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The Effect of Eight Weeks of Endurance Training with Saffron on Cytochrome C and Calmodulin Kinase 2 on the Heart Tissue of Alzheimer's Rats Treated With Trimethyl Tin (TMT)

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ABSTRACT

Introduction: A proper diet and physical activity benefit Alzheimer's disease (AD). Therefore, this study aimed to evaluate the impact of endurance training (ET) with saffron (Sa) consumption on cytochrome C and calmodulin kinase 2 in the heart tissue of AD rats.

Methods: In this experimental study, 40 rats (AD induced by intraperitoneal 8mg/kg trimethyl tin) were divided into five groups of eight animals comprising: (1) AD, (2) Endurance training (ET), (3) saffron (Sa), (4) ET+Sa, and (5) sham (Saffron solvent) groups. The ET and ET+Sa groups ran on a treadmill at 15 to 20m/min (three sessions a week for eight weeks), each lasting 15 to 30 minutes. In addition, the Sa and ET+Sa groups were given 25mg/kg saffron extract per day. The Shapiro-Wilk test, one-way ANOVA, and Tukey's *post hoc* tests were used to analyze the data ($P \leq 0.05$).

Results: ET, Sa, and ET+Sa significantly decreased cytochrome C and calmodulin kinase 2 ($P \leq 0.05$). In addition, ET+Sa significantly reduced calmodulin kinase two more than training ($P=0.04$).

Conclusion: Based on the results, Training and saffron consumption appear to have the same impacts on reducing cytochrome C and calmodulin kinase 2. However, simultaneous training and saffron consumption can improve the gene expression levels of calmodulin kinase 2.

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Introduction

Alzheimer's disease (AD) is a type of brain dysfunction that can progressively affect memory and other mental abilities, including thinking, reasoning, and judgment (1). A person's mental behavior is also affected by this disease, along with their spatial memory and cognitive abilities (2). Available data show that changes in amyloid plaques and increased levels of amyloid beta (Aβ) may affect the fibrosis of nervous system neurons. In addition to Alzheimer's, heart diseases are a significant and pervasive health issue affecting nearly 23 million people worldwide. Based on reports, a history of obesity, gender, and age are major risk factors that may develop Alzheimer's (AD) and heart disease (3).
In addition, researchers have shown a relationship between Alzheimer's and heart diseases in the elderly, so the dysfunction of peripheral vessels and the accumulation of atherosclerosis plaques are related to the incidence of stroke and cognitive disorders. Moreover, studies have indicated that disturbance in the oxidant-antioxidant system following aging and Alzheimer's disease causes disturbance in the sympathetic and parasympathetic system and plays a role in cardiac arrhythmia via disturbance in beta-adrenergic receptors.
More precisely, the increase of free radicals leads to disturbances in the number of calcium ions, calcium channels, and, eventually, the heart rhythm (4). In addition, studies have revealed that the increase of amyloid plaques in Alzheimer's disease and the resulting increase in oxidative stress in the brain tissue are related to inflammation and peripheral oxidative stress, increasing the risk of apoptosis from oxidative pathways and releasing cytochrome C from mitochondria in many peripheral organs (5).
Researchers have demonstrated that increased levels of amyloid beta can cause flaws in

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releasing acetylcholine with its toxic effects on the autonomic nervous system. Consequently, heart cells can induce defects in the calcium system (6). The signaling pathway can be attributed to calmodulin, a major calcium-binding protein involved in many cellular functions by activating calmodulin-dependent enzymes, which also regulate the activity of many ion channels, the calcium membrane pump, and the release of neurotransmitters (7). The actions of calmodulin are regulated by its enzyme, which is calmodulin kinase (8). Calcium-dependent protein kinase and calmodulin II, especially alpha and beta isoforms, play a significant role in critical neurological functions, including synthesizing neurotransmitters, releasing neurotransmitters, modifying ion channel activity, cell transfer, and cell morphology (9). Defects in the calcium system and its relationship with apoptosis have been well defined, so the decrease in calcium entry following oxidative acids causes calcium to leak into the cytosol. Then, the electron transport chain releases cytochrome C from the mitochondrial membrane and initiates apoptosis, which decreases the expression of proteins responsible for mitochondrial biogenesis, such as sirtuin-1, PPARγ, and PGC-1α (10). The incidence of AD activates the molecular pathways of apoptosis and the increase of cytochrome C (11).

Exercise has been shown to benefit cognitive disorders (12) and heart diseases (13) due to the importance of preventing heart diseases, especially in those involved. On the other hand, scholars' attention has recently been drawn to using antioxidants due to the different results following exercise activities and the adverse effects of synthetic drugs (14). As one of these drugs, saffron is regarded as having numerous pharmacological effects, and its active ingredients can be used clinically and in health care for humans. Research has shown that saffron and its active ingredients have anti-tumor, anti-inflammatory, antioxidant, and antigenotoxic effects, which enhance memory and learning, protect neurons, lowers blood pressure, reduce blood fat and insulin resistance, and reduce apoptosis and mitochondrial biogenesis in the heart tissue of diabetic rats treated with streptozotocin poison (15,16).

Although studies have investigated the impact of simultaneous training and saffron consumption on cognitive (17) and cardiac functions (13), the interactive mechanism of these two in heart tissue is not yet well known. The importance of preventing heart diseases in patients with nervous system disorders suggests that conducting these basic studies can lead to promising prevention methods and effective treatment for these people. Hence, this study attempted to examine whether eight weeks of endurance training and saffron consumption affect cytochrome C and calmodulin kinase 2 in the heart tissue of Alzheimer's rats treated with trimethyl tin (TMT).

Materials and Methods

In this experimental trial, 48 male Sprague Dawley rats aged 8-10 months and an approximate weight of 240 ± 30.65g were selected as samples. The animals had free access to food and water during the research period. The approved ethical code was IR.IAU.NAJAFABAD.REC.1401.181. A total of 40 rats received 8mg/kg neurotoxin trimethyl tin (TMT) intraperitoneally one week after adaptation to induce AD (Sigma, Germany: Cat No: 146498) (17). Memory and learning analysis were examined via a shuttle box test to detect AD in rats (18). AD rats were divided into five groups, including 1) the sham group (crocin solvent), 2) ET+Sa, 3) Sa, 4) ET, and 5) AD. Moreover, eight healthy rats were selected as the healthy control group (HC).

The induction of Alzheimer's disease was evaluated based on Yazdani et al.'s (2015) three stages habituation, memory acquisition, and memory retrieval. For this purpose, the rats were introduced to the shuttle box in the habituation phase. Rats were placed in a light compartment to learn to induce pain, and when they entered, they were shocked with 5MHz, 2mA for 3s. Next, the delay in entering the dark compartment, the duration of staying in the dark room, and the number of entering the dark compartment were measured in eight sick and eight healthy rats and compared in the memory retrieval phase 24 hours later (18).

The ET and ET+Sa groups trained at a speed of 15 to 20m/min on a treadmill for eight weeks, three sessions a week, each lasting 15 to 30min (19). In addition, the Sa and ET+Sa groups received intraperitoneally 25mg/kg aqueous extract of saffron dissolved in normal saline daily (20).

About 100mg of each tissue sample was cooled with 750 microliters of commercial Trizol

solution (Trizol Iran, YektaTajhiz Co.) and then homogenized to measure the research variables. Next, RNA was extracted using the kit protocol of CinnaGen-Iran. A spectrophotometric and optical absorption determination method was used to measure RNA's quantity and quality after extraction at 260nm wavelength. A260/A280 ratio calculation was used to determine the RNA purity level. When RNA with high purity and concentration was extracted from all studied samples, cDNA synthesis steps were performed based on the manufacturer's protocol (Biofact, South Korea). Then, the synthesized cDNA was utilized to perform the reverse transcription reaction. First, the primers designed were checked for genes (primary gene and internal control gene) using the NCBI website, and then the expression of genes was studied using the quantitative q-RT PCR method. Table 1 presents information about the primers implemented to assay each gene. The formula $2^{-\Delta\Delta Ct}$ was applied to quantify the data after completing the device activity and viewing the graphs. The sequence of the primers applied in the research is presented in Table 1.

Table 1. The Sequence of research primers in the study

Gene	Primer sequence	Sizes (Bp)
B2m	Forward:*5'- CGTGCTTGCCATTCAGAAA -3'* Reverse:*5'-ATATACATCGGTCTCGGTGG -3'*	244
Camk2a	Forward: *5'- AGCTGGTGCCTACGATTTCC -3'* Reverse: *5'- ACTTCTTCAGGCAGTCCACG -3'*	200
CytC	Forward: *5'-CTACTAATGAATAATTCCACTGCCT -3'* Reverse: *5'-CATTGTTAGCCATTCATGATCT-3'*	115

Eventually, the Shapiro-Wilk test, one-way ANOVA, and Tukey's post hoc test were run in SPSS22 software to analyze the data (P≤0.05).

Results

The mean and SD (standard deviation) of gene expression levels of cytochrome C and calmodulin kinase are presented in Figures 1 and 2, respectively. The results of a one-way analysis of variance revealed a significant difference in the gene expression levels of cytochrome C (P=0.001 and F=20.03) and calmodulin kinase (P=0.001 and F=20.04) in the research groups.

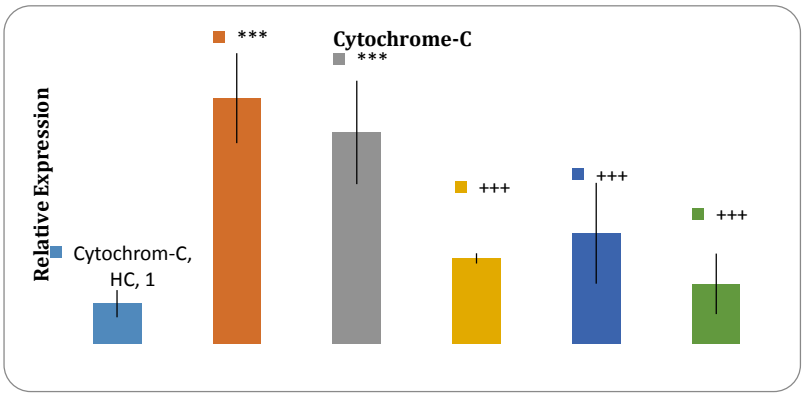

Figure 1. Cytochrome C gene expression levels in the research groups
***(P≤0.001) significant increase compared to the HC group
+++(P≤0.001) significant decrease compared to the AD group

Tukey's post hoc test results indicated that gene expression levels of cytochrome C in the AD and Sh groups were significantly higher than in the HC group (P=0.001). Nonetheless, the levels were significantly lower in the ET, Sa, and ET+Sa groups than in the HC group (P=0.001) (Figure 1). Further, gene expression levels of calmodulin kinase in the AD and Sh groups were significantly higher than in the HC group (P=0.001). However, the levels were significantly lower than the levels in the HC group in the ET (P=0.04), Sa (0.001) (P=0.001), and ET+Sa (P=0.001) groups. In addition, the levels in the ET+Sa group were significantly lower than the ET group (P=0.04) (Figure 2).

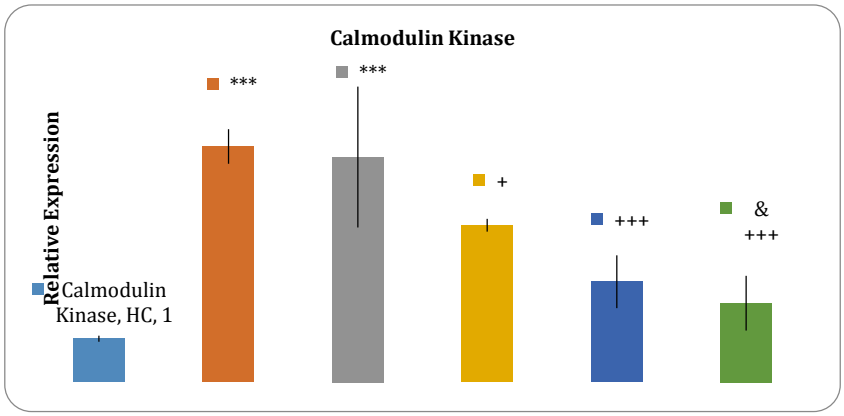

Figure 2. Calmodulin kinase gene expression levels in the research groups
***(P≤0.001) significant increase compared to the HC group
+(P≤0.05), +++(P≤0.001) significant decrease compared to the AD group
&(P≤0.05) significant decrease compared to the ET group

Discussion

The results showed that the induction of Alzheimer's significantly increased the gene expression levels of cytochrome C and calmodulin kinase in the heart tissue of rats. Organotin trimethyl tin chloride (C3H9ClSn or TMT) is a potent neurotoxin that can cause selective damage to the central nervous system. Today, animal models of AD are widely used to induce the disease (21). Increasing oxidative stress, reducing antioxidants, disrupting mitochondrial function, and causing neuronal death in different parts of the central nervous system are among the mechanisms of the neurotoxic effects of TMT (21). The growth in the accumulation of amyloid beta (Aβ) following Alzheimer's disease causes a decrease in the activity of AMPK, a decline in cytosolic Ca^{2+}, phosphorylation of Ca^{2+}-CaMKKβ, a growth in autophagy and ubiquitin-proteasome system activation and as hencea breakdown in proteins such as PGC-1α and SIRT1. These factors disrupt the function of the mitochondrial DNA of neurons and, eventually, mitochondrial cell death (21). The increase of ROS disrupts the oxidant-antioxidant balance and results in the damage of biological macromolecules, including membrane lipids, proteins, and DNA. An increase in the oxidation of membrane lipids increases MDA and oxidative stress intensifiers such as prooxidant/antioxidant balance (PAB) markers. Afterward, this process activates the caspase cascade, increases cytochrome-C oxidase, releases cytochrome-C, and induces cell death (22).

However, eight weeks of ET significantly reduces the gene expression levels of cytochrome C and calmodulin kinase 2 in the heart tissue of rats with Alzheimer's disease. Consistent with the present study, 12 weeks of aerobic training resulted in a significant decrease in cytochrome-C levels in the heart muscle of rats (23). In addition, running with different protocols (2, 4, and 6 days of running on a treadmill per week for 120min for 14 days) significantly decreased cytochrome-C in the muscle tissue of female rats (24). Additionally, three months of endurance training significantly reduced cytochrome-C in the muscle tissue of rats (25). Sports activities have been suggested to increase antioxidants by regulating the renin-angiotensin-aldosterone system (26), lower oxidative stress and apoptotic TNF receptors (27), drop Cyt-P4A, grow eNOS, activate NADPH oxidase (28), amend fat metabolism, regulate minerals, and rise mitochondrial membrane stability to improve ATP and decline Cyt-C, MDA and PAB release (26,29). Studies have indicated that the induction of ROS following sports activities is a crucial pathway for cellular biological mechanisms. However, the excessive increase in oxidative stress following sports activities can negatively affect cells, as the amount of oxidative stress induced by sports activities depends on the length of the training period and intensity (22).

In the current study, eight weeks of saffron consumption significantly reduced the gene expression levels of cytochrome C and calmodulin kinase in the heart tissue of Alzheimer's rats. Studies have revealed that

saffron and its products can favor the hippocampus, memory, and learning in animal samples with degenerative disorders. The potent antioxidant properties of crocin and crostini in saffron may help increase neurons' flexibility and minimize inflammation and death, thus enhancing neurogenesis (20). However, researchers believe that the primary physiological effects of saffron depend on its dose and duration. In confirming the findings of this study, Razavi et al. (2015) and Zeinali et al. (2019) reported that saffron, with its antioxidant effects, could significantly reduce cytochrome C (30,31). Researchers believe that saffron improves the process of enhancing synaptic stimulation in the hippocampus after applying high-frequency stimulation on presynaptic neurons and a long-term increase in synaptic potentials, thus improving the process of memory and learning.

Regarding the interactive effects, the results of the current study revealed that ET, together with saffron consumption, significantly reduced the gene expression levels of cytochrome C and calmodulin kinase in the heart tissue of rats with Alzheimer's disease. Moreover, ET combined with saffron consumption has a more significant effect than ET alone on decreasing calmodulin kinase gene expression levels in the heart tissue of Alzheimer's rats. Based on studies, training and saffron consumption with different mechanisms improve the disorders caused by Alzheimer's disease. However, the current research indicated that ET along with saffron consumption has more favorable impacts on reducing calmodulin kinase than ET. Sports activities stimulate synaptic activity in the hippocampus after applying high-frequency stimulation to presynaptic neurons, and a long-term increase in synaptic potential prevents neurodegeneration in these cells. Thus, sports activities can reduce the disorders caused by AD by increasing BDNF.

A limitation of the present study can be the lack of accurate control of the amount of food consumed by rats during the research period and measuring cytochrome C and calmodulin kinase two protein levels. Therefore, it is recommended to investigate the effects of ET and saffron consumption on cytochrome C and calmodulin kinase two protein levels in the subsequent analyses to confirm the results.

Conclusion

Based on the results, training and saffron consumption reduce the gene expression levels of cytochrome C and calmodulin kinase 2. However, simultaneous training and saffron consumption can interact with improving the gene expression levels of calmodulin kinase 2.

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