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The Effect of Fisetin Supplementation and High-Intensity Interval Training on Neurogenesis Markers in Aged Alzheimer's Model Mice

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ABSTRACT

Introduction: Alzheimer's disease (AD) is associated with a marked reduction in brain-derived neurotrophic factor (BDNF) and fibronectin 1 (Fn1). This study investigates the effects of fisetin supplementation combined with high-intensity interval training (HIIT) on these neurogenesis markers in an aged mouse model of AD.

Methods: In this experimental study, 30 aged C57BL/6 mice (weight: 30 g) with AD were randomly assigned to one of the five groups: (1) Control, (2) AD, (3) AD + Fisetin, (4) AD + HIIT, and (5) AD + HIIT + Fisetin. Alzheimer's disease was induced in the AD groups by injecting amyloid-beta (Aβ1-42) into the hippocampus. The HIIT protocol consisted of a 10-minute warm-up at 50-55% VO2 max, followed by seven intervals, each comprising 4 minutes at 80-90% VO2 max and 3 minutes at 65-75% VO2 max. Fisetin was administered at 20 mg/kg for eight weeks. Data were analyzed using one-way ANOVA with a significance level of $P \leq 0.05$.

Results: Significant differences were observed in BDNF, Fn1, and Aβ gene expression levels across the five groups of aged mice ($p < 0.001$). BDNF and Fn1 expression were significantly reduced in the AD groups compared to the healthy controls ($p < 0.001$). However, their expression levels increased significantly in the AD + Training + Fisetin, AD + Training, and AD + Fisetin groups compared to the AD-only group ($p < 0.001$). The AD + Training + Fisetin group exhibited the highest expression levels, followed by the AD + Training and AD + Fisetin groups ($p < 0.001$). Aβ expression was significantly reduced in all intervention groups, with the AD + Training + Fisetin group showing the most substantial decrease ($p < 0.001$).

Conclusion: Combining HIIT and fisetin supplementation may promote cerebral neurogenesis in AD by reducing Aβ levels and enhancing BDNF and Fn1 gene expression. Notably, the combined intervention of HIIT and fisetin exhibits a more significant effect than either HIIT or fisetin alone, with HIIT being more effective than fisetin as a standalone treatment. Thus, the combination of HIIT and fisetin appears to be the most effective complementary approach for managing this disease.

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Introduction

Alzheimer's disease (AD), the most prevalent chronic disease among the elderly, is an irreversible neurodegenerative disorder characterized primarily by memory loss and other cognitive impairments. It is the most common form of dementia (1) and is also recognized as a proteinopathy, a condition where proteins become abnormal (2). The hallmark of AD is the formation of dense amyloid plaques, known as senile plaques, in the brain (3). The primary component of these plaques is beta-amyloid (Aβ). Aβ1-42, a proteolytic product derived from the amyloid precursor protein (APP) (4), is a soluble peptide in the body that can form dense, neurotoxic aggregates through fibrillization and oligomerization (5). AD is a progressive form of dementia, neuropathologically characterized by a significant reduction in the number of neurons, particularly in the hippocampus, along with the deposition of beta-amyloid in blood vessel walls, the spread of neuritic plaques, and the formation of intracellular fibrillar tangles (6). These

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pathological changes affect the cortex, hippocampus, and medial temporal lobes. Additionally, the disease is associated with mitochondrial dysfunction, oxidative neuronal damage, synaptic loss, neuronal degeneration, and cognitive and perceptual impairment (7).

Neurotrophins are a small group of structurally and functionally related growth factors (8). The Brain-Derived Neurotrophic Factor (BDNF) has received considerable attention due to its essential role in synaptic plasticity, memory, and neurogenesis. BDNF is recognized as a key factor positively regulated by exercise (9). Low levels of BDNF are associated with cognitive impairments, depression, neurodegenerative diseases, and increased mortality. BDNF has been shown to mediate the beneficial effects of exercise on synaptic plasticity and cognitive function, potentially through its role in energy metabolism (10). Studies indicate that BDNF mRNA levels significantly increase in rats following voluntary exercise, which correlates with improvements in spatial memory (11). Exercise can enhance the expression of BDNF and CREB genes. BDNF may influence memory by inducing changes in membrane receptor expression and trafficking, as well as activating several pathways (PLC-γ, PI3K, ERK) critical for synaptic plasticity. The exercise-induced increase in BDNF levels, along with improved mitochondrial function, can reduce oxidative stress (12).

Fibronectin (FN) is derived from various cellular sources, including astrocytes, epithelial cells, fibroblasts, and mesenchymal cells, and plays a role in cell adhesion, proliferation, differentiation, epithelial tissue repair, immune regulation, nerve regeneration, and other physiological activities (13). Fibronectin 1 (*FN1*) is an effective vehicle for delivering nerve growth factors (NGF) and provides an ideal matrix for axonal repair. In animal studies, fibronectin has been used to enhance peripheral nerve repair processes (14). It forms cable-like structures that promote cellular connections and facilitate the migration of cells such as fibroblasts, neurons, macrophages, and Schwann cells, thereby contributing to peripheral nerve repair (15).

Results have shown that increasing physical activity and regular exercise, even low-intensity activities such as walking, strongly protect the nervous system and reduce the risk of dementia (16). Regular physical activity plays a positive role in aging-associated brain atrophy reduction and diseases related to cognitive impairment, thereby decreasing the incidence of Alzheimer's disease (17). Physical exercise, particularly aerobic exercise, positively impacts brain health and cognitive function, reducing the detrimental effects of neurological disorders such as Alzheimer's, Parkinson's, and depression (18). The beneficial effects of HIIT on the brain are especially pronounced in the hippocampus and dentate gyrus regions (19). These effects include increased blood flow and hippocampal volume in humans, morphological changes in dendrites and dendritic spines, and enhanced synaptic plasticity and neurogenesis in animal models (19). Recent findings suggest that combined HIIT training and herbal supplementation can produce synergistic benefits in preventing and mitigating AD (20, 21).

Fisetin (3,3',4',7-Tetrahydroxyflavone) is a natural flavonoid in various fruits and vegetables, including strawberries, apples, persimmons, grapes, onions, and cucumbers. It possesses neurotrophic, anticancer, and anti-inflammatory properties, as well as antioxidant effects against cognitive and neurological disorders, such as Alzheimer's disease (AD) (22). Additionally, fisetin has been reported to play a role in preserving neurological function during aging (23). Generally, changes in neurotrophic levels due to aging, genetic factors, and other variables have been observed in neurodegenerative diseases, contributing to neuronal destruction. Given the neuroprotective and nutritional properties of factors such as BDNF and Fn1, which enhance central nervous system function, including memory and learning, and considering the limited studies on the combined effects of exercise and fisetin supplementation on BDNF and Fn1 in neurodegenerative diseases like Alzheimer's, there is a clear need to investigate these impacts. Thus, the present study aimed to examine the effects of fisetin supplementation and high-intensity interval training on specific neurogenesis markers in the brains of aged Alzheimer's model mice.

Materials and Methods

In this experimental study, 30 aged female C57BL/6 mice were obtained from the animal breeding center at the Royan Institute, Isfahan, and were randomly assigned to the following groups: (1) healthy control, (2) AD, (3) AD +

Fisetin, (4) AD + HIIT, and (5) AD + HIIT + Fisetin. Initially, the mice were acclimated to the laboratory environment for one week. The sample size was determined using a previous study and G*Power software. During the study, mice were kept under standard conditions, including a 12-hour light/dark cycle, an ambient temperature of 20 to 22°C, relative humidity of 55%, and free access to food and water. Additionally, the study adhered to the ethical guidelines of the Helsinki Declaration, and the protocol was reviewed and approved by the Ethics Committee of Shahrekord University (approval code: IR.SKU.RERC.1402).

To induce Alzheimer's disease, Aβ1-42 oligomers (Sigma-Aldrich, USA) were prepared. Synthetic Aβ1-42 was first dissolved in cold hexafluoroisopropanol (HFIP) (Sigma-Aldrich, USA) for 20 minutes. The solution was then vortexed for 10 minutes to promote the formation of Aβ1-42 monomers. These monomers were subsequently subjected to vacuum spinning and precipitation and then dissolved in a 10% HFIP solution. The Aβ1-42 solution was incubated with continuous stirring at room temperature for 48 hours, followed by centrifugation at 4°C for 20 minutes. The supernatant was separated and transferred to pre-chilled tubes. After complete evaporation of HFIP, 50 µM Aβ1-42 oligomers were obtained. The oligomer solution was stored at 4°C until use. Female BALB/c mice were anesthetized with an intraperitoneal injection of 0.2% sodium pentobarbital (50 mg/kg) and then positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Aβ1-42 oligomers were injected into the hippocampus's bilateral dentate gyrus (DG) in C57BL/6 mice. The injection was performed with a concentration of 50 µM Aβ1-42 or 0.9% sterile saline at a volume of 1 µL, administered at a rate of 0.2 µL per minute.

High-Intensity Interval Training Protocol

The HIIT program for the rats in the exercise group will be conducted on a specialized animal treadmill (Tajhiz Gostar Iranian, 2016, Tehran, Iran). The protocol consists of a 10-minute warm-up period at 50-55% VO2max, followed by seven intervals, each comprising 4 minutes at 80-90% VO2max and 3 minutes at 65-75% VO2max, and concludes with a 1-minute cool-down period. Notably, no electrical shocks will be used during the exercise program. If needed, the animals will be encouraged to continue the exercise through manual guidance or auditory cues applied to the treadmill cover (24).

3-7- Fisetin Supplement

The fisetin supplement was obtained from Sigma-Aldrich (USA) and administered orally via gavage at a dose of 20 mg/kg for eight weeks (19).

Dissection and Sampling

Forty-eight hours after the final exercise session and following a 12-hour fasting period, the mice were anesthetized with a mixture of 50 mg/mL ketamine and 20 mg/mL xylazine. Laboratory specialists initially performed a pain response test to confirm adequate anesthesia. Once full anesthesia was verified, the animals' brains were exposed, and brain tissue was extracted. The tissue was immediately placed in liquid nitrogen for preservation.

Table 1. Primer Sequences for Research Variables

Genes	Primer Sequences	Temperature	Size (bp)
B2m	Forward: 5'- ACAGTTCCACCCGCCTCACATT -3' Reverse: 5'-TAGAAAGACCAGTCCTTGCTGAAG -3'	60	105
FN1	Forward: 5'- CCCTATCTCTGATACCGTTGTCC-3' Reverse: 5'- TGCCGCAACTACTGTGATTCGG-3'	60	110
BDNF	Forward: 5'- GGCTGACACTTTTGAGCACGTC-3' Reverse: 5'- CTCCAAAGGCACTTGACTGCTG-3'	60	123
AB	Forward: 5'- TCCGTGTGATCTACGAGCGCATC-3' Reverse: 5'GCCAAGACATCGTCGGAGTAGT-3'	59	128

Gene Expression Measurement for BDNF, Fn1, and Aβ1

RNA was extracted from hippocampal brain tissue using TRIzol reagent, following the manufacturer's protocol (Yekta Tajhiz Azma). Subsequently, cDNA synthesis was carried out according to the BIOFACT kit protocol. Gene sequences for BDNF, Fn1, and Aβ1, along with the nucleotide sequences of the forward and reverse primers, were obtained from the NCBI database (Table 1). Primers were designed using Oligo7 and Beacon Designer software. Real-time PCR was performed using SYBR Green kits on the Corbett Rotor-Gene 6000 system, with Beta-2-

microglobulin (B2M) serving as the housekeeping gene. Gene expression was analyzed and evaluated using the 2-ΔΔCT method.

Data Analysis

The Shapiro-Wilk test was used to assess normality, and Levene's test was applied to evaluate the homogeneity of variances. Data analysis was conducted using one-way ANOVA followed by Tukey's post-hoc test, with a significance level set at $p \leq 0.05$ for all tests. All statistical analyses were performed using SPSS software, version 23.

Results

One-way ANOVA demonstrated significant inter-group differences in BDNF gene expression in the brain tissue of aged mice after eight weeks of HIIT (p = 0.001). Tukey's post-hoc test revealed that BDNF expression was significantly lower in the AD group compared to the healthy control group (p = 0.001). Additionally, BDNF expression was significantly higher in the AD + Exercise + Fisetin (p = 0.001), AD + Exercise (p = 0.001), and AD + Fisetin (p = 0.001) groups compared to the AD group. Furthermore, BDNF expression was significantly higher in the AD + Exercise + Fisetin group than in the AD + Exercise (p = 0.001) and AD + Fisetin (p = 0.001) groups. Moreover, BDNF expression in the AD + Exercise group was significantly higher than in the AD + Fisetin group (p = 0.001).

Following eight weeks of HIIT, one-way ANOVA indicated significant differences in Fn1 gene expression levels in the brain tissue of aged mice across the study groups (p = 0.001). Tukey's post-hoc test demonstrated a significant reduction in Fn1 gene expression in the AD group compared to the healthy control group (p = 0.001). However, Fn1 expression was significantly elevated in the AD + Exercise + Fisetin (p = 0.001), AD + Exercise (p = 0.001), and AD + Fisetin (p = 0.001) groups compared to the AD group. Furthermore, Fn1 expression in the AD + Exercise + Fisetin group was significantly higher than in the AD + Exercise (p = 0.001) and AD + Fisetin (p = 0.001) groups. Additionally, Fn1 expression in the AD + Exercise group was significantly higher than in the AD + Fisetin group (p = 0.001).

Similarly, after eight weeks of HIIT, one-way ANOVA indicated significant differences in Aβ gene expression levels in the brain tissue of aged mice across the study groups (p = 0.001). Tukey's post-hoc test demonstrated a significant increase in Aβ gene expression in the AD group compared to the healthy control group (p = 0.001). However, Aβ expression was significantly reduced in the AD + Exercise + Fisetin (p = 0.001), AD + Exercise (p = 0.001), and AD + Fisetin (p = 0.001) groups compared to the AD group. Additionally, Aβ expression in the AD + Exercise + Fisetin group was significantly lower than in the AD + Exercise (p = 0.001) and AD + Fisetin (p = 0.001) groups. Furthermore, Aβ expression in the AD + Exercise group was significantly lower than in the AD + Fisetin group (p = 0.001).

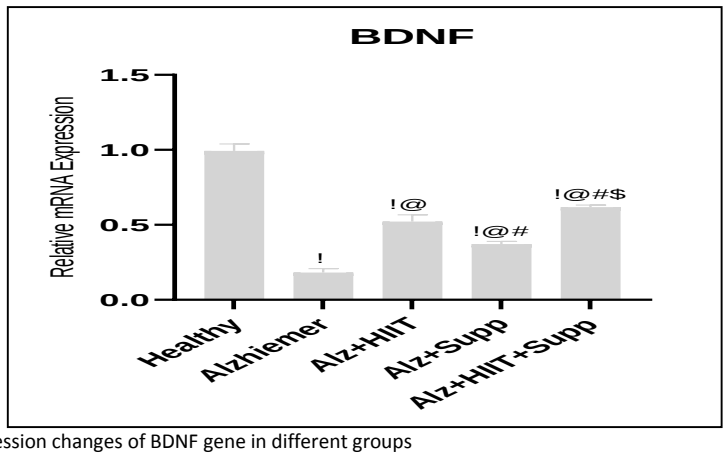

Figure 1: Relative expression changes of BDNF gene in different groups
Con: control, AD: Alzheimer's disease, Fis; Fistin, Exe; exercise
! A significant amount compared to the control group. @A significant amount compared to the Alzheimer's group# Significant level compared to the Alzheimer+HIIT group $ Significant level compared to the Alzheimer+exercise+physitin group

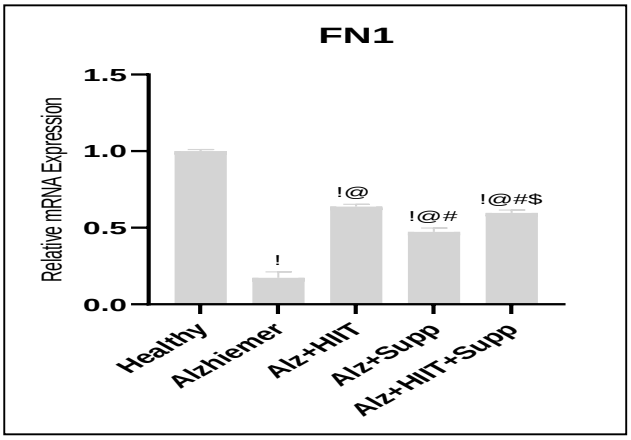

Figure 2: The relative expression changes of Fn1 gene in different groups
Con: control, AD: Alzheimer's disease, Fis; Fistin, Exe; exercise
! A significant amount compared to the control group. @A significant amount compared to the Alzheimer's group# Significant level compared to the Alzheimer+HIIT group $ Significant level compared to the Alzheimer+exercise+physitin group

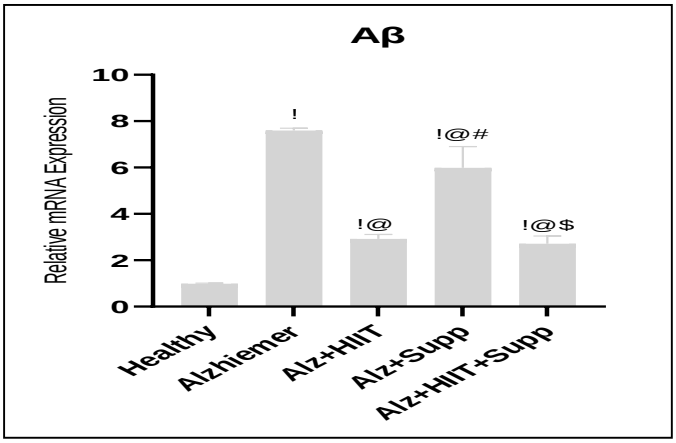

Figure 3: Changes in the relative expression of Aβ gene in different groups
Con: control, AD: Alzheimer's disease, Fis; Fistin, Exe; exercise
! A significant amount compared to the control group. @ A significant amount compared to the Alzheimer's group# Significant level compared to the Alzheimer+HIIT group $ Significant level compared to the Alzheimer+exercise+physitin group

Discussion

Aging and AD are associated with reduced brain angiogenesis and neurogenesis. This study examined the effects of HIIT combined with fisetin supplementation on BDNF, Fn1, and Aβ gene expression in elderly mice with AD. In summary, the combination of HIIT and fisetin supplementation, as well as each intervention individually, led to increased expression of BDNF and Fn1 genes and decreased expression of Aβ following eight weeks of HIIT in elderly mice with AD, compared to the Alzheimer's control group. Additionally, the combined effect of fisetin and HIIT was significantly more effective than either intervention alone, with HIIT demonstrating a more significant impact than fisetin supplementation alone.

Neurotrophic factors, such as *BDNF* and Fn1, play essential roles in supporting and promoting the growth of various brain neurons. Research indicates that exercise can indirectly influence the expression of neurotrophic factor genes by modulating the release of neurotransmitters such as acetylcholine, gamma-aminobutyric acid (GABA), and monoamines, which in turn leads to elevated *BDNF* mRNA levels in the hippocampus (25). High levels of *BDNF* in the hippocampus and cortex reflect its essential role in maintaining proper brain function. *BDNF* has a clear

neuroprotective role, and exercise enhances cholinergic activity, which is involved in neuronal plasticity induced by physical activity (26). Luo et al. demonstrated that exercise increases neurogenesis in rats and enhances BDNF mRNA production, contributing to improved brain function, learning, and memory (26). Intense physical activity also increases cerebral blood flow, potentially enhancing neuronal cell formation, angiogenesis, synaptogenesis, and neurotransmitter synthesis in various brain regions (27). In a study by Osali et al., BDNF levels and memory improved after 12 weeks of aerobic exercise (28). Zhang et al. (2023) concluded in a review that exercise, mainly treadmill running, swimming, and voluntary wheel running, significantly increases *BDNF* levels in the hippocampus and cortex of AD models, with swimming being the most effective intervention (29). However, these findings differ from studies by Betio et al. (2020) and Nakhzari et al. (2018), which reported either decreased or unchanged BDNF levels following endurance exercise (30, 31). This discrepancy may stem from differences in subjects or exercise protocols. Another possible mechanism for increased BDNF following HIIT in Alzheimer's mice may involve regulating Trk receptors in brain tissue (32). In the present study, HIIT significantly elevated BDNF levels in the hippocampus of aged Alzheimer's mice.

Fibronectin (FN) is a high-molecular-weight glycoprotein, ranging from 220,000 to 250,000 daltons. As an adhesive molecule, FN was initially isolated from human plasma and fibroblast cell surfaces and is abundantly present in the extracellular matrix (ECM). Fibronectin 1 (FN1) serves as an effective carrier for nerve growth factors (NGFs) and provides a suitable matrix for axonal repair (13). In animal studies, fibronectin has been utilized to enhance peripheral nerve repair processes (33). Research by Ping et al. (2018) highlights FN1's significant role in stimulating the growth, invasion, and survival of gliomas through the activation of the PI3K/AKT signaling pathway (34). Several studies have reported that FN modulates cellular biology via its impact on PI3K/AKT signaling (35, 36). Moreover, research by Lemanska et al. (2009) demonstrated that the molecular status of plasma FN could serve as an additional biomarker for risk assessment (33). Studies show that fibronectin (FN) has a potent angiogenic effect on endothelial cells in the central nervous system. Additionally, fibronectin enhances the survival and proliferation of brain microvascular endothelial cells, mediated by integrins α5β1 and αVβ3 through mitogen-activated protein kinase signaling (37). The mechanisms underlying the increase in FN1 in the brain after chronic HIIT are likely associated with reduced apoptosis and enhanced PI3K/AKT signaling. This exercise-induced increase in *FN1* may also involve the activation of integrins, further supporting the neuroprotective and angiogenic roles of *FN1* (33, 34) .

The results of the current study indicate that a combination of HIIT and fisetin supplementation, as well as each intervention individually, led to a reduction in *Aβ* expression after eight weeks of HIIT in aged mice with AD. Mousakhani et al. (2024) demonstrated that voluntary physical activity in an enriched environment likely improves *PI3K* and *Akt* protein mechanisms and suppresses amyloid beta accumulation through enhanced insulin sensitivity and hormone function, resulting in cognitive benefits and reduced cell death in AD (37). Additionally, Zarine Afshar et al. (2020) showed that moderate-intensity continuous exercise combined with curcumin supplementation can increase plasma soluble LRP1 levels, promoting *Aβ* clearance in the hippocampus and modulating factors affecting AD in laboratory rats (38). In contrast, Fallah et al. (2014) reported increased plasma Aβ levels in diabetic rats following voluntary exercise (39), which is inconsistent with our findings. This discrepancy may stem from differences in subjects and exercise protocols. Interval aerobic exercise may reduce brain *Aβ* plaque levels by regulating amyloid precursor protein processing or enhancing the degradation and clearance of *Aβ* (37).

In a study, fisetin supplementation significantly improved cognitive performance and memory in mice with AD (22). The current study also demonstrated that fisetin enhances BDNF and Fn1 expression in Aβ1-42 model mice, thereby supporting critical neurogenic pathways essential for neuronal survival. Notably, fisetin has been shown to reduce apoptosis and neurodegeneration induced by aluminum in the hippocampus of adult mice (40). These results suggest that fisetin plays an essential role in preventing age-related neurodegenerative disorders, including AD (22). A primary

limitation of the present study is the lack of measurement of additional factors related to cognitive performance and brain neurogenesis. Future studies should utilize different measurement techniques, such as Western blotting and ELISA.

Conclusion

Based on the results, HIIT and fisetin supplementation, either alone or in combination, can enhance neurogenesis in elderly individuals with AD by increasing the expression of BDNF and Fn1 while reducing Aβ levels. Notably, the combination of fisetin and aerobic exercise demonstrates a more substantial effect than either intervention alone, with HIIT alone proving more effective than fisetin alone. Therefore, the combined approach of HIIT and fisetin is recommended as the most effective complementary therapeutic strategy for AD. However, further and more comprehensive studies are required to validate and expand upon these findings.

Declarations

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Conflict of Interest

The authors declare that no conflicts of interest are associated with this research.

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Ethical Considerations

This manuscript is derived from a PhD dissertation in Exercise Physiology at Shahrekord University and has been registered with the Shahrekord University Ethics Committee under the code IR.SKU.RERC.1402.064.

Author Contributions

All authors equally contributed to the design, execution, analysis of results, and writing of the manuscript.

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