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Original Article

Fish Oil Attenuates Nicotine-Seeking Behavior and Oxidative Stress During Withdrawal in Stressed Mice

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Abstract

Introduction: Tobacco use remains a major cause of death worldwide, and stress significantly contributes to nicotine addiction and relapse. Emerging evidence indicates that fish oil-derived omega-3 fatty acids can reduce cravings and improve mood, offering promise for addiction management. This study examined the effects of fish oil on drug-seeking behavior and anxiety during nicotine withdrawal, particularly under stress.

Methods: Forty male NMRI mice were divided into eight groups based on stress exposure, nicotine or saline, and diet (fish oil or standard). Nicotine (0.5 mg/kg, SC) successfully established conditioned place preference (CPP) without affecting locomotor activity. Drug-seeking behavior was tracked over 8 days across conditioning, addiction, and withdrawal phases. At the end, anxiety levels, serum and brain total antioxidant capacity (TAC), and malondialdehyde (MDA) were quantified.

Results: Fish oil significantly reduced the total time animals remained in the white side during nicotine withdrawal in nicotine-treated groups ($P<0.05$), regardless of stress. Notably, this duration increased during withdrawal compared to conditioning ($P<0.05$ to $P<0.01$). In the open field test, fish oil significantly attenuated anxiety-like responses ($P<0.001$). However, this effect was not observed in the elevated plus maze. Stress-induced MDA levels were significantly decreased by fish oil ($P<0.001$).

Conclusion: Fish oil effectively diminishes drug-seeking behavior during nicotine withdrawal and reduces oxidative stress, highlighting its potential as a supportive treatment for nicotine dependence, especially under stress conditions.

Keywords: Tobacco use, Stress, Fish oils, Drug-seeking behavior, Oxidative stress

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Introduction

According to recent epidemiological data, smoking is a leading cause of death, claiming millions of lives each year worldwide.[1] Nicotine, the primary addictive component of tobacco, exerts its effects by acting on nicotinic acetylcholine receptors in the brain, leading to reinforced reward pathways and dependence.[2] Despite the rising awareness of the health risks of smoking, quitting smoking continues to be challenging due to the neurochemical and behavioral aspects of addiction, which are often further complicated by stress.[3]

Stress is a well-established factor that enhances vulnerability to nicotine addiction and relapse.[4] Repeated stress triggers neuroadaptive changes in dopaminergic and serotonergic systems, which can increase drug-seeking behavior and impede cessation efforts.[5] Moreover, withdrawal symptoms, including heightened cravings and anxiety, significantly contribute to relapse risk.[6] Consequently, identifying interventions that mitigate withdrawal symptoms and reduce relapse likelihood under stressful conditions is a major focus in addiction research.

A growing body of literature suggests that long-chain omega-3 fatty acids, widely found in fish oil, exhibit neuroprotective, anti-inflammatory, and mood-enhancing properties.[7] Several studies have indicated that omega-3 supplementation can attenuate cravings and improve mood states associated with substance withdrawal.[8,9] These effects are thought to arise from omega-3's influence on brain plasticity, neurotransmission, and oxidative stress pathways.

A state of oxidative stress is defined by an overabundance of oxidants relative to available antioxidants, and this condition is associated with neuroadaptive changes related to addiction.[10] Elevated levels of malondialdehyde (MDA), a biomarker of lipid peroxidation, are commonly observed during nicotine withdrawal and chronic stress, reflecting increased oxidative damage.[11] Interventions that reduce oxidative stress may therefore help alleviate withdrawal symptoms and decrease relapse susceptibility. Preclinical studies have begun to explore the potential of omega-3

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fatty acids to modulate addiction behaviors. Fish oil supplementation has been shown to reduce drug-seeking behaviors in animal models for cocaine and heroin.[12,13] However, data specifically addressing nicotine withdrawal, especially in the context of stress, remain limited.

This study aims to fill this gap by investigating whether fish oil can attenuate drug-seeking behavior and anxiety during nicotine withdrawal in a mouse model subjected to stress. By examining behavioral outcomes along with biochemical markers of oxidative stress, such as serum and brain MDA levels, the research seeks to evaluate the therapeutic potential of omega-3 supplementation as an adjunct in nicotine dependence treatment, particularly under stress-related conditions.

Methods

Subjects

The study involved 40 male NMRI mice, each weighing between 25–35 grams. The mice were provided with sufficient food and water and kept at a suitable temperature in a 12-hour light/dark cycle. The Ethics Committee of Animal Use at Shahrekord University of Medical Sciences approved the research (IR.SKUMS.AEC.1402.023), and all procedures adhered to the guidelines set forth for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23), as revised in 2010.

Experimental procedures

The animals were categorized into two groups: stressed (20 animals) and non-stressed (20 animals). The stressed group was further split into four sub-groups: saline + standard food, saline + fish oil supplement, nicotine + standard food, and nicotine + fish oil supplement. The non-stressed group was also divided into four sub-groups: saline + standard food, saline + fish oil supplement, nicotine + standard food, and nicotine + fish oil supplement. The mice in the supplement-receiving groups were given fish oil throughout the conditioned place preference (CPP), addiction, and withdrawal phases, which lasted 19 days. Following a 24-h withdrawal period (day 20), anxiety-like behavior was evaluated using two established paradigms: the elevated plus-maze (EPM) was administered in the morning, and after a 6-h inter-test interval, the open field test (OFT) was conducted in the evening.

Chemicals

The nicotine dose (0.5 mg/kg, free-base) was prepared accordingly using nicotine hydrogen tartrate salt (Sigma-Aldrich; N5260). The salt was dissolved in saline, and the solution was injected subcutaneously (pH 7.4, 1 mL/kg).[14] Fish oil (3 g/kg) was administered via oral gavage;[15] each gram of the supplement contained 180 mg EPA and 120 mg DHA.

Equipment

The CPP test was conducted using a custom-built three-chamber apparatus.[16] Two main sections (A and B) were identical in size (30 × 30 × 30 cm) but visually distinct: section A featured alternating black/white vertical stripes with a rough floor, whereas section B had plain white walls and a smooth floor. A smaller, red-colored tunnel (30 × 15 × 30 cm) served as the neutral start box and was connected to both conditioning chambers by removable partitions. Weight-sensitive floor sensors automatically recorded the time spent in each section. An overhead video camera (2 m above the apparatus) captured all sessions, and the footage was later analyzed using dedicated tracking software.

Behavioral Testing

Assessment of Place Conditioning

A biased protocol, which is more effective than unbiased designs for nicotine-induced place preference, was adopted for the CPP experiments.[14,17] The procedure spanned 10 consecutive days and was divided into three consecutive phases: baseline (pre-conditioning), conditioning, and testing.

Pre-conditioning

During the baseline phase (day 1), each animal was introduced into the section (C) with the guillotine doors removed, allowing free access to all chambers for 15 minutes. The time spent in each section was recorded to determine the initial preference and identify the non-preferred side for nicotine pairing.[18]

Conditioning

The animals were given alternating drug injections over 8 days to be trained to a designated location. Thus, on the second day at 9 am, after the animals were weighed, each received a subcutaneous injection of 0.5 mg/kg nicotine and was placed in the non-preferred chamber of the apparatus for 20 minutes after the guillotine valve was closed. Six hours later, they received an equal volume of saline (vehicle) and were then placed in the preferred (checkered) chamber for 20 minutes. On the third day, the order of nicotine and saline injections was switched (saline in the morning and nicotine in the evening). The injection schedule on the fourth day was identical to that of the second day.[19] This injection pattern continued up to the ninth day.

Post-conditioning

On the tenth day of the experiments (the final day of each experimental period), the guillotine valve was first removed. Each animal was then allowed to move freely in both sections of the apparatus for 15 minutes. The duration each animal spent in each area of the apparatus was recorded, particularly noting the time spent in the white chamber (the area where nicotine was administered) on the testing day.

Addiction phase

The mice in the nicotine treatment groups were administered nicotine at a daily dosage of 6 mg/kg for an additional 6 days,[20]. In contrast, the saline group was given

only saline (vehicle) during this timeframe. This dose of nicotine was delivered slowly. During this time, the mice developed a nicotine dependence, and the duration spent in the white chamber was measured once more.

Withdrawal phase

The mice were then housed in their cages for 3 days without exposure to nicotine, saline, or CPP, with free access to standard chow and water. On the fourth day of nicotine withdrawal, each mouse was placed alone in section C and allowed to explore the CPP box for 15 minutes after the sliding doors were opened; their actions were recorded on video for assessment. Overall, the drug-seeking behavior of the animals was evaluated in four phases: pre-conditioning, conditioning, addiction, and nicotine withdrawal.[21] After the experiment concluded, anxiety and locomotor activity were measured using the EPM and the OFT.

Stress protocol

Stress was created by confining animals in a modifiable restraint chamber for 2 hours each day (8:00–10:00), one week before CPP. Multiple openings in the cylinder walls allowed airflow. During this period, the animals were unable to move or turn around.[22]

Elevated plus maze (EPM) test

The EPM apparatus was composed of a 10 cm × 10 cm central platform made of dark Plexiglas, from which four arms (two open and two closed) extended: two open arms (50 cm × 10 cm) without side walls, and two closed arms (50 cm × 10 cm) with 15-cm-high side walls. The entire setup was raised 80 cm above the floor. For testing, each mouse was gently placed on the central square, facing an open arm, and allowed to move freely for 5 minutes. The time spent in the open arms was recorded and used as a measure of anxiety-like behavior.[23]

Open field test (OFT)

The open-field assessment is utilized to investigate locomotor behavior in rodent models. This evaluation was conducted in a calm setting, brightened by a fluorescent lamp positioned directly above the enclosure. In this study, a square fiberglass container was used. A 100 × 100 cm green screen made of the same material was positioned 8 cm from the bottom of the container. This screen was divided into 16 equal segments, with four segments on each side of the container. Openings with a diameter of 3 cm were made at the center of each segment and in the corners and midpoints of the middle segments. At the end of the experiment (day 20), the mice were individually placed in one corner of the container, and behavioral parameters were recorded for 5 minutes, focusing on the duration spent in the center area.[24]

Measurement of MDA level

To quantify MDA levels in serum or brain homogenate solution, 0.5 g of thiobarbituric acid was added to 80 mL of 20% acetic acid solution, and the pH was adjusted to 3.5 with sodium hydroxide (NaOH) using a pH meter. The final volume was brought to 100 mL using 20% acetic acid. Next, 100 µL of serum or brain supernatant was combined with 100 µL of 1.8% sodium dodecyl sulfate (SDS) and 2.5 mL of the previously prepared solution in glass test tubes sealed with aluminum caps. The tubes were then heated in a boiling water bath for 1 hour. Following this incubation, the tubes were allowed to cool. They were centrifuged at a speed of 4000 rpm, after which the optical absorbance of the supernatant was measured at a wavelength of 523 nm.[25]

Measurement of total antioxidant capacity (TAC)

TAC was evaluated using the FRAP (ferric reducing antioxidant power) assay. In brief, 25 µL of each sample (serum or brain homogenate) was added to 1.5 mL of freshly prepared FRAP reagent, consisting of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in 40 mM HCl), and $FeCl_3 \cdot 6H_2O$ (20 mM). The mixture was incubated at 37 °C for 10 minutes, during which the reduction of the Fe^{3+}-TPTZ complex to its blue-colored Fe^{2+} form was monitored. The absorbance was then read at 593 nm using a spectrophotometer. Antioxidant levels were expressed as µg/mL equivalents relative to the sample volume.[26]

Statistical analyses

GraphPad Prism (v. 8.0) was used for all data analyses, and the findings were presented as mean ± SEM. A three-way ANOVA (2 × 2 × 2 design) was performed with nicotine treatment (saline vs. nicotine), stress (stress vs. no stress), and diet (standard diet vs. fish oil) as independent factors. Time spent in the white chamber during the conditioning, addiction, and withdrawal phases was analyzed separately using three-way ANOVA with stress, treatment, and diet as between-subject factors. For multiple comparisons, Tukey's post hoc test was then applied following the three-way ANOVA. The time spent in the white compartment across different stages was analyzed by two-way ANOVA. The statistical inference criterion was $P < 0.05$.

Results

Pre-conditioning exploratory behavior in the CPP paradigm

One week after the stress protocol was completed, the place conditioning procedure was initiated for both groups. The first day of each session was designated as the pre-conditioning day.

During the pre-conditioning phase, referred to as the habituation day, each animal was placed inside the apparatus for 15 minutes immediately after the guillotine door was lifted. The time animals spent in each compartment was measured to assess baseline preferences.

Statistical analysis using the *t*-test indicated a significant reduction in the time spent in the white chamber by the stressed group compared to the non-stressed group ($t(38) = 3.562$, $^{**}P < 0.01$, $d = 0.25$) (Figure 1).

Time in the white CPP chamber in the conditioning phase

Based on the three-way ANOVA, fish oil had no significant

effect on mean time spent in the white chamber of the CPP apparatus across experimental groups (supplement effect: $P = 0.0509$).

Analysis revealed a significant main effect for nicotine treatment on duration of time in the white chamber (Treatment effect: $F\ (1, 32) = 11.78$, $P = 0.0017$, $\eta p^2 = 0.27$). Stress exposure also showed a significant main effect (stress effect: $F\ (1, 32) = 8.194$, $P = 0.007$, $\eta p^2 = 0.20$) (Figure 2).

A significant interaction was detected between supplement and nicotine treatment ($F\ (1, 32) = 4.214$, $P = 0.048$, $\eta p^2 = 0.17$), indicating that the effect of supplement varied depending on the injection condition.

Time in white CPP chamber in the addiction phase

Three-way ANOVA revealed that the groups receiving a high nicotine dose spent significantly more time in the white compartment than the saline-treated groups. Therefore, there was a significant main effect of nicotine treatment on time spent in the white chamber ($F\ (1,32) = 54.73$, $P < 0.0001$, $\eta p^2 = 0.63$) (Figure 3).

Time in white CPP chamber in withdrawal phase

Analysis revealed a significant main effect for fish oil on duration of stay in the white chamber during the withdrawal phase (supplement effect: $F\ (1, 32) = 26.93$, $P < 0.0001$, $\eta p^2 = 0.46$). Nicotine exposure also showed a significant main effect (treatment effect: $F\ (1, 32) = 177.8$, $P < 0.0001$, $\eta p^2 = 0.85$)

Moreover, the findings indicated that fish oil significantly reduced the time spent in the white chamber during nicotine withdrawal in the nicotine-receiving groups (*$P < 0.05$, ***$P < 0.0001$) (Figure 4).

Time in the white CPP chamber between different stages

Next, we compared time spent in the white chamber across stages in the studied groups. The results of the two-way ANOVA test revealed a significant difference in the time allocation by the mice in the non-preferred section (white) among the nicotine-receiving groups across the conditioning, addiction, and nicotine withdrawal stages. In all nicotine-receiving groups, the duration of stay in the white side during the withdrawal stage increased significantly compared to the conditioning stage (Figure 5).

Assessment of anxiety-like behavior in the studied groups

Based on the three-way ANOVA analysis, diet had no significant effect on the mean time spent by mice in the open arms of the EPM across the experimental groups ($P > 0.05$). There was a significant main effect of treatment

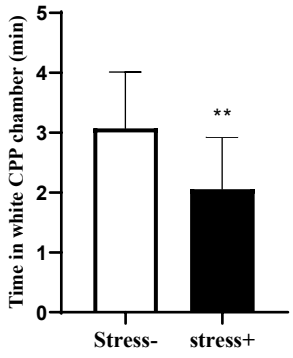


Figure 1. The average time spent in the white compartment of the CPP apparatus during the pre-conditioning phase was compared between stressed (stress+) and non-stressed (stress–) groups. Data are expressed as mean ± standard error of mean (SEM) and were statistically evaluated using an independent samples t-test

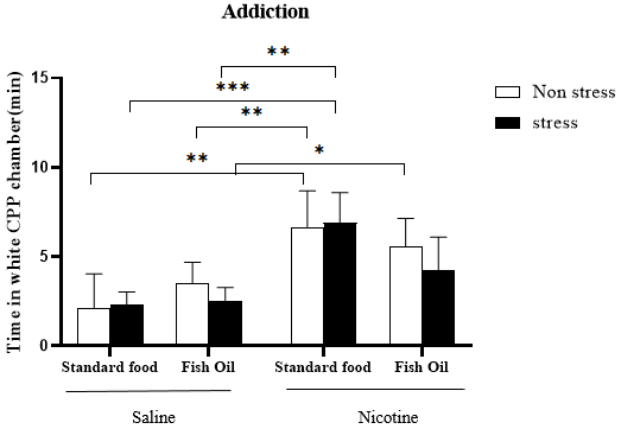


Figure 3. Mean time spent in the white compartment of the CPP for nicotine addicted mice among the studied groups after six days of subcutaneous high-dose nicotine injections. Values are presented as mean ± SEM *$P<0.05$, **$P<0.01$, ***$P<0.001$

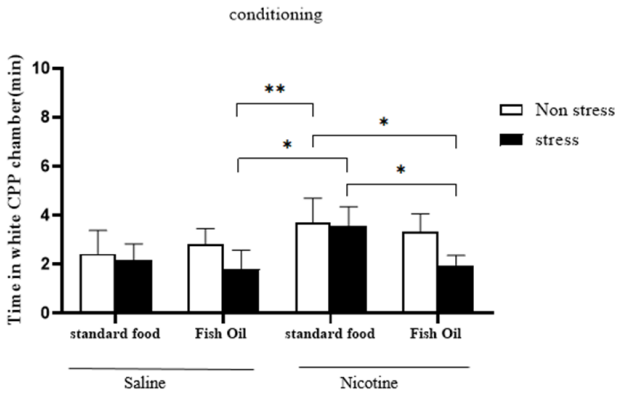


Figure 2. Mean time spent in the white compartment of the CPP across experimental groups during the 8 days of place conditioning. Values are expressed as mean ± SEM. *$P < 0.05$, **$P<0.01$

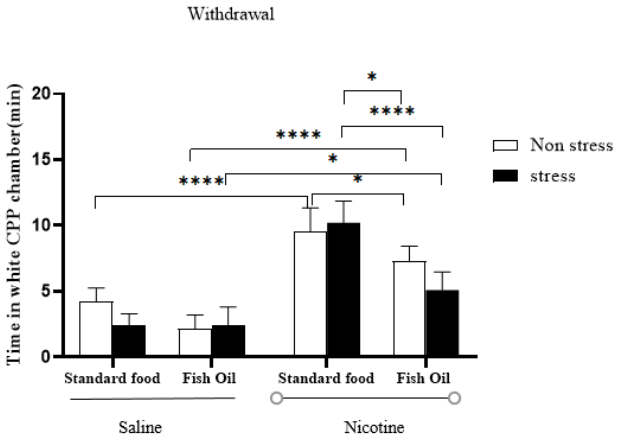


Figure 4. Mean time spent in the white compartment of the CPP across experimental groups following the nicotine withdrawal phase. Values are expressed as Mean ± SEM. *$P < 0.05$, ****$P<0.0001$

on time spent in the open arms of the EPM (nicotine effect: F (1, 32) = 130.9, $P < 0.0001$, $\eta p^2 = 0.8$). Stress exposure also showed a significant main effect (stress effect: F (1, 32) = 38.33, $P < 0.0001$, $\eta p^2 = 0.54$).

A significant interaction was found between the main effects of diet × treatment (F (1, 32) = 4.307, $P = 0.046$, $\eta p2 = 0.12$), indicating that the effect of the supplement varied depending on nicotine. Tukey post hoc tests revealed that time spent in the open arms of the EPM was significantly lower in nicotine-treated groups receiving a fish oil diet than in saline-treated groups with the same diet.

There was also a significant interaction between stress and diet (F (1, 32) = 15.11, $P < 0.001$, $\eta p^2 = 0.32$), indicating that the supplement's effect varied by stress (Figure 6).

Assessment of exploratory behavior in the Studied Groups

Analysis of the time spent in the open field central zone with three-way ANOVA showed that diet significantly influenced this behavior (Diet effect: F (1, 32) = 29.37, $P < 0.0001$, $\eta p^2 = 0.48$). Tukey's post hoc tests revealed that fish oil led to a significant increase in exploratory behavior compared to the groups fed a standard diet.

There was a significant main effect of treatment on exploratory behavior (nicotine effect: F (1, 32) = 53.45, $P < 0.0001$, $\eta p^2 = 0.62$). Stress exposure also showed a significant main effect (stress effect: F (1, 32) = 19.44, $P < 0.0001$, $\eta p^2 = 0.38$). There were no other effects or interactions ($P > 0.05$) (Figure 7).

Assessment of serum MDA levels in the Studied Groups

Three-way ANOVA of serum MDA level revealed main effects of treatment (F (1, 32) = 192.4, $P < 0.0001$, $\eta p^2 = 0.86$), stress (F (1, 32) = 76.53, $P < 0.0001$, $\eta p^2 = 0.7$) and diet (F (1, 32) = 65.77, $P < 0.0001$, $\eta p^2 = 0.67$). Tukey's post hoc tests showed that dietary supplementation with fish oil significantly decreased MDA concentrations relative to the standard diet groups.

A treatment × diet interaction was observed (F (1, 32) = 3.702, $P < 0.05$, $\eta p^2 = 0.1$), with post hoc tests showing MDA levels were significantly elevated in nicotine-exposed groups administered fish oil compared with saline-treated counterparts. The effect of fish oil differed significantly between nicotine- and saline-treated groups, suggesting that its effect varied by treatment.

A statistically significant stress × diet interaction was observed, suggesting that dietary composition modulates the impact of stress (F (1, 32) = 6.099, $P < 0.01$, $\eta p^2 = 0.16$). Stress significantly elevated MDA levels among subjects fed a fish oil-enriched diet (Figure 8).

***Assessment of brain MDA levels in* the Studied Groups**

Three-way ANOVA of brain MDA level revealed main

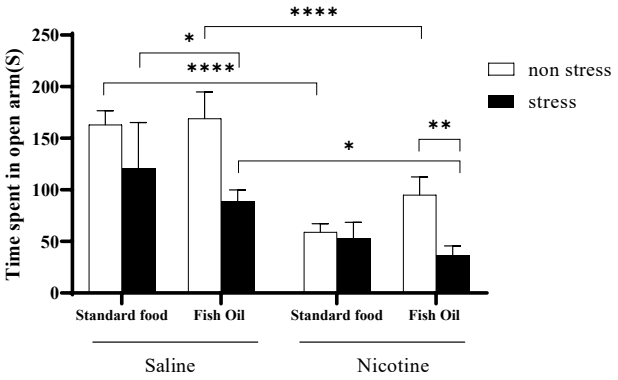


Figure 6. Comparison of the mean time spent in the open arms of the EPM among the experimental groups. A three-way ANOVA followed by Tukey's post hoc test was performed. Results are presented as mean ± SEM (number of mice per group = 5)

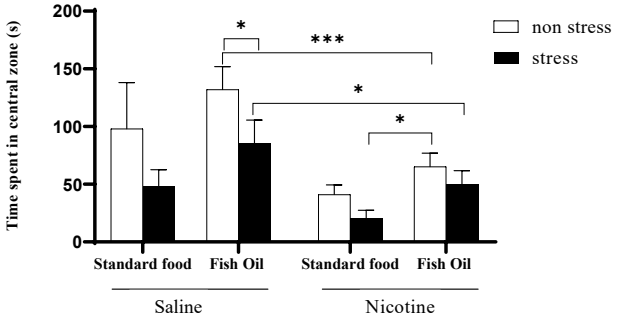


Figure 7. Comparison of the mean time spent in the central zone of the open field apparatus among the experimental groups. A three-way ANOVA followed by Tukey's post hoc test was performed. Results are presented as mean ± SEM (number of mice per group = 5)

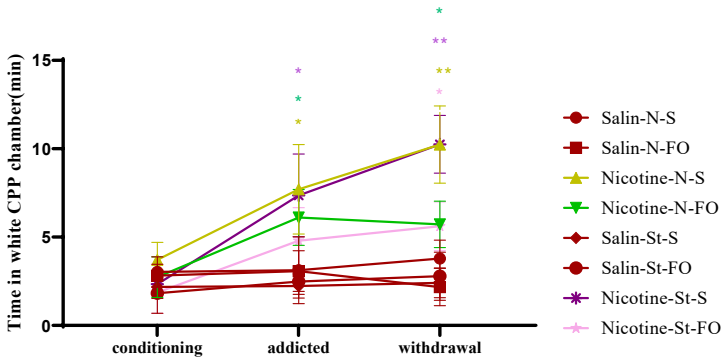


Figure 5. Comparison of the mean time spent by mice in the white compartment of the CPP apparatus across different stages of conditioning, addiction, and nicotine withdrawal in the studied groups. Values are presented as Mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ indicate comparisons with the conditioning stage. The nicotine-receiving groups included: **Nicotine-N-S** (no stress, standard diet), **Nicotine-N-FO** (no stress, fish oil supplementation), **Nicotine-St-S** (with stress, standard diet), **Nicotine-St-FO** (with stress, fish oil supplementation). The saline-receiving groups included: **Saline-N-S** (no stress, standard diet), **Saline-N-FO** (no stress, fish oil supplementation), **Saline-St-S** (with stress, standard diet), **Saline-St-FO** (with stress, fish oil supplementation)

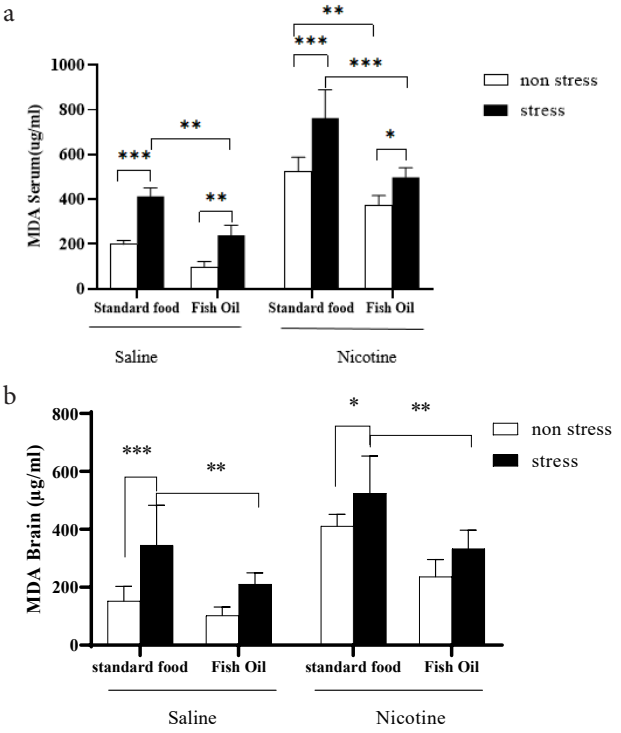


Figure 8. Comparing serum (a) and brain (b) MDA levels in the experimental groups**.** A three-way ANOVA followed by Tukey's post hoc test was performed. Results are presented as mean ± SEM (number of mice per group = 5)

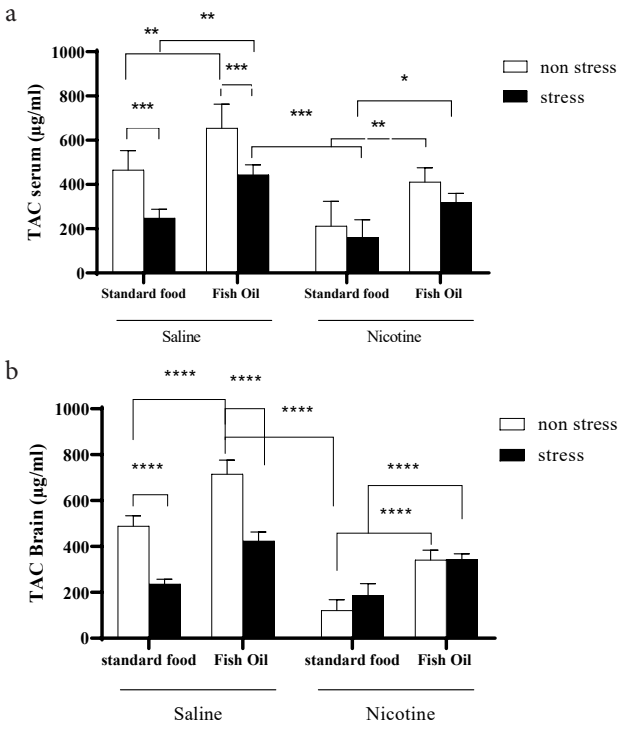


Figure 9. Comparing serum (a) and brain (b) TAC levels in the experimental groups**.** A three-way ANOVA followed by Tukey's post hoc test was performed. Results are presented as mean ± SEM (number of mice per group = 5)

effects of treatment (F (1, 32) = 46.37, $P < 0.0001$, $\eta p^2 = 0.59$), stress (F (1, 32) = 41.29, $P < 0.0001$, $\eta p^2 = 0.56$) and diet (F (1, 32) = 40.58, $P < 0.0001$, $\eta p^2 = 0.56$). Tukey's post hoc tests showed that dietary supplementation with fish oil significantly decreased MDA concentrations relative to the standard diet groups.

There was a stress × diet interaction (F (1, 32) = 6.306, $P < 0.05$, $\eta p^2 = 0.16$), and post hoc tests showed that MDA levels were significantly elevated in stressed groups administered fish oil compared with their non-stressed counterparts. The effect of fish oil differed significantly between stressed and non-stressed groups, suggesting that its effect varied depending on stress.

Assessment of serum TAC levels in the studied groups

Three-way ANOVA of serum TAC level revealed main effects of treatment (F (1, 32) = 64.69, $P < 0.0001$, $\eta p^2 = 0.67$), stress (F (1, 32) = 50.80, $P < 0.0001$, $\eta p^2 = 0.61$) and diet (F (1, 32) = 64.33, $P < 0.0001$, $\eta p^2 = 0.67$). Tukey's post hoc tests showed that dietary supplementation with fish oil significantly increased TAC levels relative to the standard diet groups.

Treatment interacted significantly with stress (F (1, 32) = 8.964, $P < 0.01$, $\eta p^2 = 0.22$). Post hoc analysis indicated that, in the absence of stress, nicotine exposure led to a notable reduction in total antioxidant capacity (TAC) levels compared to the saline-treated group. The effect of stress differed significantly between nicotine- and saline-treated groups, suggesting a treatment × stress (Figure 9).

Assessment of brain TAC levels in the studied groups

Three-way ANOVA of brain TAC levels revealed main effects of treatment (F (1, 32) = 241.1, $P < 0.0001$, $\eta p^2 = 0.88$), stress (F (1, 32) = 95.15, $P < 0.0001$, $\eta p^2 = 0.75$) and diet (F (1, 32) = 218.3, $P < 0.0001$, $\eta p^2 = 0.87$). Tukey's post hoc tests revealed that dietary supplementation with fish oil led to a significant increase in brain TAC levels relative to the standard diet groups.

Treatment interacted significantly with stress (F (1, 32) = 107.9, $P < 0.001$, $\eta p^2 = 0.77$). Post hoc analysis indicated that, in the absence of stress, nicotine exposure led to a notable reduction in total antioxidant capacity (TAC) levels compared to the saline-treated group. The effect of stress differed significantly between nicotine- and saline-treated groups, suggesting a treatment × stress.

Discussion

Nicotine addiction poses a major global health issue, leading to fatalities from cardiovascular conditions, various cancers, and respiratory diseases (WHO, 2020).[27] Owing to its high omega-3 content, fish oil appears to attenuate nicotine dependence, an effect that was especially evident during stress exposure. The prolonged time spent in the white chamber following nicotine administration indicates enhanced drug-seeking behavior and dependence. We expanded on this research by examining how fish oil supplementation, which is abundant in omega-3 fatty acids, influences drug-seeking behavior induced by nicotine withdrawal.

Our results revealed a significant difference in white chamber preference during the pre-conditioning phase between stressed and non-stressed mice ($P < 0.01$). Stressed mice spent less time in the white chamber and preferred the checkered chamber, likely as a strategy to evade stress. Stress

appears to intensify avoidance behaviors, leading mice to avoid potentially harmful environments.[28] These findings align with previous studies that demonstrate how stress reduces exploratory behavior and alters environmental preferences, with stressed mice spending less time in novel or unfamiliar settings.[29] In the conditioning phase, mice that received nicotine showed a marked increase in the duration spent in the white chamber compared to those given saline, underscoring nicotine's role as a positive reinforcer and the development of dependence. Evidence from both clinical and preclinical studies indicates that stress plays a pivotal role in multiple stages of drug addiction, including acquisition, maintenance, and relapse.[30,31] Environmental factors like stress can affect the reward system, increasing the likelihood of addiction and relapse by altering the neural circuits related to stress management and reward processing.[32-34] Both acute and chronic stress can enhance nicotine's reinforcing qualities by disrupting opioid circuits and raising sensitivity to stress. In contrast, even sub-chronic stress may provide protective or adaptive mechanisms against relapse.[35]

Activation of the HPA axis is a hallmark response to stress exposure, and subsequent elevation of glucocorticoids may enhance nicotine reward by increasing dopaminergic activity in mesolimbic pathways. Although stress has been reported to potentiate the rewarding effects of nicotine, no significant difference was observed between nicotine-treated stressed and non-stressed groups, and the stress × nicotine interaction was not significant at this stage. In the present study, stress exposure was applied prior to the initiation of the conditioning phase. The intensity and timing of the stress protocol may have influenced HPA axis activation in a manner that did not synergize with nicotine-induced dopaminergic signaling.

In contrast, a marked increase in time spent in the white compartment was observed during the addiction phase compared with the conditioning phase (Figure 5). This effect is more likely attributable to nicotine's pharmacological action than to stress exposure, as nicotine is known to strongly enhance reward-seeking behavior. A review of previous studies reveals that stress may differentially modulate morphine-induced CPP as a function of stress intensity and duration. Acute or moderate stress has been shown to attenuate morphine-induced CPP, an effect largely associated with alteration in ERK signaling. In contrast, chronic stress paradigms appear to enhance CPP through mechanisms involving the GABAergic system. Collectively, these findings highlight the complex and bidirectional nature of stress effects on opioid-related reward processing.[36,37]

A distinctive feature of our study is that, for the first time, we investigated how stress and Fish oil, a well-known source of omega-3 fatty acids, affect exploratory behavior during the stages of addiction and withdrawal in mice. Our findings showed that 19 days of fish oil supplementation significantly lowered the amount of time mice undergoing nicotine withdrawal spent in the white chamber during CPP trials, indicating a reduction in nicotine dependence and cravings. Previous studies back up these results, demonstrating that fish oil can alleviate CPP induced by drugs such as amphetamine,[15] cocaine,[12] and alcohol,[38] suggesting its potential role in preventing relapse and aiding in smoking cessation. Nevertheless, these findings are preliminary, and additional research is needed to confirm and clarify this potential. Moreover, in mice that were administered nicotine without experiencing stress or taking fish oil, the time spent in the white chamber was greatest during withdrawal, indicating increased drug-seeking behavior. Fish oil supplementation diminished this preference, likely by counteracting the adverse effects of nicotine withdrawal on learning and memory. It is well known that nicotine withdrawal can impair cognitive abilities through biochemical alterations in the hippocampus.[39] Research conducted by Ahmadi et al. (2023) indicated that omega-3 fatty acids can help avert learning and memory impairments linked to nicotine withdrawal in mice. Additionally, omega-3 was found to aid in reestablishing equilibrium in oxidative stress, inflammation, and serotonin metabolism, which are affected by nicotine withdrawal, thus alleviating behavioral issues like anxiety.[40]

Findings indicated that mice treated with nicotine and exposed to stress showed elevated anxiety levels compared to control groups. However, the relationship between stress and anxiety remains debated; some research indicates heightened anxiety and depression, while other studies report no effect or even decreases, depending on the type of stress and experimental conditions.[41,42] For instance, Hayase et al. (2013) discovered that nicotine and stress amplify each other's negative impacts on behavior, including anxiety and memory. [43] In contrast, Biala et al. (2017) indicated that nicotine alleviates anxiety and depressive behaviors induced by stress, which differs from our findings.[44] Factors such as individual differences, environmental influences, and consumption patterns also play a role in how nicotine affects anxiety.[45] In tests assessing motor activity, mice exposed to nicotine displayed significantly reduced time spent in the central area, aligning with the observation that stress diminishes locomotor activity—a symptom related to the dysregulation of the HPA axis and increased levels of stress hormones, which can disrupt various physiological functions.[46,47] Supporting this, Motaghinejad et al. (2016) found that nicotine dependence leads to decreased locomotor activity, consistent with our observations.[48] Importantly, fish oil supplementation resulted in increased time spent in the center area in OFT, but had no significant effect on the time spent in the open arms of the EPM. Although both tests can reflect anxiety-like behaviors, these paradigms assess distinct dimensions of anxiety, and multiple factors, such as testing time or locomotion, can influence the results. This effect of fish oil suggests it may enhance behavioral and psychological well-being by boosting motivation and exploratory behavior. Additionally, Pudell et al. (2014) found that fish oil reduced anxiety-like, depressive, and cognitive behaviors in mice undergoing olfactory

bulbectomy, likely by restoring key neurotrophic factors such as hippocampal brain-derived neurotrophic factor (BDNF) and serotonin—critical components of neuronal plasticity and survival.[49] Overall, fish oil appears to positively affect stress-related behaviors, potentially by modulating neurochemical pathways. Consistent with the study's aims, the results revealed that stress elevated serum and brain MDA levels, signifying increased oxidative stress, while omega-3 supplementation lowered MDA levels. This indicates that the antioxidant properties of fish oil may help alleviate oxidative stress caused by nicotine withdrawal. Stress activates the body's response systems, including the HPA axis and the sympathetic nervous system, resulting in the production of reactive oxygen species (ROS) and free radicals, which create oxidative stress when not sufficiently countered by antioxidants.[50,51] Furthermore, stress-triggered inflammation can also compromise antioxidant defenses.[52] Likewise, studies involving nicotine in combination with chronic mild stress exhibited increased MDA levels, confirming the oxidative effects linked to nicotine exposure.[44]

This study has several limitations. First, the experiments were conducted only on male mice. Using only males allows for clearer interpretation of the behavioral effects in this initial study; future research should include female subjects to examine potential sex-specific differences in nicotine reward, dependence, and withdrawal. Second, the study did not include a neutral substance (placebo oil) as a control group.

Conclusion

In conclusion, this research indicates that taking fish oil supplements can reduce nicotine-seeking behavior and lower oxidative stress during withdrawal. Although it did not have a significant effect on anxiety levels, its capacity to diminish oxidative markers and curtail drug cravings implies that fish oil may serve as a beneficial complementary method for addressing nicotine dependence. These results warrant further exploration into the role of omega-3 fatty acids as a potential adjunct therapy to enhance results in nicotine addiction treatment, especially for those predisposed to stress.

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Competing interests

The authors declare no conflicts of interest.

Ethical Approval

The Ethics Committee of Animal Use at Shahrekord University of Medical Sciences approved this research (IR.SKUMS.AEC.1402.023).

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