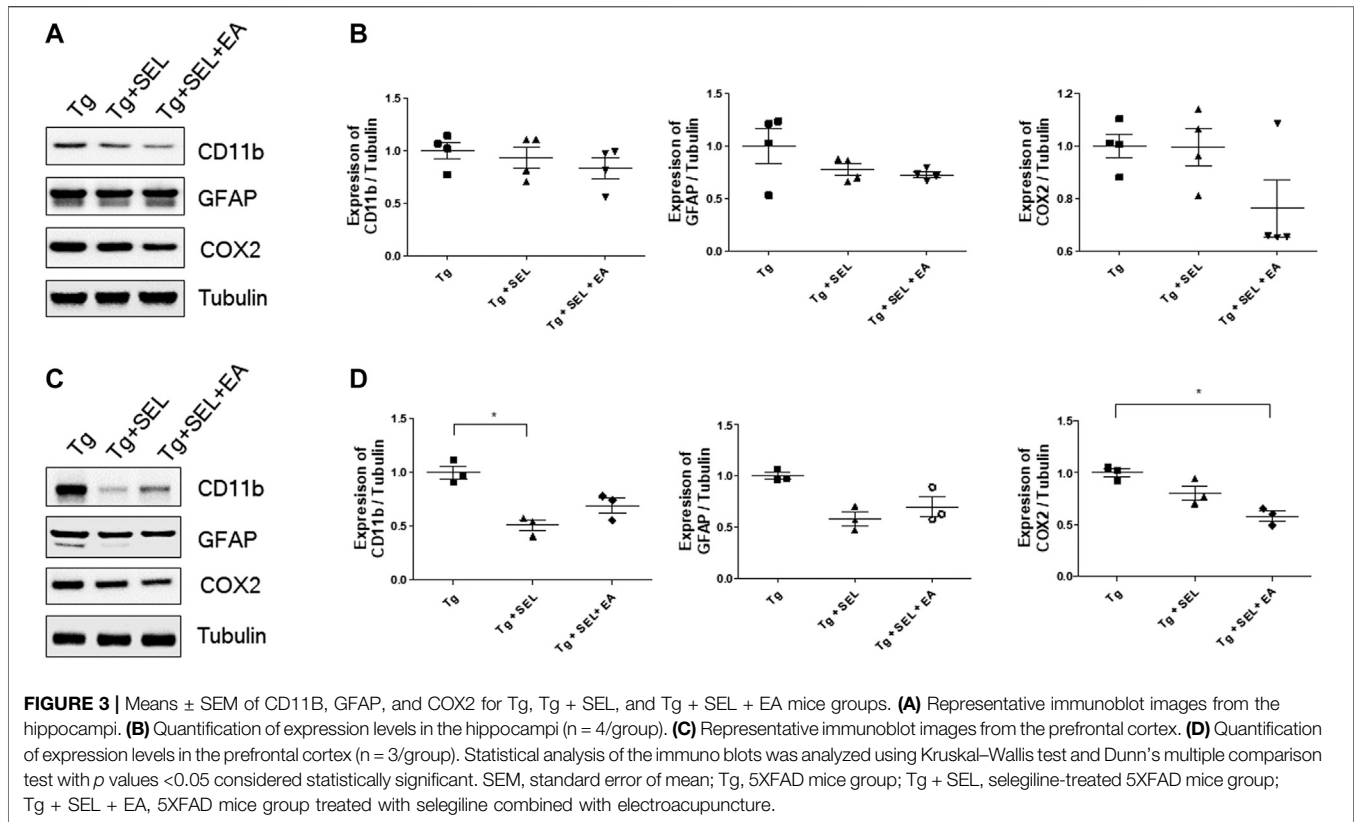
RESULTS

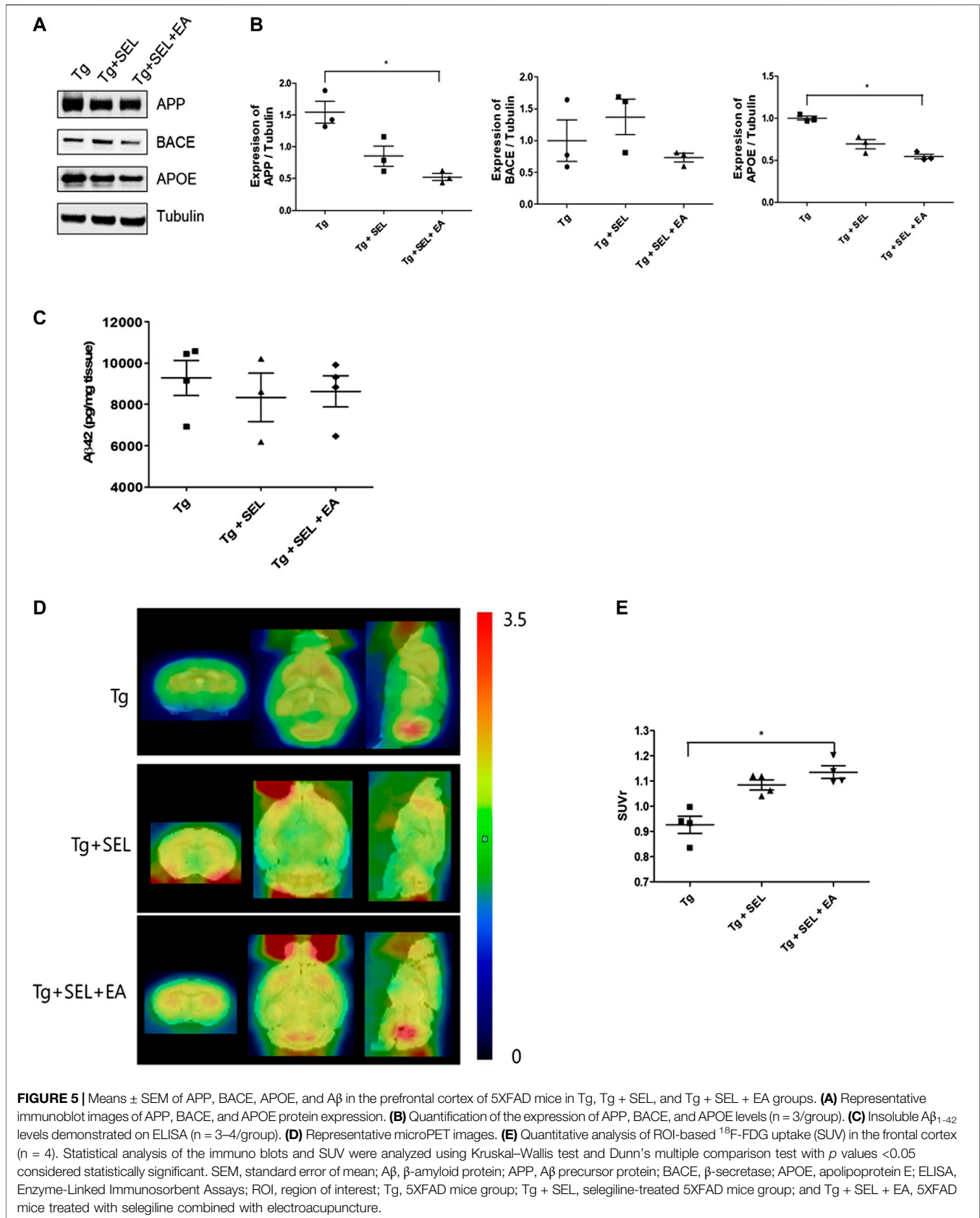
Combination Treatment With Selegiline and Electroacupuncture Increases Cognitive Function in 5XFAD Mice

SEL (10 mg/kg) was administered orally once daily for 1 week and EA treatment at KI3 was performed in 6.5-month-old 5XFAD mice three times per week for 2 weeks (**Figure 1**). To evaluate the effect of SEL combined with EA treatment on cognitive function, NOR and Y-maze tests were performed with 5XFAD mice. In the Y-maze task, the spontaneous alternation of 5XFAD mice and SEL-treated 5XFAD mice was significantly reduced by 1.3-fold compared to that of non-transgenic mice (**Figure 2A**). However, the combined treatment group demonstrated a 1.2-fold increase of spontaneous alternation compared to those in the transgenic group that did not receive any treatment and SEL-treated 5XFAD mice (Tg + SEL) (**Figure 2A**). The number of total entry was not significant between groups. In addition, exploration time of novel objects in the NOR test was 1.6-fold less in the 5XFAD mice than in the non-transgenic group (**Figure 2B**). However, exploration time of the novel object in SEL-treated 5XFAD mice (Tg + SEL) and combined treatment group (Tg + SEL + EA) were 1.4- and 1.5-fold greater than in the no treatment group (Tg) (**Figure 2B**). Consistent with this result, the exploration time of familiar objects of the 5XFAD mice was increased by 1.8-fold compared to the non-transgenic group (**Figure 2B**). The SEL-treated 5XFAD mice (Tg + SEL) and combined treatment group (Tg + SEL + EA) were significantly reduced by 1.6- and 1.4-fold compared to the transgenic group (**Figure 2B**). Total exploration times were not significant between groups.

Combination Treatment With Selegiline and Electroacupuncture Reduces Neuroinflammation in 5XFAD Mice

In AD, microglia and astrocyte activation are critical factors causing cognitive dysfunction (Fakhoury, 2018). To evaluate the effect of SEL combined with EA treatment on neuroinflammation, we examined the expression level of microglia, astrocyte, and neuroinflammatory protein, COX2, in





the hippocampus and prefrontal cortex of 5XFAD mice. As shown in **Figure 3A**, the expression levels of CD11B (for microglia) and GFAP (for astrocyte) were not significantly reduced by SEL or SEL combined with EA treatment in the hippocampus of 5XFAD mice in comparison with those in their transgenic counterparts. Though COX2 and GFAP expression levels were lower among mice treated with SEL combined with EA than transgenic mice, these differences were not significant (**Figures 3A,B**). SEL combined with EA treatment decreased the expression levels of CD11B and GFAP in the prefrontal cortices of 5XFAD mice compared to those in Tg mice (**Figures 3C,D**). Furthermore, SEL combined with EA treatment reduced the expression levels of COX2 by 1.7-fold in the prefrontal cortices of 5XFAD mice in comparison with those in Tg mice (**Figures 3C,D**).

Combination Treatment With Selegiline and Electroacupuncture Decreases the Expression Level of Oxidative Stress-Related Proteins in 5XFAD Mice

Next, we investigated the effect of SEL combined with EA treatment on anti-oxidative properties, since EA treatment has been shown to enhance anti-oxidative effects (Cai et al., 2019). The expression level of HO1 was decreased by 1.6-fold in the hippocampus of 5XFAD mice that underwent the combined treatment (**Figures 4A,B**). In addition, the expression levels of HSP70 were also reduced in the combined treatment group but it was not significant (**Figures 4A,B**).

Although the HO1 protein levels were lesser in the combined treatment group than in the transgenic group, these differences were not significant. Nevertheless, transferrin levels in the prefrontal cortex were significantly lesser 2.8-fold in the combined treatment group than in the transgenic group (**Figures 4C,D**). The expression levels of BAX were also reduced in the combined treatment group, but it was not significant (**Figures 4C,D**).

Combination Treatment with SEL and EA did not Directly Modulate A β generation but Modulated Glucose Metabolism in 5XFAD Mice

To demonstrate whether SEL combined with EA treatment regulated A β generation, we investigated the expression level of A β generation related protein and the amount of A β in 5XFAD mice. As shown in **Figure 5A,B**, we observed that the levels of APP and APOE were significantly reduced by 2.8- and 1.8-fold in SEL combined with EA treated 5XFAD mice compared to those in Tg mice. The beta-secretase (BACE) level was reduced by SEL combined with EA treatment, but this was not significant. Furthermore, the amount of insoluble A β was not changed by SEL combined with EA treatment in 5XFAD mice (**Figure 5C**). It suggests that improved cognitive function due to SEL combined with EA treatment does not directly involve A β generation. To determine the relationship between cognition and SEL combined with EA treatment, we examined the effect of combined treatment on glucose metabolism using micro PET because APOE is involved in neuroinflammation and glucose metabolism in AD pathogenesis (Jagust and Landau, 2012; Liu

et al., 2013). As shown in **Figures 5D,E**, SEL combined with EA treatment caused a 1.2-fold increase in the mean glucose metabolism of the frontal cortex involved in working memory, compared to that in Tg mice.

DISCUSSION

The number of AD patients is on the rise worldwide with aging. However, there is still no effective drug therapy for the treatment of AD. Research has shown that non-drug therapy is helpful for the maintenance or improvement of cognition in AD patients (DeVreese et al., 1996; Kukull and Ganguli, 2000; Dysken et al., 2014); however, that non-drug therapy is more effective than pharmacological therapy is controversial.

This study was designed to demonstrate the effect of a combination of SEL and EA treatment on cognition using an AD animal model. We found that the combined treatment improved cognition through the reduction of neuroinflammation and an increase in the metabolic activity of 5XFAD mice demonstrating AD biomarkers.

Remarkable factors of AD are A β plaques and phosphorylated NFT formation caused by neuroinflammation. Specifically, activated microglia induced proinflammatory cytokines, complement components, chemokines, and reactive oxygen species (ROS) lead to broken brain homeostasis (Veerhuis et al., 1999; Szczepanik et al., 2001; McGeer and McGeer, 2010). Further, research has demonstrated that A β activated microglia depend on microglial CD14 and toll-like receptors 2 and 4. Proinflammatory cytokines produced by activated microglia regulate A β clearance and A β degrading enzyme (Panchal et al., 2007; Reed-Geaghan et al., 2009). In addition, Zhang et al. and Quinn et al. suggested that A β -induced oxidative stress also lead to neuroinflammation and microglial activation in AD animal model (Quinn et al., 2005; Zhang et al., 2009). A β -induced neuroinflammation, including oxidative stress is induced by NF- κ B (Akama et al., 1998), receptor for advanced glycation endproducts (RAGE) signaling (Yan et al., 1998), NMDA-receptor, ROS (Milton et al., 2008), nitric oxide synthase (NOS), and nitric oxide (NO) (Yang et al., 2010), as well as activation of NADPH oxidase (Szaingurten-Solodkin et al., 2009).

SEL (L-deprenyl), an irreversible inhibitor of MAO-B, has been used as a drug (an antioxidant enzyme) for Parkinson's disease. In addition, SEL inhibits apoptosis induced by oxidative stress and cell death caused by the depletion of glutathione (GSH) (Qin et al., 2003). Furthermore, Knoll reported that SEL delays memory impairment in aging animals (Knoll, 1989). Jo et al. demonstrated that SEL treatment prevents impairment of synaptic plasticity and cognitive function in AD mice (Jo et al., 2014).

EA, one of acupuncture in complementary and alternative medicine (CAM), improves cognitive impairment by alleviating neuroinflammation (Feng et al., 2017) and oxidative stress (Lin et al., 2015) in the hippocampus. Our findings corroborate those of previous studies in that EA treatment was found to attenuate cognitive impairment by the reduction of neuroinflammation in 5XFAD mice (Cai et al., 2019). In addition, a recent study

reported that acupuncture improves memory better than donepezil treatment in AD patients (Huang et al., 2019). In this study, we showed that SEL plus EA treatment improved cognitive behavior and reduced neuroinflammation including oxidative stress-related proteins. This suggests that combining classical drug therapy with non-pharmacological intervention can be more effective than the exclusive use of drugs for the treatment of AD.

In terms of underlying mechanisms, our results suggest greater anti-neuroinflammation and anti-oxidative effects, and lesser autophagic dysfunction in the combined treatment group. These findings are conducive to cognitive improvements, which also align with the results of the cognitive tests performed in this study. Improved cognitive functions were also demonstrated by increased metabolic activity and lesser A β accumulation in 18 F-FDG scans, similar to previous findings (Jagust and Landau, 2012; Wang et al., 2012; Zhang et al., 2012; Cai et al., 2019; Jiang et al., 2019). Therefore, we suggest that SEL combined with EA treatment improved cognitive function by activation of frontal cortex with an increase in glucose metabolism.

Several studies demonstrated that APOE is related to AD pathology and enhanced neuroinflammation, mitochondrial dysfunction, and impaired synaptic plasticity (Chang et al., 2005; Klein et al., 2010). Interestingly, we found that while the expression of APOE was significantly lesser in the combined treatment group than that in the control group, the amount of insoluble A β and expression level of A β generation proteins such as APP and BACE did not differ significantly between the groups. Regarding Liu et al., APOE co-localizes with plaque-associated amyloid and microglia for innate immune response, and ApoE4 promotes proinflammatory responses that could exacerbate AD pathogenesis (Liu et al., 2013). In addition, APOE alters the cerebral structure and APOE ϵ 4 is involved in hippocampal CA1 apical neuropil atrophy and damaged episodic memory across the cognitive spectrum (Kerchner et al., 2014). APOE4 decreases glucose metabolism and increases A β (Jagust and Landau, 2012). EA regulates glucose metabolism in the frontal cortex of 5XFAD mice (Cai et al., 2019). Jiang et al. demonstrated an increased glucose metabolism rate in acupuncture-treated groups relative to that in the senescence-accelerated mouse prone 8 mice (Jiang et al., 2019). In addition, acupuncture increases the temporal lobe and prefrontal lobe activity associated with memory and cognitive function in functional magnetic resonance imaging (fMRI) studies (Wang et al., 2012; Zhang et al., 2012).

We found that SEL combined with EA improves cognitive function by anti-neuroinflammatory processes and increased in metabolic activity. Altogether, we suggest that the combination of

drugs with non-drug treatment could help AD symptom alleviation. Specifically, the data suggest that SEL combined with EA treatment could help improve in working and spatial memory in AD. However, this study has some limitations in using SEL combined with EA treatment for AD patients. Therefore, further research could evaluate whether EA treatment alone would be sufficiently effective in treating AD compared to the evaluated combination treatment, and whether any interactive effects exist between the drug and non-drug treatments. The synergistic molecular mechanism affecting anti-neuroinflammation and glucose metabolism between the two treatments evaluated in this study is unclear. Therefore, future studies could aim to understand the regulatory mechanism related to the signaling pathway of glucose metabolism that underlies a combination of such treatments. Additional research could also evaluate the corresponding effects on mitochondrial function. Furthermore, clinical trials could be performed to confirm the effectiveness of non-drug therapies combined with drug-based interventions for patients with AD.

DATA AVAILABILITY STATEMENT

The data supporting the conclusions of this study will be made available by the authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of KIOM.

AUTHOR CONTRIBUTIONS

MDC performed all experiments. EJY designed this study and analyzed data, and wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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