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Original Article

The effect of 8 weeks of interval training combined with the consumption of cineole, linalool, and bourbonene on MAPK/Arc gene expression and learning in rats with Alzheimer's disease

Elham Majidi[1], Khosro Jalali Dehkordi[1*], Farzaneh Taghian[1], Seyed Ali Hosseini[2]

[1]Department of Physical Education and Sport Sciences, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran
[2]Department of Sport Physiology, Marvdasht Branch, Islamic Azad University, Marvdasht, Iran

*Corresponding Author: Khosro Jalali Dehkordi, Email: khosrojalali@gmail.com

Abstract

Background and aims: This research aimed to investigate the effect of 8 weeks of interval training and the use of cineole, linalool, and bourbonene on MAPK/Arc gene expression and learning in Alzheimer's disease (AD) rats.

Methods: In this experimental study, 40 AD rats were randomized into eight groups, including (1) control, (2) AD, (3) AD + aerobic training (AT), (4) AD + linalool, cineole, and bourbonene (LCB), (5) AD + AT + LCB, (6) AT + AD, (7) LCB + AD, and (8) AT + LCB + AD. AD was induced by injecting amyloid-beta (Aβ1)-42 into the hippocampus of rats. The interval training protocol was performed five days per week for eight weeks before and after AD induction. Linalool at a concentration of 25 mg/kg, cineole at a concentration of 10 µM, and β-bourbonene at a concentration of 10 µg/mL were used for eight weeks. One-way analysis of variance was used for between-group comparisons, and Tukey's test was used for pairwise comparisons at $P \leq 0.05$.

Results: AD induction caused a significant decrease in MAPK/Arc gene expression in hippocampal tissue ($P = 0.001$). Interval exercise and consumption of three herbal drugs significantly increased gene expression of Arc ($P = 0.001$) and MAPK ($P = 0.001$). AD induction decreased learning ($P = 0.001$). Interval exercise and consumption of three herbal medicines caused a significant increase in learning ($P = 0.001$).

Conclusion: Interval exercise and using three herbal medicines have more favorable effects on improving MAPK/Arc gene expression and learning in AD than each alone.

Keywords: Interval training, Cineole, Linalool, Bourbonene, MAPK, Arc, Learning, Alzheimer's disease

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Introduction

Alzheimer's disease (AD) is a progressive neurological disorder that leads to learning and memory impairment. This disease causes the accumulation of tau protein and beta-amyloid peptides in the hippocampus, which can lead to neurological disorders. The underlying mechanism of this disease is not well understood. However, chronic neuroinflammation, increased reactive oxygen species in the brain, hypoxia, and mitochondrial dysfunction are pathological factors that lead to tau and amyloid beta (Aβ) formation as pathological indicators of AD disease (1). These pathological changes result in an impaired immune response, neurovascular dysfunction, inflammation, loss of neurons and synapses, and brain atrophy, eventually leading to severe clinical dementia (2). Exercise benefits brain health and memory and reduces the negative effects of neurological diseases such as AD (3). The positive effects of exercise on the brain are mostly seen in the hippocampus and dentate gyrus, which include increased blood flow and size of the hippocampus in humans, morphological changes in dendrites and dendritic protrusions, increased synaptic flexibility, and neurogenesis in animals with different types of exercise (4). Previous analysis of human amyloid precursor protein (hAPP) mice suggested that loss of synaptic activity-dependent proteins might play a key role in Aβ induced cognitive decline (5). Arc is primarily expressed in glutamatergic neurons of the cerebral cortex and hippocampus. It is necessary to maintain long-term improvement (LTP) and memory consolidation. In stimulated neurons, Arc mRNA expression is rapidly increased, leading to the detection of neural network activity (6). In addition, Arc mRNA is induced in clusters of activated neurons in the hippocampus that respond to specific environments and provide a potential network mechanism to encode spatial and contextual information (7). Arc may play a role in activity-dependent Aβ production since endocytic pathways are important in regulating BACE1 (β-site APP cleaving enzyme 1) activity. Furthermore, dominant-negative dynamin, which blocks

endocytosis, reduces Aβ levels in brain interstitial fluid by 70% and prevents an activity-dependent increase in Aβ (8). Previous studies indicate that expression of the immediate early gene Arc/Arg3 is required for induction of BDNF (brain-derived neurotrophic factor)-LTP and high-frequency stimulation at grain synapses of the intrinsic former pathway in the dentate gyrus, which eventually leads to brain neurogenesis (9,10). In addition, mitogen-activated protein kinase (MAPK) phosphatase (MKPs) inhibits MAPK activity by phosphorylation at tyrosine and threonine residues. Although MKPs are widely distributed in different areas of the rodent brain, including the hippocampus, cerebral cortex, ventral tegmental region, striatum, and thalamus, the mechanism of its neuronal function is not well understood (11). Recent studies have shown that MKPs are among the important regulators of synaptogenesis. Defects in MKPs regulation can impair neurodevelopment and cognitive function (12,13). A recent study showed that MKP exerts a neuroprotective role in neuritis and Aβ-induced oxidative stress and apoptosis through the inactivation of the JNK pathway (14). However, the main role of MKPs in the pathogenesis of AD remains largely unclear. Arc induction requires the activity of protein kinase A and MAP kinase. Additional evidence link the ERK phosphorylation is required for transcriptional activation of Arc/Arg3 (15). Researchers believe that in addition to exercise, a proper diet and natural antioxidants modulate immune system function, reduce inflammation, and increase neurogenesis in patients with nervous system disorders (9-12). 1,8-Cineole is an essential oil found abundantly in a variety of plants. Moreover, based on the evidence, cineole could be a potential therapeutic candidate for neurodegenerative diseases (16). In addition, (-) linalool, one of the enantiomers of a natural monoterpene, is the main volatile component of the essential oil of several species of aromatic plants such as *Lavandula angustifolia* Mill. Linalool exhibits various pharmacological effects in normal and hypertensive rats, including antimicrobial, antileishmanial, anti-inflammatory, antioxidant, and cardiovascular effects. Moreover, linalool modulates glutamatergic neurotransmission both in vitro and in vivo, possibly through *N*-methyl-D-aspartate (NMDA) receptor interactions (17). Besides, Citronella oil contains several sesquiterpenes such as β-bourbonene, β-elemene, and β-caryophyllene. β-Elemene, as a chemotherapy agent, is effective in the treatment of breast cancer, lung cancer, gastrointestinal cancer, and AD disease (18). There is limited information on the combined effects of these three plants and interval exercise on the hippocampal MAPK/Arc signaling pathway and learning in rats before and after AD induction. Therefore, the present study examined the effect of 8 weeks of interval training and the consumption of cineol, linalool, and bourbonene on MAPK/Arc gene expression and learning in AD rats.

Materials and Methods

Subjects

For this experimental study, 40 Wistar rats were purchased from the Royan Institute in Isfahan and kept in the laboratory for one week for acclimatization. During the study, animals were housed under standard conditions, including a 12-hour light-dark cycle, 55% relative humidity, room temperature of 20-22 °C, and free access to food and water. Besides, in the current study, the ethical considerations of working with animals were taken into account following the Declaration of Helsinki. Additionally, this study was approved by the Ethics Committee of the Isfahan branch of Islamic Azad University (Khorasgan). Aβ1-42 oligomer was purchased from Sigma-Aldrich. C57BL/6 mice were anesthetized by intraperitoneal administration of sodium pentobarbital 0.2% (50 mg/kg) and then placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Aβ1-42 oligomer diluted with 50 μM or 1 mM sterile 0.9% saline (1 μL per side, at 0.2 μL/min) was bilaterally injected into the dentate gyrus of the hippocampus of C57BL/6 mice (19). It should be noted that the block randomization method was used in the present study. Accordingly, the experiment was split into smaller sub-experiments (blocks), and treatments were randomly assigned to experimental units within each block. Finally, rats were randomly divided into 8 groups, including (1) the control group, (2) the rats in which AD was induced by injecting Aβ1-42 oligomer (AD), (3) AD rats treated with aerobic training (AD + AT) (4) AD rats treated with linalool + cineole + β-bourbonene (AD + LCB), (5) AD rats treated with aerobic training and linalool + cineole + β-bourbonene (AD + AT + LCB), (6) Normal rats treated with aerobic training and then AD was induced by injecting Aβ1-42 oligomer (AT + AD), (7) Normal rats treated with linalool + cineole + β-bourbonene and then AD was induced by injecting Aβ1-42 oligomer (LCB + AD), (8) Normal rats treated with aerobic training and linalool + cineole + β-bourbonene (AT + LCB + AD). It should be noted that the randomization was conducted using GraphPad online software (https://www.graphpad.com/).

Preparation of cineole, linalool, and bourbonene

Linalool and cineole compounds were purchased from Sigma-Aldrich (USA), and β-bourbonene was obtained from J&H Chemical Company. The safe dose of each compound was determined based on the articles. Accordingly, in the present study, linalool at a concentration of 25 mg/kg, cineole at a concentration of 10 μM, and β-bourbonene at a concentration of 10 μg/mL were used (16-18).

Exploration time

A square white plastic box (L × W × H, 52 × 51 × 30 cm) was used as the environment for the object recognition test. For further analysis, a video camera was used to record the behavior of the rats. Rats were placed in the

test box for 10 minutes to explore the two familiar objects. A test was conducted 24 hours later, replacing one of the known objects with a new object that differed in geometry and texture. Mice were placed individually in the test box for 5 minutes to explore the objects, and their behavior was recorded. If the nose of the mouse was within 1-2 cm of the object, it was judged to be in contact with the object.

Rats training protocol

Interval training was performed using a mouse treadmill. The exercise was started by walking slowly on a treadmill for 5 minutes at a speed of 1.8 m/min. Then, the speed was increased every 3 minutes to reach the moderate intensity (45% VO2 max). In the first week, the exercise started with 1 repetition at 45% VO2 max and a speed of 10-15 m/min and 2 recovery repetitions at 25% VO2 max until the eighth week with 6 repetitions at 45% VO2 max and a speed of 15 m/min and 3 repetitions of recovery at 20% VO2 max and a speed of 7 m/min. The total time of one session was 45 minutes (20).

Dissection and sampling

A combination of ketamine (50 mg/mL) and xylazine (20 mg/mL) was used to anesthetize the rats 48 hours after the last training session and 12 hours of fasting. First, laboratory specialists performed pain tests to ensure anesthesia. When the rats were anesthetized, their brain was removed. Finally, the harvested brain tissue was immediately placed in nitrogen. For measurement of MAPK/Arc activity, brain tissue was maintained at -80 °C.

Real-Time PCR method for molecular analysis of brain tissue

Total RNA was isolated using a Trizol reagent in accordance with the manufacturer's instructions (Thermo Scientific, USA). Sample purity and concentration of RNA were determined by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Moreover, cDNA synthesis was conducted based on the instructions (TaKaRa, Kusatsu, Shiga Prefecture, Japan). Furthermore, SYBR Green dye and quantitative real-time PCR were used to evaluate gene expression (Rotor-Gene 6000 instrument, Corbett Life Science, Mortlake, Australia). B2m was used as a reference gene for normalization. In a statistical analysis, the $2^{-\Delta\Delta Ct}$ formula was used to determine fold changes in mRNA expression. The primers used in the study are listed in Table 1.

Statistical analysis

After collecting the data, the Shapiro-Wilk test was used to check the normality of the data distribution. Finally, the statistical analysis was performed using one-way analysis of variance. Additionally, Tukey's post hoc test was used to assess the significance of differences between groups. All statistical operations were performed using SPSS at the $P \leq 0.05$ level.

Table 1. The list of primers used in this study

Genes	Primer sequences	Temperature	Size (bp)
B2m	Forward: 5′-ACAGTTCCACCCGCCTCACATT -3′ Reverse: 5′ -TAGAAAGACCAGTCCTTGCTGAAG -3′	60	105
Arc	Forward: 5′-GCTGGAAGAAGTCCATCAAGGC-3′ Reverse: 5′-ACCTCTCCAGACGGTAGAAGAC-3′	60	120
MAPK	Forward: 5′-GGCTTTCTGACGGAGTATGTGG -3′ Reverse: 5′-GTTGGAGAGCATCTCAGCCAGA -3′	60	129

Results

Data on MAPK/Arc gene expression and exploration time are shown in Figures 1, 2, and 3. The results of the ANOVA test indicated a significant difference in the expression level of MAPK ($P=0.0001$) and Arc ($P=0.0001$) genes in rat brain tissue in different study groups. Moreover, the exploration time indicated a significant difference between the groups ($P=0.0001$). The mean and standard deviation of MAPK/Arc and exploration time are shown in Tables 2 and 3.

The results of Tukey's test showed that MAPK/Arc gene expression in the AD group significantly decreased compared to other groups ($P=0.001$). The expression of MAPK/Arc gene in Alzheimer's and Training (AT + AD) and Alzheimer's groups and the combination of three plants (AD + LCB) and Alzheimer's groups and training and the combination of three plants (AD + AT + LCB) had a significant increase compared to the AD group (AD). Besides, the expression of MAPK/Arc in the groups of Training and Alzheimer's (AT + AD), combination of three herbs and Alzheimer's (LCB + AD), and Training and combination of three herbs and Alzheimer's (AT + LCB + AD) indicated a significant increase compared to other groups ($P=0.001$).

Discussion

The results of the present research showed that the use of three medicinal plants and 8 weeks of interval training is associated with a significant increase in MAPK/Arc expression in the brain tissue of mice with AD. The results showed that the MAPK/Arc signaling pathway is an important pathway in exercise-induced brain neurogenesis. Furthermore, activation of this signaling pathway in mouse models protects against brain damage and AD. However, down-regulation of the MAPK/Arc signaling pathway leads to disease progression (10,21). Expression of the MAPK/Arc is a key regulator of brain development, cell growth and proliferation, and protein synthesis. The results showed that the increase of neurogenesis in the brain is associated with the reduction of inflammation and the simultaneous interaction of MAPK/Arc in the cellular structure of the brain of AD (15). The results of the present research are in line with

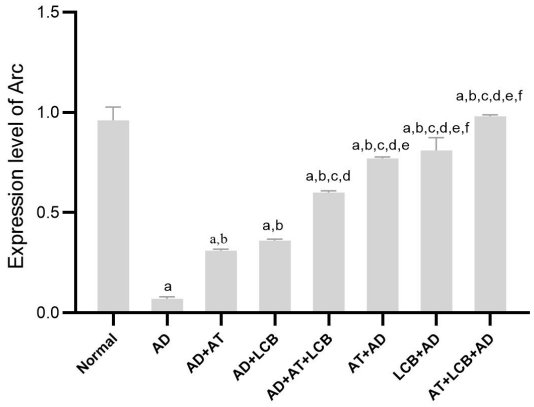

Figure 1. The mRNA expression of MAPK in the brain tissue of different study groups. The obtained values are displayed as means and standard deviation (SD). There were statistically significant differences between the mean values ($P<0.05$); (a) The significant difference of the normal group with other groups, (b) The significant difference of the AD group with other groups, (c) The significant difference of the AD+AT group with other groups, (d) The significant difference of the AD+LCB group with other groups, (e) The significant difference of the AT+AD group with other groups, (f) The significant difference of the LCB+AD group with other groups. AD, Alzheimer; AT, aerobic training; LCB, Linalool- Cineole- β-Bourbonene

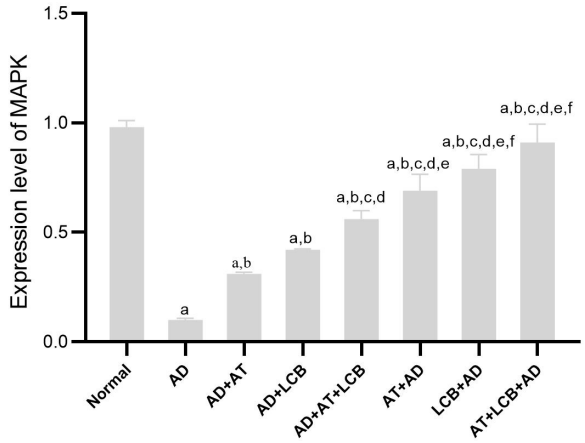

Figure 2. The mRNA expression of Arc in the brain tissue of different study groups. The obtained values are displayed as means and standard deviation (SD). There were statistically significant differences between the mean values ($P<0.05$): (a) The significant difference of the normal group with other groups, (b) The significant difference of the AD group with other groups, (c) The significant difference of the AD+AT group with other groups, (d) The significant difference of the AD+LCB group with other groups, (e) The significant difference of the AT+AD group with other groups, (f) The significant difference of the LCB+AD group with other groups. The results showed that the exploration time was not significant in Alzheimer's group (AD), but the exploration time was significant in the other groups ($P=0.001$). AD, Alzheimer; AT, aerobic training; LCB, Linalool- Cineole- β-Bourbonene

the research results of Palop et al (22), Shamsipour et al (23), Aczel et al (24), Gharari et al (25), and Leung et al (26). Palop et al investigated the susceptibility of dentate granule cells to disruption of Arc expression in hAPP transgenic mice. Results showed that Arc reduction resulted in decreased synapse function and cognitive deficits in hAPP mice and possibly in humans with AD (22). The research results of Shamsipour et al indicate that the concomitant use of *Lactobacillus plantarum* and *Bifidobacterium bifidum* combined with HIIT aerobic exercise may have a neuroprotective effect in AD (23). The researchers reported that exercise improved AD (24,25).

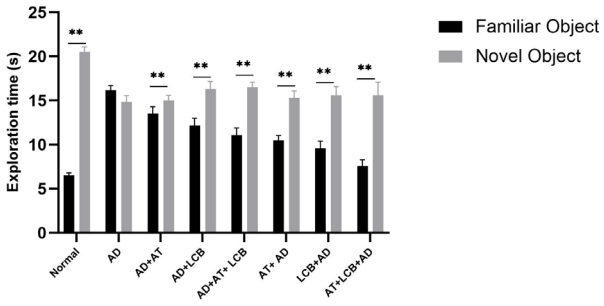

Figure 3. Exploration time in different research groups. The values obtained are presented as mean and standard deviation (SD). Statistically significant differences between means ($P<0.05$). $^{**}P\leq0.001$ Significant increase compared to other groups. AD, Alzheimer; AT, aerobic training; LCB, Linalool- Cineole- β-Bourbonene

Table 2. The expression level of the MAPK and Arc in different groups

Groups	MAPK	*P* value	Arc	*P* value
Control	0.98±0.03	-	0.96±0.07	-
AD	0.1±0.01*	<0.0001	0.07±0.01*	<0.0001
AD+AT	0.31±0.01*	<0.001	0.34±0.013*	<0.0001
AD+LCB	0.42±0.005*	<0.001	0.36±0.0013*	<0.0001
AD+AT+LCB	0.56±0.04*	<0.001	0.6±0.074*	<0.0001
AT+AD	0.69±0.08*	<0.01	0.77±0.065*	<0.0001
LCB+AD	0.79±0.07*	<0.01	0.81±0.02*	<0.0001
AT+LCB+AD	0.91±0.08	0.2476	0.98±0.05	0.9667

* The significant difference of the control group compared with other groups.

Table 3. Exploration time in different groups

Groups	Familiar object	Novel object	*P* value
Control	6.5±0. 3	20.5±0.6	<0.0001
AD	16.2±0.5	15.86±0.7	0.15
AD+AT	13.5±0.8	15.00±0.6	<0.01
AD+LCB	12.2±0.8	15.5±0.6	<0.01
AD+AT+LCB	11.1±0.8	16.5±0.6	<0.001
AT+AD	10.5±0.69	15.6±0.98	<0.001
LCB+AD	9.6±0.8	15.6±1.5	<0.001
AT+LCB+AD	7.6±0.7	24.6±1.5	<0.0001

One of the most important cellular mechanisms involved in nerve growth and neurogenesis is the expression of the Arc gene. Arc is a neuron-specific postsynaptic protein selectively expressed in Ca/calmodulin-dependent protein kinase II (CaMKII)-positive neurons. Once activated, Arc is directed to a postsynaptic density of synoptically active dendritic spines, where it binds to polysomes (26). Arc interacts with endophilin 2/3 and dynamin to help modulate the alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptor by enhancing endocytosis. Arc-mediated endocytosis suppresses neural network activity and enhances activity-dependent production of Aβ. If Arc-endosome trafficking and subsequent activity-dependent production of Aβ are left unchecked, it will generate a positive feedback mechanism in which synaptic removal results in significant loss of dendritic spines and synaptic activity,

leading to synaptic damage similar to that observed in AD (27). Arc is probably important to limit the level of neuronal excitation since Arc-mediated endocytosis will weaken neural network activity (24,25). Previous studies have shown that aerobic exercise induces its beneficial effects by increasing brain oxygen consumption, increasing brain neurotransmitters, and increasing the regulation of neurotrophic factors in the brain (28). Aerobic exercise increases Arc expression in the hippocampus through increased MAPK expression (15). MAPK is one of the most important proteins involved in learning and memory and brain neurotransmitter in the hippocampus. The results of the present research are in line with the research results of Sun et al (29), Blüthgen al (15), and Ang et al (30). Sun et al showed that exercise activity modulates the expression of ERK, p38, and JNK, which were identified as MAPK signaling agents related to hippocampal neuronal functions. In conclusion, exercise acts as an effective strategy to prevent the development of AD by regulating adult neurogenesis and enhancing brain immune activity through controlling MAPK signaling (29). Ang et al also showed that forced running for 12 weeks with high intensity can increase memory and learning and simultaneously influence the number of cholinergic neurons (30). In line with these findings, the results of the present study indicate that Aβ1-42 injection decreased MAPK gene expression in AD mice. Taken together, these studies indicate that MAPKs may accelerate the development of AD. Preventing excessive MAPK activation can reduce Aβ deposition, tau hyperphosphorylation, neuronal apoptosis, and memory impairment (31). MAPK signaling consists of several transduction factors involved in neuronal plasticity and inflammatory regulation. Therefore, it is possible that exercise exerts an anti-AD effect by regulating MAPK signaling in hippocampal tissue. The importance of the MAPK signaling pathway in the regulation of many physiological processes related to learning and memory has been emphasized in several studies. Blüthgen et al found that Arc induction requires protein kinase A and MAPK activity. Another body of evidence links the activation of ERK in granule cells to the transcriptional activation of Arc/Arg3 (15). The present research indicated that interval aerobic training increased MAPK/Arc of the hippocampus in the AD rat model. Furthermore, AD has been mainly associated with cognitive decline in various species including humans and rats. In a study, five months of exercise activity led to a decrease in amyloid platelets in the frontal cortex and hippocampus and the time to reach the platform and an increase in the memory and learning rate in mice with AD (31). The present research indicated that the use of three medicinal plants and 8 weeks of interval training improved memory and learning in AD rats. Using the combination of three plants (cineole, linalool, and bourbonene) with anti-inflammatory and neurogenerative properties together with aerobic exercises can increase the expression of the MAPK/Arc gene and improve learning memory in rats with AD. Cineole has medicinal properties including antioxidant, anti-inflammatory, and pain-relieving properties. The results of a study by Paul et al showed the therapeutic role of 1, 8-cineole in preventing AD (32). In addition, linalool has high antioxidant properties and fights oxidative stress in brain tissue caused by hydrogen peroxide. Linalool crosses the blood-brain barrier. Linalool also improves beta-amyloidosis and tauopathy in the hippocampus and amygdala of linalool-treated 3xTg-AD mice compared to untreated mice (33). The results of a previous study showed that bourbonene has anti-inflammatory and neuroprotective effects and improves memory and learning. Additionally, they showed that the consumption of bourbonene improved cognitive memory in Alzheimer's rats (34). The limitation of this study was the control of food and calorie intake. In addition, considering the isoforms of MAPK/Arc in the hippocampus and their role during exercise and the lack of measurement of different isoforms, other measurement methods such as Western blot and ELISA are recommended to be used in future studies.

Conclusion

In general, it can be concluded that the use of the combination of three plants (cineole, linalool, and bourbonene) along with interval aerobic exercises with positive regulation of MAPK/Arc in the hippocampus tissue is effective in controlling brain damage and ultimately leads to improvement of learning and memory. Therefore, it is recommended that a combination of three herbs along with interval training should be used in case of AD.

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Authors' Contribution

Conceptualization: Khosro Jalali Dehkordi.
Data curation: Elham Majidi, Khosro Jalali Dehkordi.
Formal analysis: Khosro Jalali Dehkordi, Farzaneh Taghian.
Funding acquisition: Elham Majidi.
Investigation: Elham Majidi, Khosro Jalali Dehkordi, Farzaneh Taghian, Ali Hoseyni.
Methodology: Elham Majidi, Khosro Jalali Dehkordi.
Project administration: Khosro Jalali Dehkordi.
Software: Elham Majidi, Khosro Jalali Dehkordi.
Supervision: Khosro Jalali Dehkordi.
Writing–original draft: Elham Majidi, Khosro Jalali Dehkordi.
Writing–review & editing: Khosro Jalali Dehkordi, Farzaneh Taghian, Seyed Ali Hosseini.

Competing Interests

The authors declare that there is no conflict of interests.

Ethical Approval

Ethical considerations in this study included obtaining permission

from the Ethics Committee of Isfahan (Khorasgan) Branch, Islamic Azad University (IR.IAU.KHUISF.REC.1401.391) and obtaining written consent to participate in the study from the participants.

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