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Original Article

Possible involvement of the PI3K/Akt/mTOR signaling pathway in the antidepressant-like effects of modafinil in a mouse model of maternal separation stress

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Abstract

Background and aims: Depression is a widespread mental disorder. Maternal separation (MS) stress during early life increases the risk of depression. Modafinil (MOD) exhibits potential antidepressant-like properties, but its underlying molecular mechanisms remain unclear. The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway is implicated in neuropsychiatric disorders. This study aimed to investigate the role of the PI3K/Akt/mTOR pathway in the antidepressant-like effects of MOD in MS mice, offering potential insights into new therapeutic targets for depression treatment.

Methods: In this study, 50 male NMRI mice were divided into five groups (n = 10). The control group received normal saline, while the MS groups received normal saline and MOD at 25 mg/kg, 50 mg/kg, and 75 mg/kg for seven days. The counting open field test (OFT), forced swimming test (FST), and splash test were conducted to assess depression in rodents. The expression of *PI3K*, *AKT*, and *mTOR* genes was evaluated in the hippocampal tissue with real-time polymerase chain reaction (RT-PCR).

Results: The results revealed that MS could induce depressive-like behaviors in mice. MOD administration significantly diminished immobility time in the FST at all doses ($P < 0.001$) and amplified cleaning time in the splash test at doses of 25 mg/kg ($P < 0.01$), 50 mg/kg ($P < 0.001$), and 75 mg/kg ($P < 0.001$). Additionally, MOD noticeably downregulated the expression of *PI3K*, *AKT*, and *mTOR* genes in the hippocampus at all doses ($P < 0.001$).

Conclusion: MOD possessed antidepressant-like properties in maternally separated mice. MOD could effectively ameliorate depressive-like behaviors and suppress the hippocampal expression of *PI3K/AKT/mTOR* genes. However, future studies are warranted to find the exact and more mechanisms of action of MOD in depression.

Keywords: Maternal separation stress, Depressive disorder, PI3K/AKT/mTOR pathway, Modafinil, Mouse

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Introduction

Depression is a widespread and incapacitating mental health condition that impacts a substantial number of individuals globally, creating a significant burden on global mental health (1). Despite the wide range of antidepressant medications that are accessible, there remains considerable demand for more potent treatments because approximately one-third of individuals diagnosed with major depressive disorder do not experience positive responses to the antidepressant medications currently on the market (2).

Animal models offer valuable resources for investigating the neurobiological mechanisms behind depression and assessing potential therapeutic interventions (3). One particular model, known as maternal separation (MS) stress, represents a form of early-life stress (ELS). MS stress has been linked to an elevated susceptibility to developing psychiatric disorders in later stages of life, including depression (4-6). Research findings suggest that ELS can result in long-lasting modifications in the hypothalamic-pituitary-adrenal axis in response to stress throughout adulthood. This mechanism can increase vulnerability to depression that is unresponsive to standard antidepressant drugs (7).

Modafinil (MOD), a wake-promoting agent, has been approved for the treatment of narcolepsy and other sleep disorders (8). However, accumulating evidence suggests that MOD may also possess antidepressant-like properties (9). MOD acts on multiple neurotransmitter systems, including dopamine, norepinephrine (NE), and serotonin (5-HT), which are known to be dysregulated in depression (10,11). Moreover, MOD exhibits the ability to inhibit the production of proinflammatory cytokines, suggesting its potential to mitigate neuroinflammation (12). Nonetheless, the detailed molecular mechanisms mediating the antidepressant-like effects of MOD remain unclear.

In recent years, intracellular signaling pathways have emerged as critical regulators of neuronal function and synaptic plasticity, with potential implications

for depression (13). One such pathway is the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway. The PI3K/Akt/mTOR participates in various cellular processes, encompassing growth, cell survival, and synaptic plasticity (14). Disturbance in this pathway has been associated with various neuropsychiatric disorders, including depression (15). Furthermore, recent research has highlighted the role of the PI3K/Akt/mTOR pathway in the antidepressant-like effects of various compounds and medications, including Matrine, ketamine, and valproic acid (16-18).

As a result, this study aims to explore the probable participation of the PI3K/Akt/mTOR pathway in the antidepressant-like effects of MOD in a mouse model of MS stress. Understanding the role of this signaling cascade in MOD's antidepressant-like effects could offer valuable insights into the underlying molecular mechanisms and potentially uncover new therapeutic targets for depression treatment.

Materials and Methods

Animals

Pregnant NMRI mice (25–30 g weight) were bought from the Pasteur Institute in Tehran, Iran, on the first day of pregnancy. They were kept in a standard laboratory environment with controlled temperature (22 ± 1 °C), a 12-hour light/dark cycle, and ad libitum access to food and water. Full efforts were made to optimize animals' comfort.

Maternal separation

The day of birth was designated as postnatal day 0 (PND 0). From PND 2 to PND 14, the mouse pups were separated from their mothers for three hours each day. Following PND 14, the pups were reintroduced to their mother's cages and reserved together until day 21. On day 21, the animals were weaned and housed in groups of 10 in separate cages until PND 53. The control group mice were housed in the same cage as their mothers without any intervention from PND 0 to PND 21, and then they were divided into groups of 10 from PND 21 to PND 53. All experimental assessments were conducted between 9:00 AM and 12:00 PM to maintain consistency in timing (19,20).

Study design

A total of 50 male NMRI mice, weighing between 25–30 g, were allocated into five groups, each consisting of 10 mice. The control group (group 1) did not undergo the separation paradigm but received normal saline at a dosage of 1 mL/kg. Groups 2–5 were maternally separated mice treated with normal saline (1 mL/kg) and/or MOD at doses of 25 mg/kg, 50 mg/kg, and 75 mg/kg, correspondingly. All administrations were conducted intraperitoneally over 7 consecutive days. The doses and duration of administrations were selected in accordance with our pilot study as well as relevant previous research (21,22). Behavioral experiments were performed after the final injection on PND 60. Subsequently, mice were euthanized under deep anesthesia using ketamine (40 mg/kg) and xylazine (10 mg/kg), according to previous studies (23,24). The hippocampi were carefully isolated and deposited at -70 °C in a freezer until further molecular assays. The expression of *PI3K*, *Akt*, and *mTOR* genes was estimated using real-time polymerase chain reaction (RT-PCR) in hippocampi samples (25,26). Throughout the research process, utmost care was taken to minimize any potential suffering experienced by the animals, and full determination was applied to diminish the overall number of animals utilized, ensuring their welfare and well-being.

Behavioral experiments

Forced swimming test

The length of immobility was recorded during the forced swimming test (FST), which is used as an indicator of depressive-like behavior. To conduct the test, the experimenter carefully lowered the mice into a glass container (25 × 12 × 15 cm) filled with water at a temperature of 25 °C from a height of 20 cm. The duration of the test was considered 6 minutes, with the initial two minutes allocated for the mice to acclimate to the new environment. Subsequently, the immobility time was calculated for the next 4 minutes (27).

Open field test

This study examined the impact of treatments and MS on the locomotion of mice. Prior to the FST, the open field test (OFT) was conducted to assess the mice's exploratory behavior. The OFT apparatus was constructed using matte white Plexiglas, measuring 60 × 60 × 40 cm, and was softly illumined. Mice were gently placed in the central zone of the open field, individually, and their locomotion was recorded by a camera for 5 minutes. The recorded data were subsequently analyzed using EthoVision software, version 8. The horizontal (distance moved) and vertical (number of rearing) activities in the open field were quantitatively investigated to evaluate the locomotor activities of mice (19,28).

Splash test

The motivational and self-care behaviors in rodents were assessed with the splash test. In this test, a 10% sucrose solution was sprayed onto the dorsal coat of mice within their home cage. Subsequently, the grooming time was recorded by a camera for 5 minutes. The focus of this experiment was to evaluate grooming activities, such as nose and face cleaning, head washing, and body grooming, as indicators of self-care behavior in the mice (20,28).

Real-time polymerase chain reaction method

In this investigation, RT-PCR was employed to examine the expression of *PI3K*, *Akt*, and *mTOR* genes in

hippocampi samples. These samples were harvested, and total RNA was extracted by RNX-plus. Subsequently, the RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). Gene-specific primers and fluorescent probes were designed and optimized for PI3K, Akt, and mTOR (Table 1). RT-PCR amplification was conducted on cDNA samples utilizing a light cycler instrument (Roche Diagnostics, Mannheim, Germany; Takara Bio). To determine the relative gene expression of *PI3K*, *Akt*, and *mTOR*, the resulting data were investigated based on the $2^{-\Delta\Delta Ct}$ method in the hippocampus. The β-2 microglobulin (B2M) was employed as the housekeeping gene (29,30).

Data analysis

The data collected in this study were recorded and analyzed using PRISM statistical software (version 7). Statistical examination was performed using one-way ANOVA and Tukey's post hoc test. The obtained data are expressed as means ± standard error of the mean (SEM). A significance level was considered at $P<0.05$.

Results

Effect of modafinil on immobility time in forced swimming test

The results (Figure 1) revealed a substantial increase in the immobility time in MS mice treated with saline compared to the control group ($P<0.001$). Treatment of MS mice with MOD at doses of 25 mg/kg, 50 mg/kg, and 75 mg/kg exhibited a notable decrease in the immobility time when compared to the saline-received MS group ($P<0.001$).

Effect of modafinil on locomotor activity in forced swimming test

The findings indicated that MS stress did not make any substantial changes in horizontal activities in comparison to the control group. Moreover, the administration of MOD had no considerable effect on horizontal activities during the OFT compared to the saline-treated MS group (Figure 2). Additionally, MS stress did not elicit alterations in vertical activities when compared to the control group. However, a significant increase ($P<0.05$) in vertical activity was observed in the group that received MOD (75 mg/kg) in comparison to the saline-treated MS group (Figure 3).

Effect of modafinil on grooming behavior time in the splash test

According to the results (Figure 4), there was a substantial decline in the grooming action time among mice in the MS group treated with saline in comparison to the control group ($P<0.001$). The duration of grooming activity in the groups receiving MOD at doses of 25 mg/kg ($P<0.01$), 50 mg/kg ($P<0.001$), and 75 mg/kg ($P<0.001$) significantly increased when compared to the MS group treated with saline.

Table 1. Primer sequences utilized in PCR amplification

Primer	Forward sequence	Reverse sequence
B2M	GGAAGTTGGGCTTCCCATTCT	CGTGATCTTTCTGGTGCTTGTC
PI3K	GCAACTCCTGGACTGCAACT	CAGCGCACTGTCATGGTATG
Akt	TAGCCATTGTGAAGGAGGGC	CCTGAGGCCGTTCCTTGTAG
mTOR	GCTCCAGCACTATGTCACCA	CGTCTGAGCTGGAAACCAGT

Note. PCR: Polymerase chain reaction; PI3K: Phosphatidylinositol-3-kinase; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin.

Modafinil reduced the expression of PI3K, Akt, and mTOR genes in the hippocampus

The findings of our study (Figure 5) revealed a significant decrease in the expression of *PI3K*, *Akt*, and *mTOR* genes in the hippocampus of MS mice receiving MOD at doses of 25 mg/kg, 50 mg/kg, and 75 mg/kg when compared to the saline-treated MS group ($P<0.001$).

Discussion

The present findings provide evidence that exposure to MS stress induces depressive-like behaviors in adult male mice, as evidenced by augmented immobility time in the FST and reduced grooming time in the splash test. Notably, our observations confirmed that MOD exhibits antidepressant-like properties in MS mice by reducing the immobility time in the FST and increasing the grooming activity time in the splash test. Additionally, the downregulation of *PI3K*, *AKT*, and *mTOR* gene expression was observed in the hippocampus following MOD treatment.

Previous research extensively documented the negative impacts of ELS on adult individuals, particularly in relation to the development of depressive symptoms and alterations in brain structures and neurotransmission (31,32). The MS paradigm has been widely utilized as an experimental model of ELS, effectively inducing anxiety and depressive-like behaviors in animals (33,34). Consistent with these findings, our findings confirmed that MS mice exhibited reduced grooming activity time in the splash test and increased immobility time in the FST compared to the control group. Furthermore, to ensure that alterations in motor activity did not confound the immobility measurements in the FST, the OFT was performed prior to the FST to assess the ambulatory behavior of the mice.

Numerous studies have established the beneficial effects of MOD on memory enhancement across various experimental models (35). Cao et al revealed that MOD administration in sleep-deprived mice exerted neuroprotective effects by suppressing autophagy and apoptosis in hippocampal neurons (36). Additionally, experiments conducted in mouse models have shown that MOD possesses antiepileptic properties, as evidenced by its ability to mitigate epileptic episodes (37). With regard to depression, clinical and experimental studies have consistently demonstrated the antidepressant properties of MOD (38-40). Despite these advancements, the particular underlying mechanisms mediating the

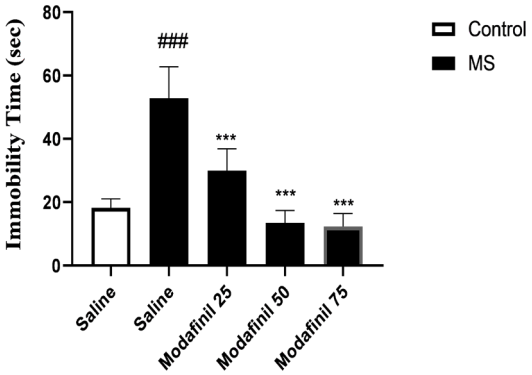

Figure 1. The length of immobility in the forced swimming test. *Note*. SEM: Standard error of the mean; ANOVA: Analysis of variance; MS: Maternal separation. The data are reported as the mean ± SEM obtained from 10 mice. Statistical analysis involved the use of one-way ANOVA, followed by Tukey's post hoc test. $^{###}P<0.001$ compared to the saline-treated control group and $^{***}P<0.001$ compared to the saline-treated MS group

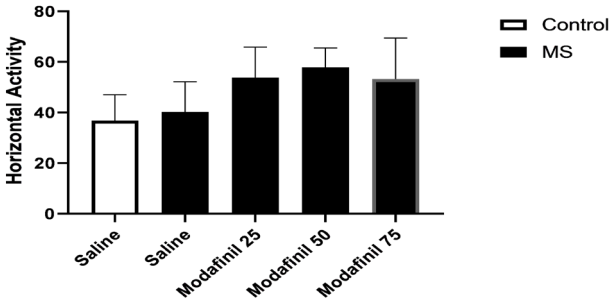

Figure 2. Assessment of horizontal activities in the forced swimming test. *Note*. SEM: Standard error of the mean; ANOVA: Analysis of variance. The data are reported as the mean ± SEM obtained from 10 mice and analyzed with one-way ANOVA and Tukey's post hoc test

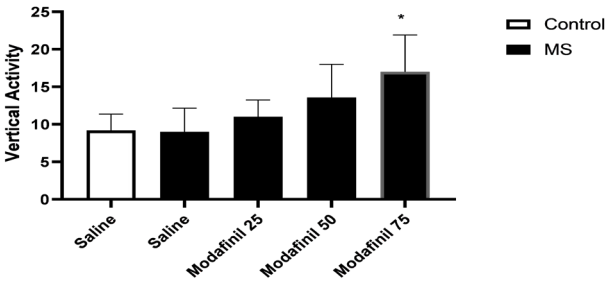

Figure 3. Assessment of vertical activities in the open field test. *Note*. SEM: Standard error of the mean; ANOVA: Analysis of variance; MS: Maternal separation. The data are presented as the mean ± SEM from 10 mice and analyzed using one-way ANOVA and Tukey's post hoc test. $^{*}P<0.05$ compared to the saline-treated MS group

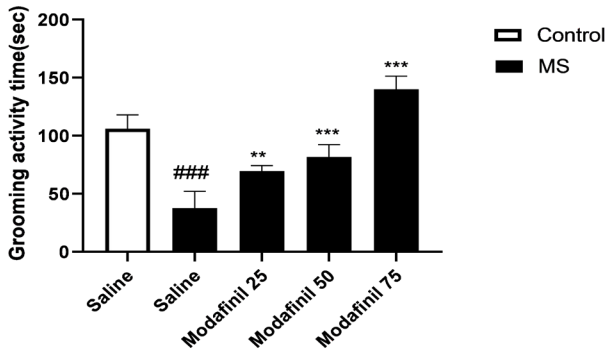

Figure 4. Grooming activities in the splash test. *Note*. SEM: Standard error of the mean; ANOVA: Analysis of variance; MS: Maternal separation. Data are expressed as the mean ± SEM from 10 mice and analyzed with one-way ANOVA and Tukey's post hoc test. $^{###}P<0.001$ compared to the saline-treated control group. $^{**}P<0.01$ and $^{***}P<0.001$ related to the saline-treated MS group

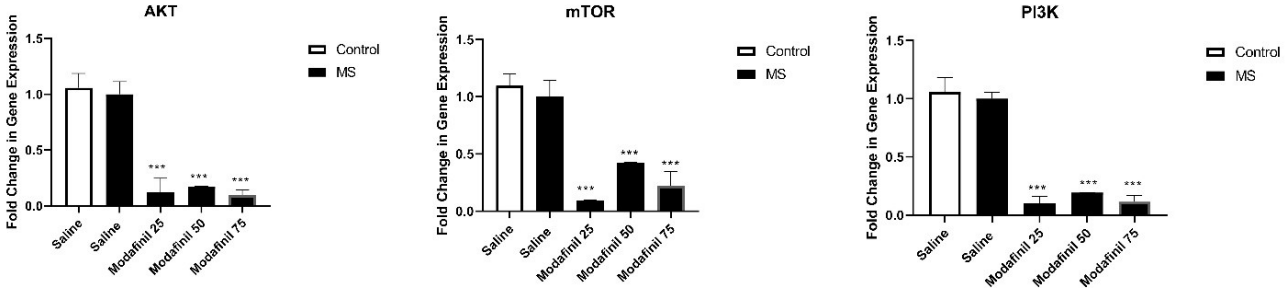

Figure 5. The expression of *PI3K*, *Akt*, and *mTOR* genes in the hippocampus. *Note*. PI3K: Phosphatidylinositol-3-kinase; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin; SEM: Standard error of the mean; ANOVA: Analysis of variance; MS: Maternal separation. The data are demonstrated as the mean ± SEM from 10 mice and analyzed using one-way ANOVA and Tukey's post hoc test. $^{***}P<0.001$ compared to the MS group treated with saline

antidepressant-like effects of MOD remain elusive. In line with the aforementioned research, our findings provide further support for the antidepressant-like effects of MOD. Specifically, our results indicated that MOD treatment significantly increased the duration of immobility time in the FST and grooming activity time in the splash test, corroborating its potential as an effective therapeutic intervention for depressive disorders.

Extensive evidence confirms the implication of the PI3K/Akt/mTOR signaling pathway in the underlying mechanisms of depression (41). For instance, Feng et al provided evidence that the negative regulation of the PI3K/Akt/mTOR signaling network mediates depressive behaviors (42). Further, Lu et al discovered that the prompt emergence of antidepressant-like effects caused by GLYX-13 is connected to the activation of the PI3K/AKT/mTOR signaling pathway in the hippocampus of mice (43).

In contrast to the aforementioned studies, our findings demonstrated that MOD administration leads to a decrease in the expression of the PI3K/Akt/mTOR pathway in the hippocampus, aligning with the observations made by Tao et al. in their investigation of liquiritigenin's effects on unpredictable chronic mild stress-induced depressive-like behaviors (44). Interestingly, despite the downregulation of this signaling

pathway, MOD still manifests antidepressant-like properties. These consequences challenge the notion that the antidepressant-like properties of MOD are solely attributed to the upregulation of the PI3K/Akt/mTOR pathway, as previously postulated. Based on our findings, no significant differences were observed in the gene expression of *PI3K*, *Akt*, and *mTOR* in the hippocampus between the control and MS groups. This suggests that the alterations in the PI3K/Akt/mTOR signaling pathway may not be specifically localized to the hippocampus in the context of MS-induced depressive-like behaviors.

Further studies are warranted to unravel the underlying mechanisms involved in the antidepressant-like effects of MOD and to determine the role of the PI3K/Akt/mTOR signaling pathway in MS-induced depressive-like behaviors. Specifically, future investigations should explore the gene expression of *PI3K*, *Akt*, and *mTOR* in other brain regions following MOD treatment in the context of MS or other models of depressive-like behaviors. Such studies will contribute to a comprehensive understanding of the complex mechanisms underlying the antidepressant-like effects of MOD and shed light on the potential therapeutic targets associated with MS-induced depressive-like behaviors.

Conclusion

Overall, the findings confirmed the influence of MS stress in eliciting depressive-like behaviors. Moreover, the administration of MOD exhibited significant antidepressant-like effects, as evidenced by improvements observed in the FST and splash test. Furthermore, our outcomes indicated that MOD treatment is linked to the downregulation of gene expression related to the PI3K, AKT, and mTOR signaling pathways, specifically within the hippocampus.

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Competing Interests

The authors have no conflict of interests to declare regarding the study and the preparation of the article.

Ethical Approval

The experiments conducted in this study strictly adhered to ethical guidelines outlined in the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press) established by the National Institutes of Health. The study protocols received approval from the Ethics Committee of Shahrekord University of Medical Sciences (Ethical code IR.SKUMS.REC.1398.100).

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