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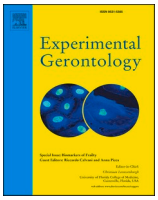

Swimming exercise and clove oil can improve memory by molecular responses modification and reduce dark cells in rat model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is marked by reduced acetylcholine receptor (AChR) density and an increase in nucleotide oligomerization domain (NOD)-like receptors NLR family, pyrin domain containing 1 (NLRP1). We examined the effect of swimming and consumption of clove supplements on memory, dark cells, and α7nAChR and NLRP1 mRNA and protein expression in the hippocampus of the rat model of AD. Forty-eight rats were divided into six groups: sham (sh), healthy-control (HC), Alzheimer (-control (AC), -training (AT), -training-supplement (ATS), and -supplement (AS)). Alzheimer was induced by injection of amyloid β_{1-42} ($A\beta_{1-42}$). Swimming exercise protocol (30 min) and gavaging clove supplement (0.1 mg/kg) were administered daily for three weeks. The results indicated that in response to AD, α7 nicotinic acetylcholine receptor (α7nAChR) mRNA and protein rate ($p = 0.001$) and memory ($p = 0.003$) were significantly decreased. In contrast, NLRP1 mRNA and protein rate ($p = 0.001$) and dark cells ($p = 0.001$) were significantly increased. This is while exercise and clove supplementation improved Alzheimer-induced changes in α7nAChR, NLRP1, memory, and dark cells ($p < 0/05$). The present study indicated that exercising and consuming clove supplementation could improve memory by increasing α7nAChR and decreasing NLRP1 and dark cells.

1. Introduction

AD is the common age-related cause of dementia (Jia et al., 2019), recognized by dark cells, cognitive dysfunction, inflammatory responses (Saresella et al., 2016), and memory loss (Jia et al., 2019). AD results in many economic, medicinal, and social problems. Despite the expanded studies in improving remedial drugs, treatment strategies remain insufficient (Esfandiarei et al., 2019). There is growing support reporting that nutrition and exercise can positively affect neurodegenerative diseases (Alkhouli et al., 2019). The Clinical researches demonstrated that physical activity could limit the beginning or delay the progression of age-related human diseases such as AD (Esfandiarei et al., 2019; Alkhouli et al., 2019). Physical activity and a healthy diet are harmless, cheap, and helpful interventions that can improve living quality in patients with AD (Esfandiarei et al., 2019). Exercise training may affect cognitive function (Bernardo et al., 2020), the expression of neurotrophic factors (Jiang et al., 2014), and the levels of inflammatory responses by affecting the cholinergic anti-inflammatory pathway (Cechella et al., 2014).

The importance of eugenol-rich diets to manage neuronal cells of anti-inflammatory responses was reported (Taher et al., 2015). Previous data have shown that eugenol, as a significant part of clove oil, is well-known to involve glutamatergic and cholinergic systems (Cortés-Rojas et al., 2014; Wie et al., 1997). Eugenol also can promote anti-inflammatory properties (Abbasi et al., 2019). Besides, clove oil has shown links with anti-oxidant properties and neuroprotective effects tightly (Baghshahi et al., 2014). It may improve cognitive performance (Han and Parker, 2017) and reduce AChE activity and oxidative damage. Additionally, clove oil can delay the progression of AD (Kumar et al., 2016).

Inflammatory mediators are central to the neuroinflammation in AD (Saresella et al., 2016). Stimulation of the α7nAChR, as ligand-gated ion channels (Ni et al., 2013; Parri et al., 2011) and a member of the cholinergic anti-inflammatory pathway (Neumann et al., 2015), can reduce the inflammatory responses (Huang et al., 2019). On the other hand, high levels of the NLRP1 inflammasome were found in the brain

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(Tan et al., 2014). NLRP1, as a component of the inflammasome, can active caspase-1 to induce the inflammatory response and cell death (Tan et al., 2014). Besides, inhibition of the NLRP1 inflammasome could decrease the innate immune response and improve cognitive deficits (Tan et al., 2014). There is growing evidence that NLRP1 inflammasome increase in AD (Tan et al., 2014), while a loss of α7nAChR has been reported in the brain of Alzheimer's patients (Ni et al., 2013). Activation of α7nAChRs decreases the inflammation complex level (such as NLRPs) and reduces the pro-inflammatory cytokines secretion (Abbasi et al., 2019; Kelley et al., 2019).

According to the mentioned studies on the effect of physical activity and also clove oil on the physiological and cognitive factors of the brain, the present study aimed to investigate if clove oil treatment and exercise, either alone or together, could be considered a treatment to improve the anti-inflammatory pathway and subsequently reduce AD progression. In this study, we examined the efficacy of clove oil and/or exercise on spatial memory, dark cells, and α7nAChR and NLRP1 mRNA and protein levels in the brain of the rat model of AD.

2. Results

2.1. Confirmation of the Alzheimer's model

10 days after the induction of AD pathology, obtaining the Alzheimer's model was confirmed by the immunofluorescence study (Fig. 1). There was a significant difference between the mean expression of (amyloid β) Aβ plaques in HC (256.7 ± 34.80) and AC (12,949 ± 441.8) groups ($p = 0.001$, $t = 28.64$).

Swimming exercise and clove oil supplementation improved spatial learning and spatial memory in rat model of AD in the Morris water maze test.

Memory and learning impairments are consequences of AD. We used the MWM to evaluate the effect of exercise and clove oil on learning and spatial memory in our rat model of AD. A two-way ANOVA was used to investigate the difference between groups and days on escape latency time. These results showed that the difference between days ($F = 28.079$, $p = 0.000$, $df = 3$), and groups ($F = 63.163$, $p = 0.000$, $df = 6$) were significant, but no interaction between groups*day ($F = 0.478$, $p = 0.957$, $df = 18$).

According to Fig. 2A, in four days of the learning test, a significant difference in spatial learning ability was observed among the studied groups ($F = 13.873$, $p = 0.001$). Also, the percentage of spatial learning ability of the AC group was significantly lower than the other groups ($p < 0.05$). There was no difference in pair-wise comparison of Sh, HC, AT, ATS, and AS groups in four days ($p \geq 0.05$).

The mean time elapsed in the quadrant containing the hidden platform was considered spatial memory (Fig. 2B). A significant difference in spatial memory ability was observed among the study groups ($F = 16.699$, $p = 0.001$). Also, the mean time elapsed in the quadrant containing the hidden platform of the AC group was significantly lower than HC ($p = 0.003$) and, in the ATS group, it was significantly higher than that in the AT group ($p = 0.022$). Although time elapsed in the quadrant containing the hidden platform of the ATS group was higher than the AS group, there was no significant difference between the ATS and AS groups ($p = 0.321$). Moreover, there was no significant difference between the HC group with Sh ($p = 1.00$), AT ($p = 0.682$), ATS ($p = 0.325$), and AS ($p = 1.00$) groups. It means that although AD reduces spatial memory, exercise, clove oil, or both can compensate for the behavioral deficit. According to the results of this study swimming and the consumption of clove oil have a desirable effect on spatial memory in the Rat model of AD.

2.2. Swimming exercise and clove oil supplementation decrease the degree of AD-induced dark cells percentage in the hippocampus

The percentage of dark cells in the CA1 region of the hippocampus in the Rat model of AD in different groups is presented in Fig. 3. Results showed there was no difference between dark cells percentage in the hippocampus in the HC and Sh groups (1.00).

AD induction increased the percentage of dark cells in the CA1 region of the hippocampus of the AC group compared to HC ($p = 0.001$). In contrast, there was no difference between percentage of dark cells in the AT group ($p = 0.491$) and the AS group ($p = 0.491$) compared to the AC group. However, swimming and the consumption of clove oil (in the ATS group) ($p = 0.039$) significantly reduced the percentage of dark cells compared to the AC group. The percentage of dark cells in AT ($p = 0.009$) remained significantly higher than HC group but not in AS ($p = 0.056$) and ATS ($p = 0.221$) groups. Also, there was no difference between the percentage of dark cells in the ATS group compared to AT ($p = 0.169$), and the AS ($p = 0.491$) groups.

2.3. Swimming and clove oil improved the expression of reduced α7nAChR mRNA and protein levels in the hippocampus of the Rat model of AD

Inflammation is involved in AD. It has been reported that α7nAChR is affected by an inflammatory response. In this study, we examined if the expression of α7nAChR mRNA and protein levels are affected by

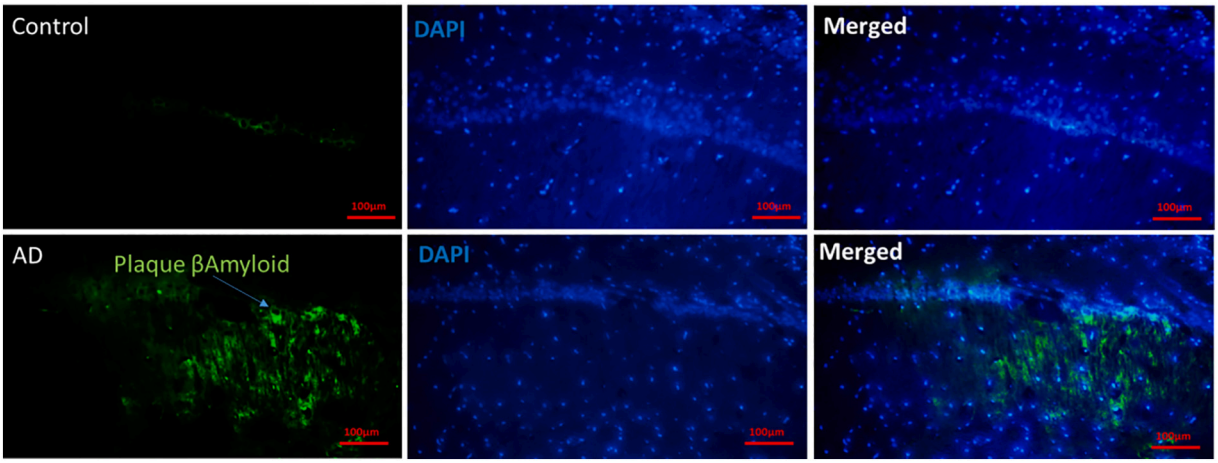


Fig. 1. Photomicrographs of immunofluorescent staining of Aβ plaques in the CA1 region of the hippocampus (control and Alzheimer's rats). Green structures indicate the accumulation of Aβ plaques. Magnification: 100×, 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

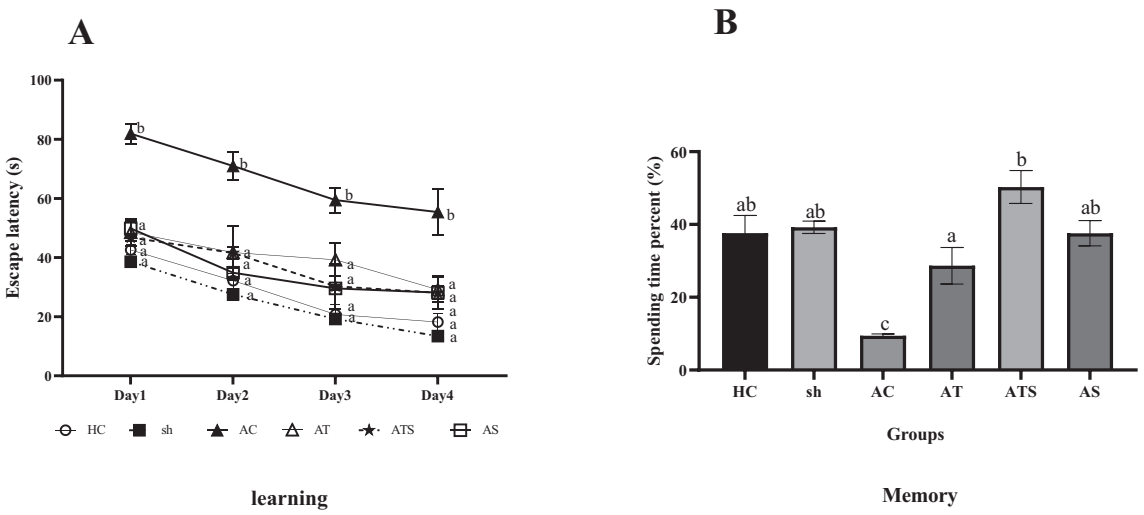


Fig. 2. Effect of swimming exercise and clove oil on spatial learning (A) and spatial memory (B) in the MWM test. The groups consisted of healthy-control (HC), Alzheimer control (AC), Alzheimer-training (AT), Alzheimer-supplement (AS), Alzheimer-training-supplement (ATS), and Sham (Sh). Data are expressed as the mean ± SD. Significant level: $p < 0.05$. Bar with different letters indicates a significant difference (ANOVA and subsequent Tukey's HSD, $p < 0.05$).

Alzheimer, exercise, and clove oil. Photomicrographs of immunofluorescence staining and the mean expression of α7nAChR mRNA and rate of protein are shown in Fig. 4. There is a significant difference in the expression of α7nAChR mRNA and its protein levels observed among the studied groups ($F = 48.766$, $p = 0.001$) and ($F = 172.414$, $p = 0.001$), respectively. There was no difference between the mean expression of α7nAChR mRNA and protein in the HC and Sh groups ($p = 0.964$, $p = 1.00$). The mean expression of α7nAChR mRNA and protein levels of the AC group was significantly lower than the HC group ($p = 0.001$). The mean expression of α7nAChR mRNA and protein levels in group AT ($p = 0.001$), ATS ($p = 0.001$), and AS ($p = 0.001$) was significantly higher than the AC group.

The level of α7nAChR protein in the hippocampus of AS and AST group rats was higher than the AT group ($p = 0.001$). But this significant increase in the protein level was not associated with a significant increase in the gene expression level because the increase in α7nAChR mRNA of AS and AST groups to the AT group was not significant ($p = 0.998$, $p = 0.937$). However, differences in the α7nAChR mRNA and protein levels between HC and the treated groups were significant ($p < 0.05$). Our results illustrate that the swimming, and clove oil improved the expression of reduced α7nAChR mRNA and protein levels in the rat model of AD.

2.4. Swimming and clove oil supplementation reduced the increased expression of NLRP1 mRNA and protein levels in the hippocampus of the Rat model of AD

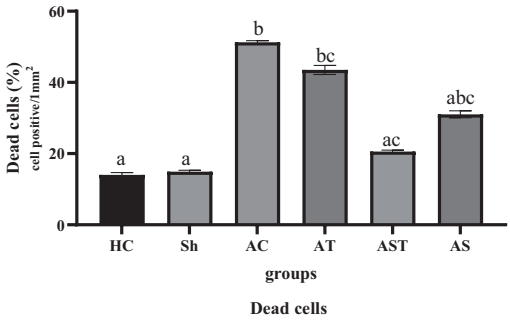
NLRP1 induces inflammatory responses and is recognized to affect AD and dark cell percentage. This study examined the effect of AD, exercise, and clove oil on the expression of NLRP1 mRNA and its protein level. Photomicrographs of immunofluorescence staining and the mean expression of NLRP1 mRNA and protein level are shown in Fig. 5. This study showed a significant difference in NLRP1 mRNA and its protein level was observed among the studied groups ($F = 52.025$, $p = 0.001$) and ($F = 188.402$, $p = 0.001$), respectively. This study showed no difference between the HC and Sh groups ($p = 1.00$). $A\beta_{1-42}$ injection caused an increase of NLRP1 mRNA and its protein level in the AC group compared to the HC group ($p = 0.001$). The mean NLRP1 mRNA and its protein level in the ATS ($p = 0.001$) and AS ($p = 0.001$) groups were lower than in the AC group; nevertheless, no difference was observed between AT and AC groups ($p = 0.191$, $p = 0.055$). This showed that clove oil supplement (with or without exercise) could balance the increased expression of NLRP1 protein level induced by $A\beta_{1-42}$ injection. A comparison between treated groups showed that treatment with clove oil and exercise (ATS group) resulted in a more considerable decrease in NLRP1 protein rate than exercise (AT; $p = 0.001$) and clove oil (AS; $p = 0.006$) alone among the Alzheimer groups. Nonetheless, no reduction was found in the NLRP1 mRNA of the ATS group compared to AT ($p = 0.108$) and AS ($p = 1.000$) groups.

3. Discussion

The present study showed that spatial learning and memory were impaired in the Rat model of AD. In treated Aβ1–42 injected rats, the protein rate and mRNA expression of α7nAChR were comparable to those in the healthy control group. α7nAChR expression has been reported to be reduced in the brains of Alzheimer's patients (Oz et al., 2013). Because of AD and at high doses of Aβ, a cross-link between Aβ and α7nAChR is established and ultimately leads to blockage and dysfunction of α7nAChR (Ni et al., 2013). While according to the study by Tan et al., α7nAChR can restrain the formation of interleukin-1β and interleukin-18 by inhibiting NLRP1 and preventing inflammation-related injuries (Tan et al., 2014). In the current study, due to the Aβ1–42 injections, the increase in mRNA expression and protein rate of NLRP1 was found in the CA1 region of the hippocampus. It seems Aβ can activate inflammation (Masters and O'Neill, 2011). Increasing Aβ activates caspase-1, leads to the secretion of pro-inflammatory cytokines, and finally, increases pyroptosis (Lamkanfi and Dixit, 2014). Therefore, the increase in NLRP1 is probably due to a rise in Aβ and a decrease in α7nAChR.

In addition, the increase in dark cells following AD has been confirmed by some researchers (Tan et al., 2014; Lamkanfi and Dixit, 2014). We have seen an increase in dark cells in the CA1 region of the hippocampus following $A\beta_{1-42}$ injection in the rat model of AD. One of the significant causes of dark cells is the activation of caspases (Song et al., 2018). The activation of caspases can be due to inflammasomes such as NLRP1, which eventually leads to an increase in dark cells (Song et al., 2018). Increased dark cells can cause memory impairment (Lee et al., 2003). Researchers have previously stated that excessive release of AChE, and afterward, the reduction in the level of acetylcholine, is one of the leading causes of impaired spatial memory (Farioli-Vecchioli and Tirone, 2015; Amirahmadi et al., 2022). The results illustrated that the reduced α7nAChR expression, increased NLRP1 expression, and activated dark cells pathway had contributed to cognitive impairment and

A



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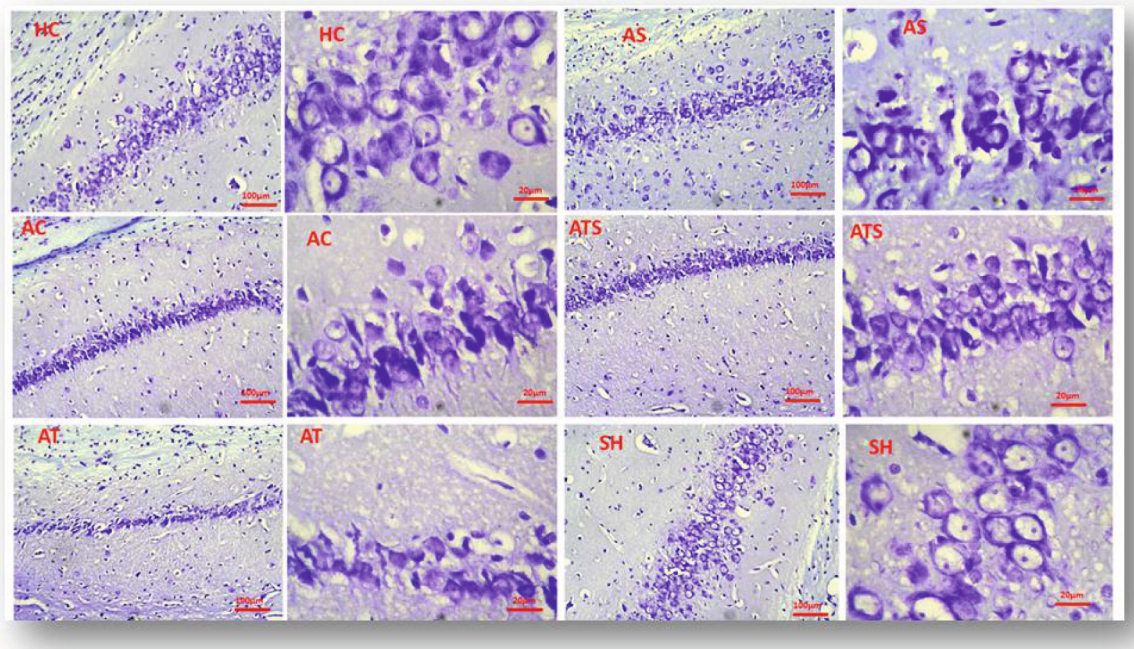


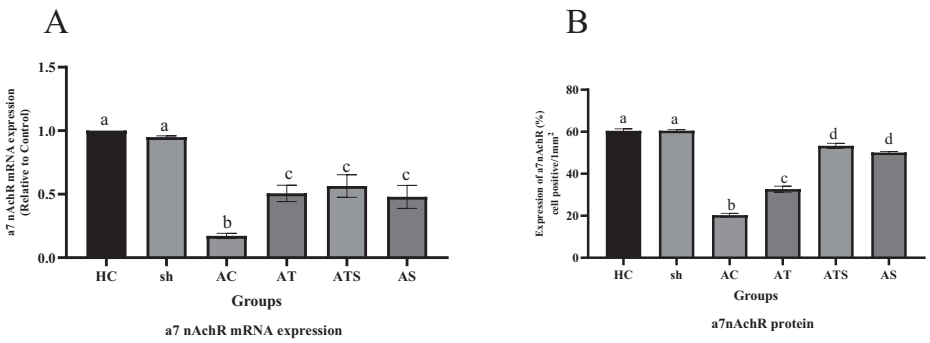
Fig. 3. Effect of swimming exercise and clove oil on the Percentage of dark cells in the CA1 region of the hippocampus in the rat model of AD in different groups. (A) The dark cells Percentage in the CA1 region of the hippocampus in other groups. Significant level: $p < 0.05$. (B) Purple plaques indicate dark cells. Bar with different letters shows a significant difference (ANOVA and subsequent Tukey's HSD, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

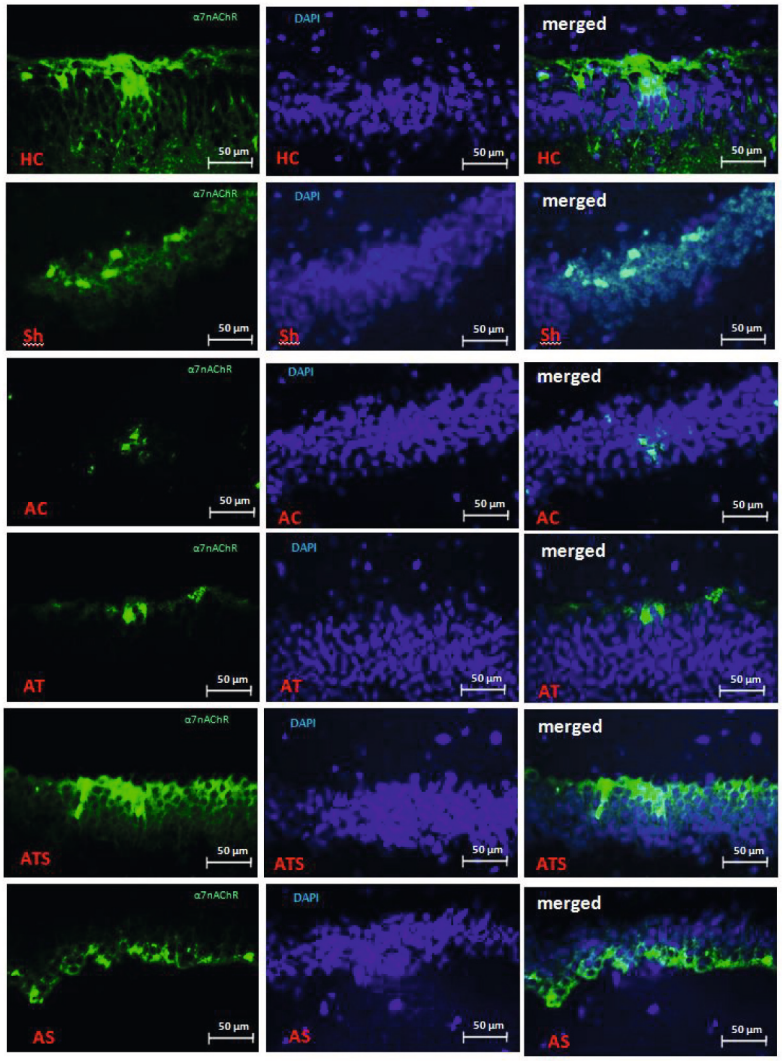
the reduction of spatial memory caused by AD.

An active lifestyle may prevent Alzheimer's (Brasure et al., 2018) and delay the onset of AD. This study discovered that exercise was a positive and influential factor in increasing the protein expression level and mRNA of α7nAChR in Alzheimer's groups. The findings also emphasized the role of exercise in reducing NLRP1 mRNA and protein levels. In the study of Khakroo Abkenar et al. (2019), high-intensity aerobic exercise increased interleukin-1β, interleukin-18, and NLRP3 and subsequent neuronal death, while low-intensity exercise has backfired. According to moderate-intensity aerobic exercise, NLRP1 changes to physical exercise are associated with exercise intensity; high-intensity exercise may increase NLRP1 levels and worsen AD symptoms. However, confirmation or rejection of this hypothesis requires further investigation. Due to the direct relationship between Aβ and NLRP1 in the hippocampus (Tan et al., 2014), the exercise by reducing Aβ levels in the rat model of AD (Alkadhi and Dao, 2018) may potentially reduce NLRP1 expression and prevent dark cells and memory impairment.

Furthermore, Farzi et al. have reported that exercise improves spatial memory by reducing AChE activity in the rat model of AD; As a result, decreased AChE activity can be considered as one of the mechanisms to enhance cognition and memory in AD (Farzi et al., 2018). Accordingly, the reduction in dark cells in the ATS group scan is attributed to the effect of exercise on increasing decreased α7nAChR levels and decreasing increased NLRP1 levels in patient groups. In addition, reducing dark cells after exercise can improve memory.

Researchers have already shown that aerobic exercise reduces NLRP and interleukin (Khakroo Abkenar et al., 2019). Eight weeks of resistance training reduces NLRP3 gene expression and serum caspase-1



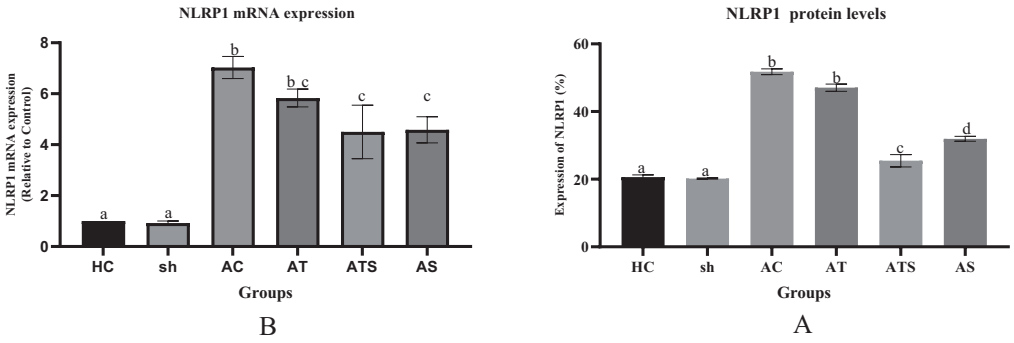


C

Fig. 4. Effect of swimming exercise and clove oil on the expression of α7nAChR mRNA (A) and protein levels (B, C) in the CA1 region of the hippocampus in the rat model of AD in different groups. Green immunostaining indicates α7nAChR expression. Significant level: $P < 0.05$. (A) Percentage of α7nAChR protein expression in other groups. (B) α7nAChR mRNA. (C) Immunofluorescence-stained images (Green dots express α7nAChR protein). Magnification: 400×. Bar with different letters indicates a significant difference (ANOVA and subsequent Tukey's HSD, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

levels (Mejías-Peña et al., 2017). On the other hand, it is known that eugenol decreases interleukin (Abbasi et al., 2019). Exercise seems to improve memory and learning by enhancing the role of eugenol in suppressing inflammation.

Clove oil has antioxidant, anti-inflammatory, and anti-viral properties (Han and Parker, 2017). In this study, clove oil consumption partially compensated for the decreased protein expression level and mRNA of α7nAChR in the CA3 region of the hippocampus of the rat model of AD. The effect of clove oil on reducing AChE was confirmed in the study by Dalai et al. (Dalai et al., 2014). They demonstrated eugenol to be helpful in cognitive diseases such as Alzheimer's by affecting AChE (Dalai et al., 2014). Since the protein expression and mRNA of α7nAChR in the rat model of AD increased after consumption of clove oil and according to previous research (Wie et al., 1997; Dalai et al., 2014),



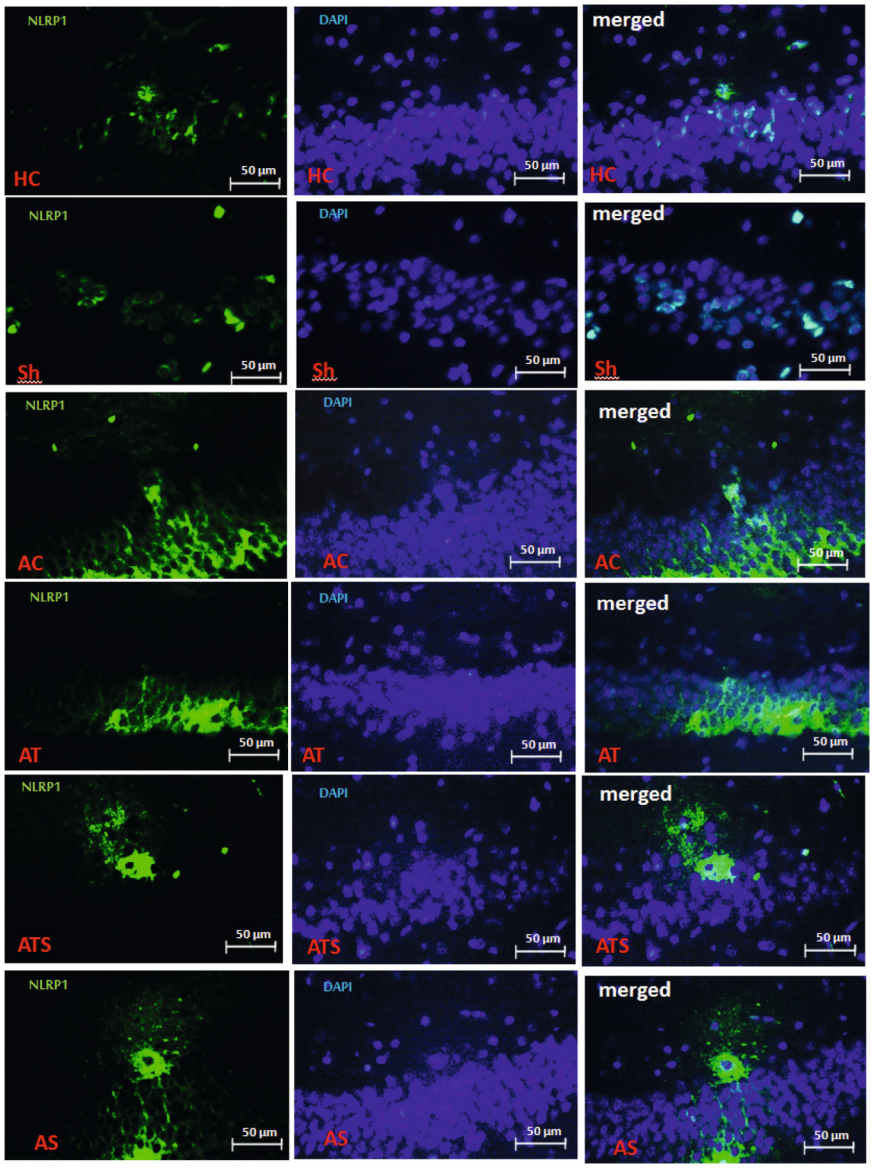


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Fig. 5. Effect of swimming exercise and clove oil on the expression of NLRP1 mRNA (A) and protein levels (B, C) in the CA1 region of the hippocampus in the rat model of AD in different groups. Green immunostaining indicates NLRP1 expression. Significant level: $p < 0.05$. (A) Percentage of NLRP1 protein expression in different groups. (B) NLRP1 mRNA. (C) Immunofluorescence-stained images (Green dots express NLRP1 protein). Magnification: 400×. Bar with different letters indicates a significant difference (ANOVA and subsequent Tukey's HSD, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clove oil may effectively improve the protein expression level and mRNA of α7nAChR by reducing AChE activity.

According to previous research, eugenol alters the nuclear factor kappa B (NF-κB) pathway, and it reduces interleukin-6 production (through its anti-inflammatory activity on (COX)-2 and 5-LOX) Also, eugenol improves learning and memory by altering neurotransmitters in the brain in Kunming mice (Wie et al., 1997). Eugenol also effectively suppresses inflammation and subsequently can reduce neuronal death (Taher et al., 2015). In the present study, eugenol may promote learning and memory and reduce neuronal death by affecting the inflammatory pathway.

Consuming clove oil also reduced the protein expression level and mRNA of NLRP1 in the CA1 region of the hippocampus of the rat model of AD. Clove oil has anti-inflammatory activities by activating microglia (Taher et al., 2015). NLRP1 is recognized as one of the most crucial components of inflammation, and clove oil appears to reduce NLRP1 mRNA and protein expression by affecting the NF-κB pathway and interleukin-6 production (Wie et al., 1997).

As the results showed, clove oil reduced the percentage of dark cells in the CA1 region of the hippocampus (although it was not significant). Previously, it has been found that clove oil effectively suppresses inflammation (Taher et al., 2015). In this study, clove oil stops dark cells by reducing inflammation. The results showed that consuming clove oil improved learning and memory in the ATS group, which improved the cognitive function of the ATS group to the level of the healthy control group. Clove oil can modify cognitive function by reducing AChE activity, reducing oxidative damage, and improving the neuroprotective effect (Song et al., 2018).

The role of clove oil consumption in reducing NLRP1 (as a component of the inflammation pathway) was confirmed. On the other hand, inflammation can reduce cognitive function by intensifying the process of dark cells. Therefore, clove oil seems to improve learning and memory by altering α7nAChR levels and subsequently affecting the inflammatory pathway.

Generally, it seems that Aβ is dose-dependent. Under normal conditions, the low-level Aβ triggers the PI3K/MAPKERK current by binding to α7nAChR and forming Aβ-α7nAChR complexes (Parri et al., 2011). Under these conditions, α7nAChR can also inhibit the formation of interleukin-1β and interleukin-18 by inhibiting NLRP1; in this way, it prevents inflammatory-related injuries (Tan et al., 2014).

In AD, an increased level of Aβ decreases α7nAChR efficiency (Ni et al., 2013); up to previous research, we know it reduces α7nAChR results in the increase of NLRP1 and ultimately leads to inflammation (Abbasi et al., 2019; Ni et al., 2013), Conversely, increasing caspase-1 causes inflammation (Khakroo Abkenar et al., 2019), enhancing dark cells percentage (Khakroo Abkenar et al., 2019).

According to previous research, clove supplementation and physical activity reduce Aβ levels (Brasure et al., 2018). The combination of training with clove oil supplementation is probably associated with further reductions in Aβ, caspase-1, interleukin-1β, and interleukin-18 levels (Khakroo Abkenar et al., 2019). In addition, it will likely decrease dark cells (Khakroo Abkenar et al., 2019)-clove supplementation and physical activity double effect reducing NLRP1 levels. Decreased NLRP1 levels are likely associated with decreased caspase-1 levels and eventually reduced dark cells.

Clove oil supplementation and physical activity can increase reduced α7nAChR levels and reduce elevated NLRP1 levels in AD by decreasing levels of caspase-1, interleukin-1β, interleukin-18 (Khakroo Abkenar et al., 2019), AChE, and Aβ (Brasure et al., 2018). Hence, clove oil can potentially improve the quality of life of Alzheimer's patients. However, confirmation of the details of this pathway requires more future extensive research.

4. Conclusion

In conclusion, the present study showed that endurance training and clove oil supplementation could enhance spatial memory by modifying α7nAChR and NLRP1 levels and dark cells. Endurance training and clove oil supplementation caused an increase in α7nAChR and a decrease in NLRP1 in the hippocampus of the Rat model of AD. Positive changes in these factors improve spatial learning and memory probably.

This study showed that the combination of endurance training and clove oil supplementation caused a significant improvement in studied factors in the Rat Model of AD because the training and supplementation group rats experienced better memory performance than the other rats. Therefore, the results of this study present evidence to investigate new therapeutic options in the rat models of AD.

5. Methods

5.1. Animals

Forty-eight male Wistar rats (6 weeks old) were purchased from the Pastor Institute (Tehran, Iran). The rats were housed (4 rats per cage) at 22 ± 2 °C, with cycles of light/dark 12:12 h, and were fed a pellet diet (Behparvar, Thran, Iran) and water ad libitum. The rats have been cared for following the International Association for the Study of Pain (IASP). The Lorestan University Animal Ethics Committee approved the study in Khorramabad, Iran (LU.ECRA.2018.15). This Committee confirms that all experiments were performed under relevant guidelines and regulations. Further, the present study complied with the Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines. After one week of adaptation to the new environment, rats were familiarized with the swimming protocol (30 s swimming/2 periods). After that, randomly, the animals were divided into six groups with eight rats in each group, as follows: healthy-control (HC), Alzheimer control (AC), Alzheimer-training (AT), Alzheimer-supplement (AS), Alzheimer-training-supplement (ATS), and Sham (Sh) (Fig. 6). Eight samples (same animals) were used for each immunofluorescence/histological and PCR analysis.

5.2. Hippocampal injections of $A_{\beta 1-42}$ to induce AD pathology

$A\beta_{1-42}$ was provided from Sigma-Aldrich, USA. Next, $A\beta_{1-42}$ aggregation was prepared by dissolving that in sterile saline. The solution was incubated to a final 1 mg/ml concentration at 37 °C for seven days. This allowed the peptide to fibrillize associated with toxicity and stored at −20 °C. The $A\beta_{1-42}$ solution containing insoluble precipitates of both fibril-like structures and different-sized oligomers facilitates marked learning deficits. After that, the rats were anesthetized with ketamine (75 mg/kg, Interchemie, Netherland) and Xylazine (10 mg/kg, Bremer/pharmaGmBH, Germany) and placed onto a stereotaxic frame (RWD68505, Brazil). The skull was positioned in the frame, and a sagittal midline incision was made along the midline. The area surrounding the bregma was cleaned and dried. After that, the incubated $A\beta_{1-42}$ solution was injected into the CA1 region with a volume of 5 μl containing 5 μg $A\beta_{1-42}$ very slowly (over 2 min) bilaterally. Stereotaxic injection coordinates were as anterior-posterior (AP), −3.3 mm; medial-lateral (ML), +−1.8 mm; ventral (V), −2.5 mm; by measuring the distances from bregma base on the stereotaxic Atlas of Paxinos and Watson (2006). To prevent $A\beta_{1-42}$ reflux out of the needle tract and maximize diffusion, the needle remained at the injection site for 1 min after injection (Limón et al., 2012). Injections of $A\beta_{1-42}$ (Limón et al., 2012) or isotonic saline solution (Panahzadeh et al., 2022) (Vehicle) for AD or Sham groups were infused with a Hamilton syringe. Following surgery, the rats were returned to their cages and frequently monitored during recovery.

5.3. Evaluation of the Alzheimer's model

In addition to 48 rats in the main groups, in the first phase, four animals were euthanized ten days after injection to perform histological

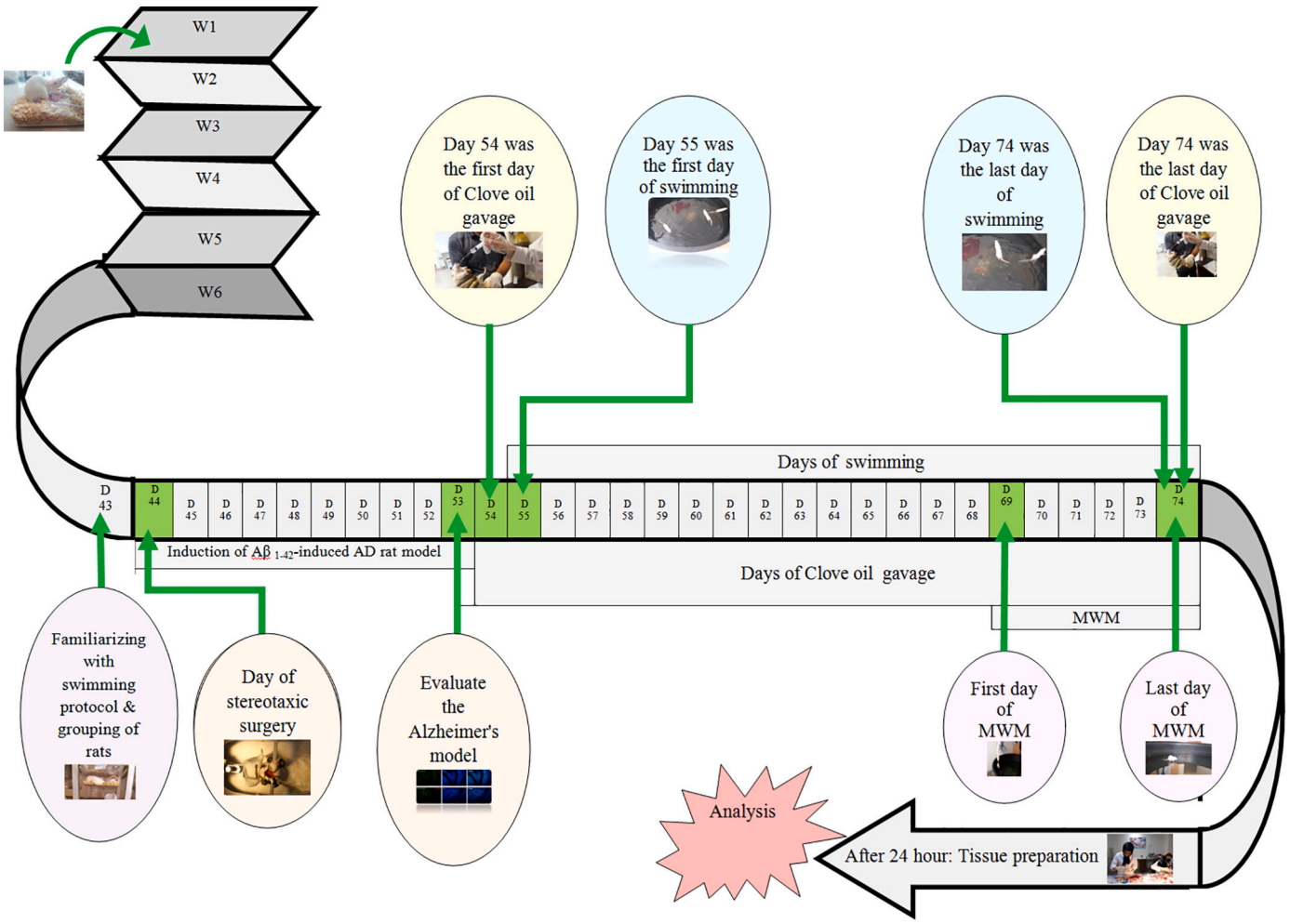

Fig. 6. Experimental design: AD was induced by injection of $A\beta_{1-42}$, and after 10 & 11 days, treatment with clove oil and swimming began for three weeks. MWM was performed on the 17th day of exercise interventions; finally, 48 h after the last intervention, animals were euthanized for more study.

analyses to evaluate the Alzheimer's model 32. The remainder rats were used for the next phase. Immunofluorescence was used for evaluating the Alzheimer's model.

5.4. Supplement treatment

Clove oil was purchased from the Shafa Kurdistan Company (Kurdistan, Iran). The GC–MS method was used to determine the quantity of the active compounds in the clove oil (Hewlett-Packard, HP-6890, USA). The column used was HP-1MS (methyl silicon-cross link). Column (60 m × 0.20 mm, the film thickness 0.25 μm). The oven temperature was as follows: Initial temperature: 160 °C, Final temperature 230 °C, at a rate of 7 °C/min. The carrier gas was Helium (99.9999 %), with a 1 ml/min flow rate. Injector temperatures were 250 °C. For Mass Spectrometry-MS, HP-59-70 (Hewlett-Packard, USA) was used. The gas chromatographic analysis of clove oil is shown in Table 1. The supplement group was treated with 0.1 ml/kg of clove oil daily for 21 days (Kumar et al., 2016).

5.5. Swimming protocol

Rats were placed in a box containing 40 cm water at 37 °C (45 cm long, 25 cm wide, and 45 cm-high plastic containers). 1 ml of soap was added to decrease surface tension and to avoid floating behavior. The swimming protocol had two phases: The adaptation phase and the primary phase. Rats were submitted to swimming, according to Table 1. This swimming protocol was a high-intensity training model, and according to previous studies, blood lactate concentration remained more than 1 mmol/l (Table 2) (Kuphal et al., 2007) (with some minor modifications).

Table 1
Chemical composition of clove oil.

Compound	PA (%)[a]	KI[b]
Eugenol	85/5	2350
αCaryophyllene	7/5	2460
Caryophyllene	0/8	2580
Eugenyl acetate	1/3	759
Others	4/9	different

[a] Kovats index.
[b] Peak area percentage in relation to peak total area.

Table 2
Swimming protocol.

Phase	Adaptation phase				Main phase
Days	Day 1	Day 2	Day 3	Day 4	Day 5–20
Time	2	2	3	2	1
Repetition	30 s	2 min	10 min	15 min	30 min
Interval between swimming	2 h	2 h	5 min	5 min	no interval

5.6. Morris Water Maze test

The Morris Water Maze method (MWM) tested spatial learning and memory. MWM was performed on the 17th day of exercise interventions. MWM consisted of four successive trials and one probe day. This method used a water tank (50 cm in height and 130 cm in diameter). The tank was filled up with water (to a depth of 30 cm). The temperature of the water was 26 ± 2 °C. The MWM tank hypothetically was parted into four quadrants. During the successive trials, a movable hidden escape platform (8 × 6 cm^2 area) was located underwater surface in the fourth quadrant of the tank during the swimming time. In successive trials and probes, days were measured using the Noldus Ethovision system, version 5 (USA). Rats were trained for four successive trials with a maximum of 120 s daily to locate the platform. After finding the hidden escape platform, the animal was permitted to stay there for 30 s. Each animal's time to detect and locate the platform was considered escape latency time. If an animal could not find the platform, it was placed by hand on the platform. During the probe day, the platform was removed, and the animals were allowed to search the platform for 60 s. Each rat's time in the target quadrant was noted and analyzed later (Rahman et al., 2019).

5.7. Tissue preparation

After the end of the MWM, animals were anesthetized with ketamine (100 mg/kg) and Xylazine (5 mg/kg) and flowed by transcardial perfusion with saline (100 ml) and 4 % paraformaldehyde (250 ml) in phosphate buffer (0.1 mg) with pH 7.4 (Keshvari et al., 2020). The brain was immediately removed, and the hippocampus was dissected out. After that, half part of the hippocampus was frozen and the other part was fixed in paraffin for further studies.

5.8. RNA extraction and cDNA synthesis

About 50 mg of the hippocampus was homogenized. Then chloroform was added, and the microtube was shaken vigorously (for 15 s). Subsequently, the microtube was centrifuged (at 4 °C for 15 min at 12,000 PRM). The RNA was presented in the aqueous phase. Lastly, the extracted RNA was washed in ethanol and dissolved in 20 μL RNAS-free water. After that, its concentration was checked. For cDNA synthesis, 1 μg of total RNA was used. Up to the manufacturer's protocol, synthesize using the Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, USA). The cDNA was kept at −20 °C for further use (Zhang et al., 2015).

5.9. Real-time-PCR

For α7nAChR and NLRP1 mRNA expression analyses, quantitative real time-PCR was performed using an ABI Step-One real-time PCR system and software (Applied Biosystems, USA). B-ACTIN was used as the reference gene. The amplification reactions were detected using the Real Q Plus Master Mix Green high ROX (Ampliqon, Denmark). The standard PCR conditions were 95 °C (10 min), 95 °C (15 s), and 60 °C (1 min) for 40 cycles. The expression levels of α7nAChR and NLRP1 transcripts were analyzed by the $2^{-\Delta\Delta}$ CT method. Results were obtained from ratios between the target gene (α7nAChR and NLRP1 mRNA) and the B-ACTIN housekeeping mRNA (Saresella et al., 2016). The primer sequences in the qRT-PCR were as follows: B-ACTIN, F➔GTGTGATGGTGGGTATGGGT, and R➔GGTCATTGTAGAAAGTGTGGTG; α7nAChR, F➔ATTGAAGATGTGGAATGGGAGGT, and R➔AGGTTGACGATGTAGAAGGAGGA; NLRP1, F➔CAAGAGGGAAAGGTGGACAG, and R➔GGAAGTGATGGGGATGAAGTGT.

5.10. Immunofluorescence

For immunofluorescence, the dissected hippocampus was immersion fixed in 10 % formalin for 48 h (Rahman et al., 2021) Blocks of the hippocampus were embedded in paraffin and then cut into 5 μm thick. Paraffin was removed, and the sections were blocked with goat serum (10 %) and Triton X-100 (3 %) in PBS for 30 min. Subsequently, a primary antibody was added in diluting 1:100 in PBS. Antibodies included α7nAChR (cat. no. sc-5544; Santa-Cruz Biotechnology Inc., USA) and NLRP1 (cat. no ab3683; Abcam, UK). Sections with primary antibodies were incubated at 2 to 8 °C overnight. After washing with PBS (4 times for 5 min), the secondary antibody (Goat Anti-Rabbit IgG H&L; cat. no. ab6717; Abcam, UK) was diluted 1:150 in PBS and incubated with sections at 37 °C for 1.30 h in darkness. After washing four times, DAPI was added (Zhan et al., 2015). 500 μm after and before of injection, the section was selected. Each sample was sectioned 5 μm with a 25 μm interval between each section based on neuron size [each neuron is usually 15–25 μm]. For each rat, 20 tissue sections with a thickness of 5 μm were examined; the average of every 20 tissue stained and counted. All of them were calculated in the statistics. The sections were investigated with a fluorescence microscope (LABOMED, USA) and a camera (Delta Pix, Denmark). The images were analyzed using the software Image J 1.5.

5.11. Cresyl violet

We sectioned the CA1 region of the hippocampus at 5-μm thickness, using the Paxinos and Watson atlas (2006) as a reference. After that, hippocampal sections were Nissle-stained for 20 min, with 0.1 % Cresyl violet solution. Subsequently, the sections were washed in water, then in 70 % ethanol (2 steps/min), cleared in Xylenol (2 steps/5 min), and dried in the fume hood. The percentage of dark cells in the CA1 region of the hippocampus was evaluated. After staining, counting each section in three incisions was done with a minimum distance of 50 μm. An area of 1350 μm^2 was considered for counting neurons in each group. The counting of dark cells was examined using a light microscope (LABOMED, USA) with a magnification of ×400. Image J software v1.8 (NIH, Wayne Rasband, USA) was used (Azad et al., 2011). Apoptotic cells were evaluated based on cell shrinkage, loss of uniformity of Nissl body, cytoplasm, nucleus density, and pyknotic nucleus. Viable cells were considered cells with orderly arranged cells with normal morphology, abundant cytoplasm, and evident nucleus and nucleolus (Pakravan et al., 2022).

5.12. Statistical analysis

Shapiro-Wilk Test investigated the normality of data. The one-way ANOVA was utilized to compare groups with different variables. Tukey's post hoc test was used as the supplementary test. Kruskal-Wallis analysis was used to evaluate the dark cells. To examine the interaction of the day*group on spatial learning, we used a two-way ANOVA test.

The independent *t*-test was utilized for the evaluation of the Alzheimer's model. Data was defined according to the mean ± SD. The significance level was considered as $p \leq 0{:}05$. SPSS 20 was used for data analysis.

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CRediT authorship contribution statement

Z. GK. And M. F. designed this study; Z. GK. Analyzed the data. Z. GK., M. F., R. M. and E. VZ. Performed research and wrote the paper.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2023.112192.

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