The Effect of Menthol on Anxiety and Related Behaviors in Mice

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Objective: To evaluate menthol in the reduction of symptoms of anxiety in mice.

Design: An Experimental Animal Study.

Setting: Animal House, College of Medicine and Medical Sciences, Arabian Gulf University.

Method: Male BALB/c mice (8 weeks old, 15-20g), underwent different stressors to exhibit anxiety-like behaviors in two different periods (7 days to induce acute and 30 days for chronic stress). Six groups of animals (control, control+menthol, acute stress, chronic stress, acute stress+menthol, chronic stress+menthol) were tested in the elevated plus-maze and forced swimming test as well as in behavioral spectrometer. The cortisol level in the animals of all the groups was tested. Menthol as crystals dissolved in water and administered by oral gavage technique 30 minutes before test.

Result: Administration of menthol decreased the level of cortisol in the blood and showed less anxiety behavior by spending more time in the open arms of the elevated plus-maze and reduced the immobility time in the forced swimming test.

Conclusion: Administration of menthol decreased the level of cortisol in the blood and showed less anxiety behavior. In behavioral spectrometry tests, mice treated with menthol showed an increase in grooming behavior (still, paw, nose, and hand) and a notable decrease in locomotor behavior (walk, trot, run).

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Anxiety is an emotion expressed by the human body in response to the different stressors in daily life. It can affect a person mentally and physically. People with anxiety disorders usually have recurrent masterful thoughts, feelings of tension and may experience some physical symptoms such as sweating, trembling, dizziness, or a rapid heartbeat[1].

Anxiety could be due to genetic and environmental factors. The autonomic hyperactivity of the hypothalamic-pituitary-adrenal axis has been found to play a major role in the pathogenesis of anxiety. Obsessive-Compulsive Disorder, generalized anxiety disorder, separation anxiety, social anxiety disorder and panic disorder are types of anxiety disorders. People often have more than one anxiety disorder[2]. Major depressive disorder, personality disorder, and substance use disorder may lead to anxiety[3]. Hyperthyroidism, heart disease, caffeine, alcohol or cannabis use and withdrawal from certain drugs mimic the symptoms of anxiety.

Treatment of anxiety may include lifestyle changes, counseling, and medications, such as antidepressants, benzodiazepines, or beta-blockers[4]. Moreover, new drugs are being investigated for the ability to alleviate symptoms of anxiety, such as Vortioxetine which is a serotonin transporter (SERT) inhibitor. Another drug is Guanfacine which is a noradrenergic alpha-2 receptor agonist that leads to a reduction in the aberrant noradrenergic signaling[5].

Menthol is used to treat minor conditions such as sore throat and muscle aches due to its local anesthetic effect[6]. Menthol has been found to reduce neuronal excitation by specifically enhancing GABAAR-mediated inhibition. A study found that menthol also can inhibit in vivo hyperactivity[7].

A study showed increased nicotine intake in the male group when given menthol. Whereas in the female mice group, menthol did not influence nicotine intake[8]. However, menthol had an influence on the psychostimulation in adult male mice exhibited as decrease in locomotion despite increase in nicotine intake[9]. In another study, menthol smokers have greater up-regulation of nicotinic acetylcholine receptor (nAChRs) than non-menthol smokers[10]. Another study showed kappa-opioid receptors disruption produces anti-stress effects and under some conditions can prevent the development of stress-induced adaptations[11].

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The aim of this study is to evaluate the ability of menthol to reduce the symptoms of anxiety in mice.

METHOD

Male BALB/c mice (8 weeks old, 15-20g) were used in this study. They were kept in the animal house under appropriate conditions with free access to food and water. They underwent different stressors to exhibit anxiety-like behaviors in different periods (7 days to induce acute stress and 30 days to induce chronic stress). Six groups of animals (control, control+menthol, acute stress, chronic stress, acute stress+menthol, chronic stress+menthol) were tested in the elevated plus-maze and forced swimming test as well as in behavioral spectrometer. The cortisol level in the animals of all the groups was tested.

Different stressors were applied to the mice to exhibit anxiety-like behaviors[12]. The stressors that were used consisted of a 1-minute tail pinch, 5-min thermal stimulation in a 45°C environment, restraint stress, and 24 hours reversed light/dark cycle. One stimulus was selected every day and applied to the mice for a period of 30 days. The interchange between different stressors was adopted to avoid stress adaptation.

Restraint and immobilization stress was induced by keeping the animals in a semi-cylindrical tube with ventilation holes for 180 min. The procedure was used for 7 days to induce acute stress[11].

Menthol crystals were dissolved in water. A dose of 200 mg/kg of menthol was administered to mice via oral gavage technique.

The elevated plus-maze tests for anxiety-like behavior consists of two open arms (30m x 5 cm) and two enclosed arms which are equal in size with 15-cm-high walls[13]. The arms were elevated 60 cm above the floor. To reduce the probability of animals falling from the apparatus, a 4-mm-high wall surrounded the sides of the open arms. Each mouse was individually placed in the center of the maze (5cmx5cm), facing one of the closed arms and was allowed to explore the maze during a 10-minute test period. The number of open and closed arm entries and the time spent in each arm were analyzed. Entering the open arms less frequently and spending less time in them were indicative of anxiety-like behavior. The total number of open and closed arm entries reflect the general activity during the 10 minutes test[14].

Each mouse was placed in an inevitable glass tank filled with water and their escape related mobility behavior was measured. Behaviors during the swim test were recorded using a smartphone camera and scored manually by trained, experimentally-blind observers[16].

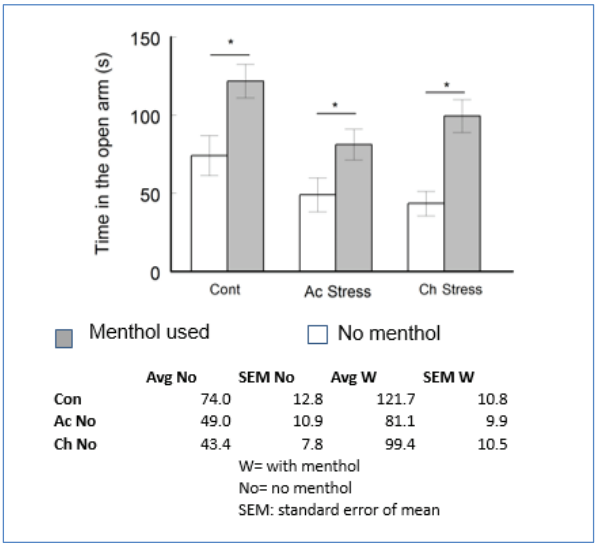
Grooming behaviors were assessed using a behavioral spectrometer chamber which captured everything the mouse did. An automated behavioral identification software that combines vibration, animal weight, and infrared beams was used to determine grooming and locomotor behaviors in mice. Mice were placed in the center of the field and data were collected over a 30-minute test. Between sessions, the chamber was wiped with 70% ethanol[15].

The antidepressant effect of menthol was assessed with the ELISA kit by measuring the quantity level of cortisol in stressed mice before and after the administration of menthol. ELISA test started one hour after administration of menthol. All mice were sampled by withdrawing blood from an orbital vein (4ml/mouse), blood was collected in serum separator tubes (SST), and samples allowed to clot overnight at 4 degrees Celsius before centrifugation for 15 minutes at 1000×g. The serum was removed for assay immediately.

RESULT

In the elevated plus-maze test, mice treated with menthol showed less anxiety-like behavior by spending more time in the open arms compared to the mice group which were not treated with menthol, see figure 1.

Figure 1: Time Spent in the Open Arms by Mice Treated with Menthol and Mice Not Treated with Menthol



	Avg No	SEM No	Avg W	SEM W
Con	74.0	12.8	121.7	10.8
Ac No	49.0	10.9	81.1	9.9
Ch No	43.4	7.8	99.4	10.5

W= with menthol
No= no menthol
SEM: standard error of mean

Menthol treated mice showed less anxiety-like behavior in the EPM test compared to the mice which were not treated with menthol. Improvement of anxiety-like behavior was significantly (P-value 0.0073) seen among mice treated with menthol, see table 1.

Table 1: Comparison between Control Group Not Treated with Menthol and Control Group Treated with Menthol

	Variable 1	Variable 2
Mean	121.7142857	74
Variance	818.9047619	1143.66667
Observations	7	7
Pooled Variance	981.2857143	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	2.849605306	
P(T<=t) one-tail	0.007316746	
t Critical one-tail	1.782287556	
P(T<=t) two-tail	0.014633492	
t Critical two-tail	2.17881283	

The acute stress group treated with menthol showed a slight decrease in anxiety-like behavior by spending more time in the open arms of the EPM test (P-value =0.025), see table 2.

Table 2: Comparison between Acute Stress Group Not Treated with Menthol and Acute Stress Group Treated with Menthol

	Variable 1	Variable 2
Mean	49	81.1428571
Variance	839	691.47619
Observations	7	7
Pooled Variance	765.2380952	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	-2.173803128	
P (T<=t) one-tail	0.025225203	
t Critical one-tail	1.782287556	
P (T<=t) two-tail	0.050450406	
t Critical two-tail	2.17881283	

The chronic stress group treated with menthol had significantly lower anxiety-like behavior compared to the chronic stress group not treated with menthol (P-value=0.0005), see tables 3-6.

Table 3: Comparison between Control Group Not Treated with Menthol and Acute Stress Group Not Treated with Menthol

	Variable 1	Variable2
Mean	74	49
Variance	1143.66667	839
Observations	7	7
Pooled Variance	991.333333	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	1.48547099	
P (T<=t) one-tail	0.08160506	
t Critical one-tail	1.78228756	
P (T<=t) two-tail	0.16321013	
t Critical two-tail	2.17881283	

Table 4: Comparison between Control Group Not Treated with Menthol and Chronic Stress Group Not Treated with Menthol

	Variable 1	Variable 2
Mean	74	43.4285714
Variance	1143.66667	427.619048
Observations	7	7
Pooled Variance	785.642857	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	2.04050269	
P (T<=t) one-tail	0.03196706	
t Critical one-tail	1.78228756	
P (T<=t) two-tail	0.06393412	
t Critical two-tail	2.17881283	

Table 5: Comparison between Chronic Stress Group Not Treated with Menthol and Chronic Stress Group Treated with Menthol

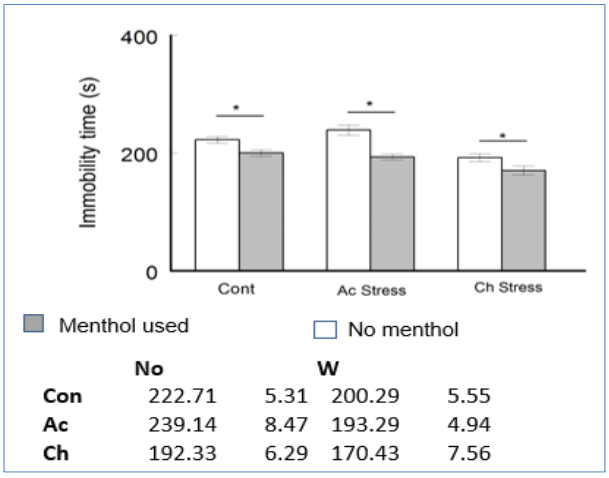
	Variable 1	Variable 2
Mean	43.4285714	99.4285714
Variance	427.619048	766.285714
Observations	7	7
Pooled Variance	596.952381	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	-4.28797461	
P (T<=t) one-tail	0.00052702	
t Critical one-tail	1.78228756	
P (T<=t) two-tail	0.00105405	
t Critical two-tail	2.17881283	

Table 6: Comparison between Acute Stress Group Not Treated with Menthol and Chronic Stress Group Not Treated with Menthol

	Variable 1	Variable 2
Mean	49	43.4285714
Variance	839	427.619048
Observations	7	7
Pooled Variance	633.309524	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	0.4141833	
P (T<=t) one-tail	0.34302358	
t Critical one-tail	1.78228756	
P (T<=t) two-tail	0.68604717	

In the forced swim test, mice treated with menthol showed less anxiety-like behavior by spending lower immobility time compared to the mice not treated with menthol, see figure 2.

Figure 2: The Immobility Time in the Forced Swim Test in Mice Treated with Menthol and Mice Not Treated with Menthol



The control group treated with menthol showed a higher rate of struggling time (P-value= 0.0063) compared to the control group not treated with menthol, see table 7.

Table 7: Comparison between Control Group Treated with Menthol and Control Group Not Treated with Menthol

	Variable 1	Variable 2
Mean	200.2857143	222.7142857
Variance	215.2380952	197.2380952
Observations	7	7
Pooled Variance	206.2380952	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	-2.921804745	
P (T<=t) one-tail	0.006398672	
t Critical one-tail	1.782287556	
P (T<=t) two-tail	0.012797344	
t Critical two-tail	2.17881283	

The acute stress group treated with menthol showed less anxiety-like behavior (P-value= 0.00026) in FST by spending lower immobility time compared to the acute stress group not treated with menthol, see tables 8-9.

Table 8: Comparison between Control Group Not Treated with Menthol and Acute Stress Group Not Treated with Menthol

	Variable 1	Variable 2
Mean	222.7142857	239.1428571
Variance	197.2380952	501.8095238
Observations	7	7
Pooled Variance	349.5238095	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	-1.643975874	
P (T<=t) one-tail	0.063052561	
t Critical one-tail	1.782287556	
P (T<=t) two-tail	0.126105123	
t Critical two-tail	2.17881283	

Table 9: Comparison between Acute Stress Group Not Treated with Menthol and Acute Stress Group Treated with Menthol

	Variable 1	Variable2
Mean	239.1428571	193.2857
Variance	501.8095238	170.9048
Observations	7	7
Pooled Variance	336.3571429	
Hypothesized Mean Difference	0	
Degrees of freedom	12	
t Stat	4.677789634	
P (T<=t) one-tail	0.000267146	
t Critical one-tail	1.782287556	
P (T<=t) two-tail	0.000534292	
t Critical two-tail	2.17881283	

The chronic stress group treated with menthol had significantly lower anxiety-like behavior compared to the chronic stress group not treated with menthol, see tables 10-12.

Table 10: Comparison between Control Group Not Treated with Menthol and Chronic Stress Group Not Treated with Menthol

	Variable 1	Variable 2
Mean	222.7142857	192.3333
Variance	197.2380952	237.4667
Observations	7	6
Pooled Variance	215.5238095	
Hypothesized Mean Difference	0	
Degrees of freedom	11	
t Stat	3.719689815	
P (T<=t) one-tail	0.001691802	
t Critical one-tail	1.795884819	
P (T<=t) two-tail	0.003383605	
t Critical two-tail	2.20098516	

Table 11: Comparison between Control Group Not Treated with Menthol and Chronic Stress Group Treated with Menthol

	Variable 1	Variable 2
Mean	192.3333	170.4286
Variance	237.4667	400.2857
Observations	6	7
Pooled Variance	326.2771	
Hypothesized Mean Difference	0	
Degrees of freedom	11	
t Stat	2.179708	
P (T<=t) one-tail	0.025946	
t Critical one-tail	1.795885	
P (T<=t) two-tail	0.051892	
t Critical two-tail	2.200985	

Table 12: Comparison between Chronic Stress Group Not Treated with Menthol and Chronic Stress Group Treated with Menthol

	Variable 1	Variable 2
Mean	192.3333	239.1429
Variance	237.4667	501.8095
Observations	6	7
Pooled Variance	381.6537	
Hypothesized Mean Difference	0	
Degrees of freedom	11	
t Stat	-4.30678	
P (T<=t) one-tail	0.000621	
t Critical one-tail	1.795885	
P (T<=t) two-tail	0.001242	
t Critical two-tail	2.200985	

There was a significant increase in grooming behavior (still, paw, nose and head) and a decrease in locomotor behavior (walk, trot and run), see figure 3 (A-B).

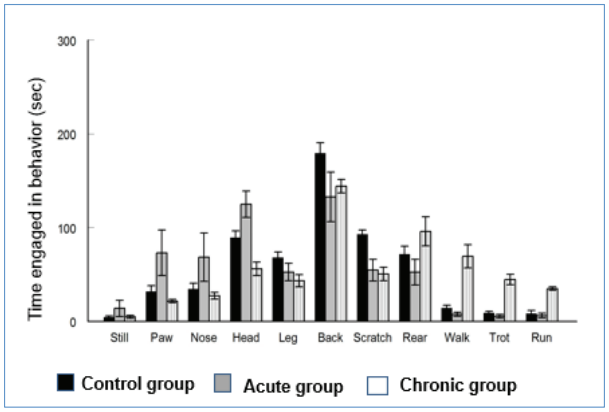


Figure 3 (A): Time Engaged in Each Behavior: Grooming Behavior (Still, Paw, Nose, and Hand) and in Locomotor Behavior (Walk, Trot, Run) in Mice Not Treated with Menthol

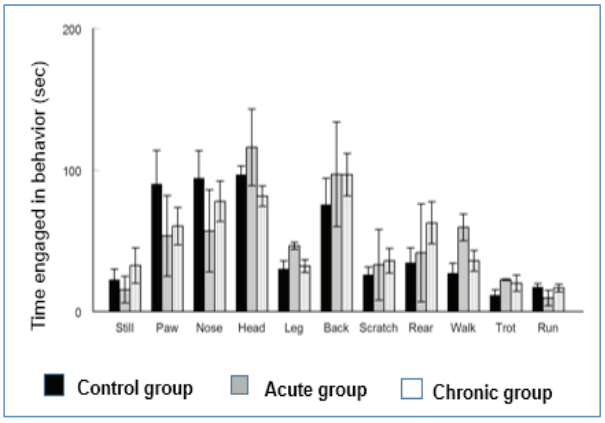


Figure 3 (B): Time Engaged in Each Behavior: Grooming Behavior (Still, Paw, Nose, and Hand) and in Locomotor Behavior (Walk, Trot, Run) in Mice Treated with Menthol

The cortisol level significantly dropped after the administration of menthol, see figure 4.

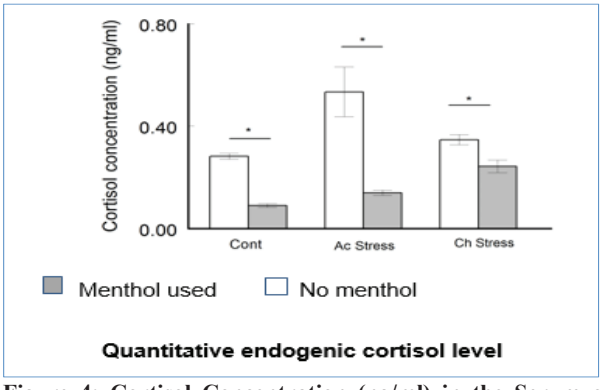


Figure 4: Cortisol Concentration (ng/ml) in the Serum of Mice Treated with Menthol

DISCUSSION

Anxiety is one of the mental illnesses that have devastating outcomes on the patients' health. Patients with anxiety have different presentations and variable degrees of severity, but generally, the chronic nature of the disease causes debilitating health problems[17].

Antidepressant drugs have been used for the treatment of anxiety disorders despite their ineffectiveness in some patients. Several studies have established the effectiveness of using nutritional and herbal treatments for treating depression and anxiety disorders[18,19,20].

In this study, the level of cortisol was significantly decreased by the administration of menthol at a dose of 200 mg/kg which produces an anxiolytic activity by regulating the pituitary-hypothalamic adrenal axis leading to less production of cortisol, thus, reducing the symptoms of anxiety[21,22].

In the elevated plus-maze, mice treated with menthol spent more time in the open arms than expected compared to the non-treated mice. In the forced swim test, the administration of menthol exhibited an antidepressant-like activity by decreasing the immobility time in mice treated with menthol compared to the control group.

In the behavioral spectrometry test, mice treated with menthol showed an increase in grooming behavior (still, paw, nose, and hand) and a notable decrease in locomotor behavior (walk, trot, run). In the acute stressed mice group who were not given menthol, the behavioral spectrometer detected an increase in grooming of some body parts (paw, nose and hand) and a moderate decrease in locomotor behavior (rear, walk, trot and run) compared to the control group. However, the chronic stressed group who were not given menthol showed a significant increase in locomotor behavior (walk, trot and run) and a mild decrease in grooming (paw, nose, and head) compared to the control group.

CONCLUSION

Administration of menthol decreased the level of cortisol in the blood and showed less anxiety. In the behavioral spectrometry test, mice treated with menthol showed an increase in grooming behavior (still, paw, nose and hand) and a notable decrease in locomotor behavior (walk, trot, run).

Our result suggests a direct relation between menthol administration and anxiety-like behavior change. Future studies are needed to evaluate the effect of menthol on various hormones.

Author Contribution: All authors share equal effort contribution towards (1) substantial contributions to conception and design, analysis and interpretation of data; (2) drafting the article and revising it critically for important intellectual content; and (3) final approval of the manuscript version to be published. Yes.

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Competing Interest: None.

Sponsorship: None.

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