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Original Article

Opposing Effect Against Oxidative Stress Imbalance in the Prefrontal Cortex Partly Mediates Analgesic Properties of Anethole in Maternally Separated Female Mice

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Abstract

Background and aims: Oxidative stress (OS) is involved in the physiopathology of pain response, and maternal separation (MS) stress is accompanied by OS imbalance in the brain. Anethole is a natural compound with neuroprotective effects. Accordingly, this trial study aimed to estimate the analgesic effect of anethole in female mice undergoing MS stress, with a focus on OS imbalance in the prefrontal cortex (PFC).

Methods: In general, 28 female mice were used in this experimental study. MS mice received saline (10 mL/kg) or anethole (62.5 mg/kg and 125 mg/kg), while control mice received saline (10 mL/kg). One hour after treatment, the animals underwent pain assessment tests, including the tail flick and hot plate tests. Moreover, malondialdehyde (MDA) and total antioxidant capacity (TAC) were assessed in the PFC. Finally, one-way ANOVA followed by Tukey's post hoc test was employed for statistical analysis.

Results: MS in female mice was linked with a decrease in latencies in the tail flick ($P=0.0133$) and hot plate ($P=0.014$) tests, a rise in MDA ($P=0.0001$), and a reduction in the TAC ($P=0.0001$) in the PFC. Anethole increased the latencies in the tail flick ($P=0.0001$ for both doses) and hot plate ($P=0.002$ and $P=0.0002$ for 62.5 mg/kg and 125 mg/kg, respectively) tests. However, anethole decreased MDA ($P=0.0017$ for a dose of 125 mg/kg) while increasing TAC ($P=0.0018$ for a dose of 125 mg/kg) in the PFC.

Conclusion: It was revealed that anethole, partly through OS diminution in the PFC, increased latencies to pain stimuli in MS female mice.

Keywords: Anethole, Maternal separation, Pain, Oxidative stress, Prefrontal cortex

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Introduction

Oxidative stress (OS) plays an important role in pain physiopathology, particularly in chronic circumstances (1). More precisely, OS is involved in neuropathic pain, where a disproportion between reactive oxygen species and antioxidants causes tissue damage (2). It has been found that OS promotes hyperalgesia as indicated by enhanced pain sensitivity and activation of pain-related genes (3). In addition, increased OS markers in the spinal cord are linked to pain hypersensitivity (4). Growing evidence has revealed that agents with potential analgesic properties exert their effects through combating OS (5, 6).

According to preceding studies, maternal separation (MS) stress in infancy can deleteriously influence rodents' brain areas, including the prefrontal cortex (PFC) (7). Further, MS can cause abnormalities in neurotransmission and disrupt the normal functioning of the brain (8, 9). Moreover, MS is accompanied by an OS imbalance in the brain (10, 11). According to research, there is a complex association between MS and pain sensitivity in adulthood. While some studies reported an increase in pain sensitivity following MS (12, 13), others indicated that MS led to resilience against pain (14). MS-induced changes in pain sensitivity may be mediated by changes in glutamatergic signaling, glial cell activation, and neurotrophin expression (14). Likewise, pain sensitivity may be influenced by decreased activity of noradrenergic and serotonergic neurons in the locus coeruleus and dorsal raphe nucleus of maternally separated animals (12). These findings demonstrate a complex link between early life stress and pain sensitivity in adulthood.

Anethole, a chemical found in star anise, has shown promising effects on the experimental models of neurological disorders (15). It has been found that anethole exerts antioxidant effects (16). Although this chemical has antinociceptive effects in experimental paradigms, the exact underlying mechanism has not been understood (17). Furthermore, anethole exhibited neuroprotective properties in a rat model of Parkinson's disease through enhancing cognitive performance, raising pain threshold, and lowering OS in the hippocampus (18). These findings all point to the antioxidant and neuroprotective characteristics of anethole, making it a promising agent for treating pain and related pathophysiological diseases. Therefore, to suggest anethole in pain control, it is critical to identify underlying mechanisms. Thus, this study seeks to investigate the possible analgesic effects of anethole in female mice subjected to MS stress, considering its potential anti-OS capability in the PFC.

Materials and Methods

Animals and Maternal Separation

This experimental study included Naval Medical Research Institute mice. The animals were obtained from the Pasteur Institute, Iran. Ordinary preservation conditions encompassed a temperature of 21 ± 2°C, a 12-hour light and dark cycle, and unrestricted admission to food and water. In addition, male and female mice were co-housed for mating (3 female mice co-housed with 1 male mouse). The MS paradigm included untying the offspring from their mothers for 180 minutes every day from postnatal day (PND) 2 to PND 14 (13, 19). After PND 14, the offspring came together with their mothers pending PND 25. On PND 25, the pups were weaned and kept in groups. The control mice, which were not exposed to the MS model, persisted with their mothers from PND 0 to PND 25 without handling. It is important to note that the pups were handled by one experienced researcher in order to minimize bias among groups.

Study Design

Twenty-eight female Naval Medical Research Institute mice were randomly allocated to four experimental groups (n = 7). MS mice were divided into 3 groups receiving either normal saline (10 mL/kg) or anethole at 62.5 mg/kg and 125 mg/kg. Moreover, mice from different mothers were randomly assigned to each group so that each group contained mice from different mothers. Control mice (group 4) received normal saline (10 mL/kg). It should be noted that anethole and saline were injected as a single dose via the intraperitoneal (i.p.) route at PND 50. The dosage of the injections was established based on previous research (17, 20). One hour after injections, the animals were exposed to pain assessment tests, including hot plate and tail flick tests, by an experimenter who was blinded to the groups. All tests were performed between 8:00 am and 02:00 pm, and the interval between the tests was 15 minutes. Figure 1 presents a schematic of the study design. Four days before behavioral tests, mice were placed on the apparatus for habituation (by a fixed experimenter) while no painful stimuli were applied. Subsequently, they underwent deep anesthesia using 60 mg/kg of pentobarbital intraperitoneally (21). PFC was dissected on an ice surface, rapidly snap-frozen in liquid nitrogen, and then deposited at -70°C pending biochemical assessments.

Behavioral Tests

Hot-plate and tail-flick trials, as valid tests, were employed to investigate the analgesic effects of anethole. The hot plate test shows the supraspinal reply, whereas the tail-flick test assesses the nociceptive reply mostly at the spinal level (22, 23).

Hot Plate Test

The mice were placed on a 52 ± 0.2°C heated plate, and the time it took to lick the forepaw, hind paw, or jump was recorded (24). An increase in this time indicated an analgesic effect. The latency measurement was performed once per mouse. A cut-off time of 60 seconds was set to sidestep any tissue damage.

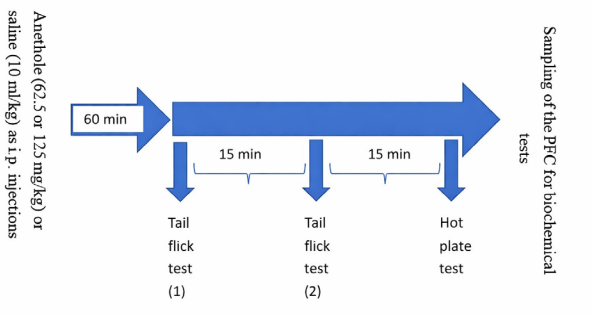

Figure 1. A Schematic of the Study Design
Note. PFC: Prefrontal cortex

Tail Flick Test

The tail-flick test was completed based on the criteria designated by D'amour and Smith (25). Radiant heat was applied to the tail (1–3 cm from the base) by means of a tail-flick device. In addition, tail-flick dormancy was measured as the time break between the application of a standardized ray fixated on the tail and the abrupt subtraction of the tail from the painful stimulus. Moreover, latencies were calculated twice per mouse with 15-minute intervals, and average values were reported accordingly. The cut-off time of 15 seconds was considered to prevent damage to the tail.

Measuring the Antioxidant Capacity of the Prefrontal Cortex

The total antioxidant capacity (TAC) of the PFC was documented by the ferric-reducing ability of the plasma process. In brief, according to the capacity to change Fe^{3+} to Fe^{2+}, producing a blue Fe^{2+} complex (the Fe^{3+} tripyridyl s triazine complex), and employing an automatic plate reader (LQ-300 + II-Epson, USA) at 37°C with a pH value of 3.6, the absorbance was measured at 593 nm (26).

Measuring Malondialdehyde of the Prefrontal Cortex

To measure the MDA level in the PFC, 900 µL of Tris-KCl buffer was added with 100 µL of the supernatant of PFC aliquots, followed by adding 500 µL of 30% TCA. Then, 500 µL of thiobarbituric acid (0.75%) was mixed and warmed for 45 minutes at 80°C in warm water, and the combination was centrifuged (3,000 rpm, 5 minutes). Eventually, the supernatant's absorbance was detected at 562 nm using an enzyme-linked immunosorbent assay reader (27).

Statistical Analysis

GraphPad Prism software (version 8) was employed for data analysis. Data were expressed as means ± standard deviations (SDs). One-way analysis of variance test and Tukey's post hoc test were utilized for statistical analysis, respectively, and $P < 0.05$ was considered statistically significant. Additionally, an α error of 0.05 and power (1 − β) of 0.8 were set, and the required total sample size per

group was calculated as 6–8 in behavioral tests and 3–5 for molecular evaluations. According to previous research, the in vivo and in vitro assay groups of 6–8 animals and 3–5 samples are decidedly reproducible and would suit to obtain dependable results (28).

Results

The Effect of Anethole on the Latency to Pain Response in the Tail Flick Test

The results (Figure 2) revealed that the latency of the MS mice (mean ± SD: 4.374 ± 0.6275) was significantly ($P = 0.0133$) shorter than that of the control group (mean ± SD: 6.593 ± 1.540). Furthermore, the latency to pain response of the MS mice that received anethole at doses of 62.5 mg/kg (mean ± SD: 9.264 ± 0.5280) and 125 mg/kg (mean ± SD: 8.648 ± 1.072) significantly increased compared to the saline-treated MS mice ($P = 0.0003$ for both doses).

The Effect of Anethole on the Latency in the Hot Plate Test

Based on the data (Figure 3), the latency of the MS mice was considerably shorter than that of the control animals (mean ± SD: 6.376 ± 1.887 and mean ± SD: 10.81 ± 1.971, respectively, $P = 0.014$). Moreover, the latency of the MS mice receiving anethole at doses of 62.5 mg/kg (mean ± SD: 11.94 ± 2.531) and 125 mg/kg (mean ± SD: 12.92 ± 2.140) represented a significant increase, in comparison to the MS group receiving saline ($P = 0.002$ and $P = 0.0002$, respectively).

The Effect of Anethole on Total Antioxidant Capacity in the Prefrontal Cortex

The results (Figure 4) also demonstrated that TAC in the PFC of MS mice (mean ± SD: 690.5 ± 36.21) was remarkably shorter than that of the control group (mean ± SD: 987.6 ± 62.68) ($P = 0.0001$). Likewise, the TAC of the MS mice receiving anethole at a dose of 125 mg/kg (mean ± SD: 845.8 ± 58.92) could significantly increase compared to the saline-receiving MS group ($P = 0.0018$).

The Effect of Anethole on Malondialdehyde Level in the Prefrontal Cortex

The results (Figure 5) confirmed that MDA in the PFC of MS mice (mean ± SD: 110.7 ± 14.86) was significantly higher than that of the control animals (mean ± SD: 53.98 ± 5.159) ($P = 0.0003$). In addition, the MDA of the MS mice that received anethole at a dose of 125 mg/kg (mean ± SD: 77.18 ± 13.24) was noticeably reduced compared to MS animals that received saline ($P = 0.0017$).

Discussion

Based on the results, anethole had antinociceptive effects on MS female mice, showing increased latencies in the hot plate and tail flick tests. Furthermore, anethole increased TAC while decreasing MDA in the PFC of MS mice. These results indicate that reducing OS markers in the PFC, may, in part, mediate the antinociceptive effects of anethole in female MS mice.

It was found that facing early-life adversities, such as stress, has an enduring and lifelong effect on the development of the brain in adulthood (29). As an early-life stress, MS triggers neurochemical, neurohormonal, and neurostructural changes in the central nervous system (30). Additionally, it has been shown that early-life stress disrupts the opioidergic neurotransmission and alters the analgesic response by the downregulation of opioid receptors (31). MS-induced changes in pain sensitivity may be mediated by alterations in glial cell activation, glutamatergic signaling, and neurotrophin expression (14). Pain sensitivity may be affected by reduced activity of serotonergic and noradrenergic neurons in the dorsal raphe nucleus and the locus coeruleus of maternally detached animals (12). Our results confirmed that MS reduced the latencies in the hot plate and tail flick tests.

Extensive evidence highlights the role of OS in the

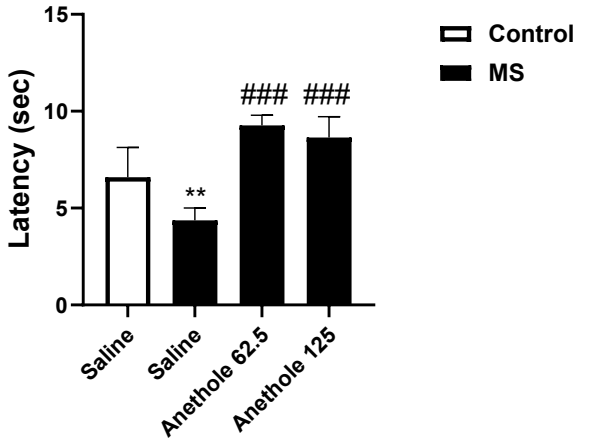

Figure 2. Comparison of Latency in the Tail Flick Test in Experimental Groups
Note. MS: Maternal separation; ANOVA: Analysis of variance; SD: Standard deviation. Data are expressed as means ± SD (n = 7) and analyzed with one-way ANOVA and Tukey's post hoc tests. $^{*}P = 0.0133$ compared with the control group, and $^{###}P = 0.0003$ in comparison with the saline-received MS groups

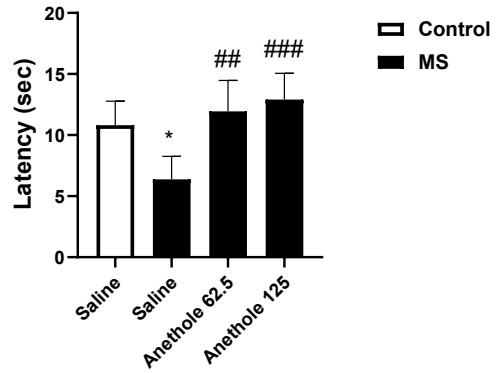

Figure 3. Comparison of Latency in the Hot Plate Test in Experimental Groups
Note. MS: Maternal separation; ANOVA: Analysis of variance; SD: Standard deviation. Data are expressed as means ± SD (n = 7) and analyzed with one-way ANOVA and Tukey's post hoc tests. $^{*}P = 0.014$ compared with the control group, and $^{##}P = 0.002$ and $^{###}P = 0.0002$ in comparison with the saline-received MS group

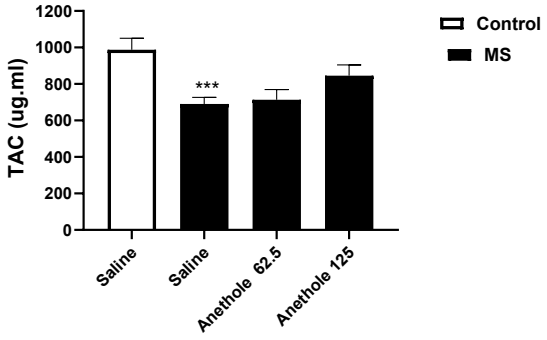

Figure 4. Comparison of the TAC in the PFC in Experimental Groups
Note. TAC: Total antioxidant capacity; PFC: Prefrontal cortex; MS: Maternal separation; SD: Standard deviation; ANOVA: Analysis of variance. Data are expressed as means ± SD (n = 5) and analyzed with one-way ANOVA and Tukey's post hoc test. *** $P = 0.0001$ compared with the control group, and ## $P = 0.0018$ in comparison with the saline-treated MS group

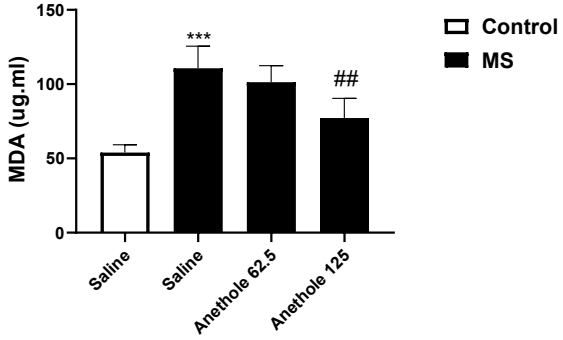

Figure 5. Comparison of the MDA in the PFC in Experimental Groups
Note. MDA: Malondialdehyde; PFC: Prefrontal cortex; MS: Maternal separation; ANOVA: Analysis of variance; SD: Standard deviation. Data are expressed as means ± SD (n = 5) and analyzed with one-way ANOVA and Tukey's post hoc test. *** $P = 0.0003$ compared with the control group, and ## $P = 0.0017$ in comparison with the saline-received MS group

pathophysiology of pain (32, 33). It has been found that OS promotes hyperalgesia as indicated by increased pain sensitivity and activation of pain-associated genes (32, 34). Based on the growing evidence, agents with potential analgesic properties have their effects through combating OS (5, 6). Previous studies demonstrated that an increase in the MDA level is associated with nociceptive behaviors, and the mitigation of the MDA level is linked with an antinociceptive response (35, 36). Furthermore, a direct association was found between reduced TAC and pain response, thus agents with increased TAC have possessed analgesic effects (37). Moreover, MS stress is related to a decrease in the TAC and an increase in the MDA level (38). Our findings revealed that MS in female mice was associated with an increase in the MDA but a decrease in the TAC in the PFC. These biochemical changes are, at least in part, accompanied by a decline in latencies to pain stimuli in the hot plate and tail flick tests.

Anethole has been shown to have antioxidant effects (16, 39). Likewise, it exerted its neuroprotective effects by alleviating OS (40). Some studies have also reported the neuroprotective effects of anethole (18, 41). Additionally, this compound has antinociceptive effects in experimental paradigms (17). However, there are no clear data on the exact mechanisms underlying the antinociceptive effect of anethole. The results of this study revealed that anethole had antinociceptive effects on MS mice, demonstrating an increase in latencies to painful stimuli in the hot plate and tail-flick tests. Previous research has shown that there are differences between female and male subjects in response to painful stimuli (42). In addition, there are differences regarding the effects of MS in female and male mice (13, 43).

This study had some limitations. Only female mice were used in this study. Thus, further studies should evaluate the effects of anethole on painful stimuli in male mice in order to clarify possible gender differences. Anethole increased the TAC and decreased the MDA level in the PFC of MS female mice. Indicating the important role of PFC in processing the pain response (44). Another limitation was that only MDA and TAC were evaluated to assess the OS state. Therefore, more studies should investigate more oxidative/antioxidative markers. Nonetheless, the findings of this study provided evidence about the possible effect of anethole on nociceptive behavior in MS female mice, with a focus on the OS state in the PFC.

Conclusion

Overall, anethole, through decreasing the MDA level and enhancing the TAC in the PFC, could partly increase latencies to pain stimuli in the tail flick and hot plate tests in MS female mice. However, more studies are warranted to clarify the exact mechanisms underlying the antinociceptive effect of anethole in female MS mice.

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Authors' Contributions

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Competing Interests

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

Data Availability

The data used to support the findings of this study are available upon reasonable request from the corresponding author.

Ethical Approval

All steps of experimentation were performed in accordance with the regulations of the Shahrekord University Guide for the Care and Use of Laboratory Animals (ethical code: IR.SKU.REC.1404.049). Full determinations were made to reduce the use of animals while maintaining their comfort.

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