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Effects of Chronic Levamisole Administration on Anxiety-Like Behaviors in Adult Male Rats *

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Levamisole is an antihelminthic drug originally developed for veterinary medicine, and it is also used in human medicine due to its immunomodulatory effects. Recently, it has been outstanding for its reported benefits in the treatment of COVID-19. Furthermore, levamisole inhibits monoamine oxidase-A (MAO-A), which plays an essential role in the pathophysiology of anxiety. This study aimed to investigate the effects of levamisole on anxiety-like behaviors assessed in adult male rats by the light-dark box test (LDB) and the open field test (OFT). The animals were divided into two groups (n=10 per group): vehicle and levamisole. The vehicle group was administered distilled water, while the levamisole group received levamisole (2 mg/kg) by oral gavage daily for 4 weeks. After the last levamisole treatment, LDB and OFT experiments were performed. Levamisole significantly enhanced the latency to enter the dark compartment (p=0.025). Moreover, the number of transitions decreased with levamisole treatment (p=0.021). Nevertheless, levamisole did not alter the time spent in the light and dark compartments (p=0.363 for both). In the OFT, the number of entries into the central area (p=0.782), the total number of crossed lines (p=0.250), the number of fecal pellets (p=0.336), the total number of rearings (p=0.367), and the duration of grooming (p=0.187) remained unchanged with levamisole administration. However, levamisole increased the time spent in the central area, while it decreased the time spent in the peripheral area (p=0.049 for both). Chronic levamisole administration may stimulate anxiolytic-like effects in adult male rats, without affecting locomotor and exploratory activities.

Key Words: *Levamisole, MAO-A, light-dark box test, open field test, anxiolytic-like effects*

Yetişkin Erkek Sıçanlarda Kronik Levamizol Uygulamasının Anksiyete Benzeri Davranışlar Üzerindeki Etkileri

Levamizol, temelde veteriner hekimlikte geliştirilmiş bir antihelmintik ilaçtır ve immünomodülatör etkileri nedeniyle beşeri tıpta da kullanılmaktadır. Son zamanlarda, COVID-19 tedavisinde bildirilen faydalarıyla öne çıkmaktadır. Ayrıca, levamizol, anksiyete patofizyolojisinde önemli bir rol oynayan monoamin oksidaz-A'yı (MAO-A) inhibe etmektedir. Bu çalışma, levamizolün yetişkin erkek sıçanlarda aydınlık-karanlık kutu testi (AKT) ve açık alan testi (AAT) ile değerlendirilen anksiyete benzeri davranışlar üzerindeki etkilerini araştırmayı amaçlamaktadır. Hayvanlar iki gruba ayrıldı (grup başına 10 hayvan): Taşıyıcı ve levamizol. Taşıyıcı grubuna distile su verilirken, levamizol grubuna 4 hafta boyunca günlük oral gavaj yoluyla levamizol (2 mg/kg) verildi. Son levamizol uygulamasından sonra, AKT ve AAT deneyleri gerçekleştirildi. Levamizol, karanlık bölmeye giriş gecikmesini önemli ölçüde arttırdı (p=0.025). Ayrıca, geçiş sayısı levamizol uygulamasıyla azaldı (p=0.021). Bununla birlikte, levamizol aydınlık ve karanlık bölmelerde geçirilen süreyi değiştirmedi (p=0.363). AAT'de, merkezi alana giriş sayısı (p=0.782), toplam geçilen çizgi sayısı (p=0.250), dışkı peleti sayısı (p=0.336), toplam şahlanma sayısı (p=0.367) ve temizlenme süresi (p=0.187) levamizol uygulamasıyla değişmeden kaldı. Ancak, levamizol merkezi alanda geçirilen süreyi artırırken (p=0.049), periferik alanda geçirilen süreyi azalttı (p=0.049). Kronik levamizol uygulaması, yetişkin erkek sıçanlarda lokomotor ve keşifsel aktiviteleri etkilemeden anksiyolitik benzeri etkiler oluşturabilir.

Anahtar Kelimeler: *Levamizol, MAO-A, aydınlık-karanlık kutu testi, açık alan testi, anksiyolitik benzeri etkiler*

Introduction

Levamisole is a broad-spectrum antihelminthic agent used against parasitic worm infections in veterinary medicine—and in human medicine outside the U.S. and Canada (1, 2). It was withdrawn from the U.S. market in 2000 owing to reports showing serious side effects in humans (e.g., agranulocytosis, vasculitis, and leukoencephalopathy (3) and then in 2003 from the Canadian market due to reports of agranulocytosis (4). Levamisole has been widely used to adulterate cocaine, and the Drug Enforcement Agency estimates that up to 80% of U.S. seizures contain it (5). However, it is included on the World Health Organisation's (WHO) list of essential medicines since it has a wide spectrum of activity and is safe, and a single oral dose can cure soil-transmitted helminth infections, and it is inexpensive (6). Additionally, it has been used successfully as monotherapy for the treatment of various skin diseases (7). More importantly, in the treatment of COVID-19, levamisole offers clinical benefits in improving health status

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among patients with mild to moderate cases (8). Additionally, it has been demonstrated to reduce hospital length of stay and improve certain clinical outcomes in hospitalized patients with severe COVID-19 (9).

Levamisole can cross the blood-brain barrier thanks to its high lipophilicity (10). It can suppress monoamine oxidase (MAO) (11) and also inhibit monoamine transporters for serotonin (5-HT), noradrenaline (NA), and dopamine (DA) (12). MAO is the responsible enzyme for the deamination of monoamines (13), and it has two isoforms, MAO-A and MAO-B (14). MAO-A mainly metabolizes adrenaline, NA, and 5-HT, while MAO-B degrades phenylethylamine and benzylamine (15). DA can be metabolized by both isoenzymes. The dysfunction of MAO-A activity has been associated with anxiety disorders (14).

Monoamines [(DA), (NA), and (5-HT)] and their metabolites are involved in a pivotal group of biogenic amines (16). Serotonergic dysregulation (17), dysfunction of dopaminergic receptors (18), and noradrenergic mechanisms (19) collectively contribute to the pathogenesis of anxiety. Considering the effects of levamisole on MAO and monoamine transporters, together with the important role of monoamines and MAO-A in anxiety disorders, we hypothesized that levamisole may modulate anxiety-like behaviors in male rats. To our knowledge, even though various properties of levamisole have been studied in several studies in recent years, its effects on anxiety-like behavior in male rats have been scarcely investigated.

The purpose of the current study was to investigate the effects of levamisole on anxiety-like behavior in adult male rats using different experimental designs: the light/dark box (LDB) and the open field test (OFT). The LDB test is a paradigm that allows determining the levels of innate aversion in rodents against brightly illuminated fields and the conflicting propensity of animals to explore novel spaces (20). In OFT, rodents come across a new environment, which elicits behavioral and physiological responses associated with anxiety (21).

Materials and Methods

Research and Publication Ethics: This study received approval from the Ethics Committee for Experimental Animals Research at Fırat University on July 14, 2020, with approval number 10 (Elazığ, Turkey).

Animals: Twenty naive adult male Sprague-Dawley rats (10-12 weeks old; 200–250 g) were supplied by the Experimental Research Unit of Fırat University (Elazığ, Turkey). The animals were housed in Plexiglas cages under standard laboratory conditions, with a 12-h light/12-h dark cycle (lights on at 8:00 AM) and a stable temperature (21±1°C) and humidity (55±5%).

Levamisole Treatment: Animals were randomly divided into two groups: a vehicle (n=10) group and a levamisole group (n=10). An a priori power analysis was not performed, and the sample size (n=10 per group) was determined based on group sizes commonly used in previous experimental animal studies investigating anxiety-like behaviors (22–24). Vehicle animals received distilled water. Levamisole was administered by oral gavage to the levamisole group at a dose of 2 mg/kg between 11:00 and 11:15 AM, once daily for 4 weeks, (25). The duration of levamisole administration was determined based on the estimated duration of action of MAO inhibitors (MAOIs) (26). Levamisole was prepared daily by dissolving it in distilled water.

Behavioral tests

Light/Dark Box Test (LDB): The Plexiglas light/dark box apparatus, divided into two equal-sized compartments (80 cm length × 40 cm height × 40 cm width) by a barrier that contained a doorway, was used in the present study. The light compartment is transparent, and the dark compartment is black and opaque, with a black lid cover. On the test day, the light compartment was illuminated with a 60-watt white light bulb placed 40 cm above the box, based on previous studies. At the start of the experiment, each rat was placed individually at the center of the lit compartment, facing away from the other compartment, and allowed to freely explore the environment for 5 min, and recorded by a camera system placed on the apparatus (27, 28). Between tests, the chambers were cleaned with 70% ethanol (29). For each rat, the latency to enter the dark compartment, the number of transitions, and the time spent in the light and dark compartments were measured. Animals were considered in a compartment when all four paws crossed into another compartment (30). All behavioral records were analyzed by a trained observer blind to the treatments. The LDB experiments were performed after the last levamisole treatment at the end of the fourth week.

Open Field Test (OFT): The open-field test apparatus was a square-shaped box made of opaque white Plexiglas (80 cm length × 40 cm height × 80 cm width). It contained a total of 25 squares, 9 in the center and 16 in the periphery. The apparatus was illuminated by a 60-watt white light bulb located 60 cm above the center (31). At the start of the experiment, each rat was individually positioned at the center of the open-field arena and observed for 10 minutes (29). The following behavioral parameters were recorded by a camera system placed on the apparatus: the number of entries into the central area, the total number of crossed lines (i.e., horizontal activity), the number of fecal pellets, the total number of rearings (i.e., vertical activity, defined as standing on the hind limbs, with both forepaws lifted off the floor), the duration of grooming (rapid cleaning movements of the forepaws towards the face and/or the body), the amount of time spent in the central area, and the amount of time spent in the peripheral area. Between experimental phases, the arena was wiped with a paper towel soaked in 70% ethanol (32). All behavioral records were analyzed by a trained observer blind to the treatments. The OFT experiments were performed on the same day as the last levamisole treatment, following LDB.

Data Analysis: For data analysis and graph generation, SPSS 21.0 (SPSS Inc., Chicago, IL) and GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, CA, U.S.) were used. The Shapiro-Wilk test was used to evaluate the normality of the data. After normality was confirmed, Student's t-test was used to assess normally distributed data, while the Mann-Whitney U test was used to analyze non-normally distributed data. For normally distributed parameters, data were expressed as mean ± standard error of the mean (S.E.M.), and for non-normally distributed data, as median (interquartile range). The significance level was defined as $p<0.05$. No formal correction for multiple comparisons (including Bonferroni adjustment) was applied. Effect sizes were calculated in addition to *p*-values. Cohen's d was calculated when the Student's t-test was used, whereas r was calculated for parameters analyzed using the Mann–Whitney U test. Statistical tests used for each parameter are summarized in Table 1.

Table 1. Statistical tests used for data analysis

Parameter	Statistical test
LDB Test	
Latency to enter the dark compartment	Mann-Whitney U
Number of transitions	
Time spent in the light compartment	Student's t-test
Time spent in the dark compartment	
Number of entries into the central area	
OFT	
Number of entries into the central area	Mann-Whitney U
Total number of crossed lines	
Number of fecal pellets	
Total number of rearings	Student's t-test
Duration of grooming	
Amount of time spent in the central area	
Amount of time spent in the peripheral area	

LDB: light-dark box test, OFT: open field test.

Results

The Effects of Levamisole on the Anxiety-Like Behavior in the LDB Test: Figure 1 illustrates the effects of levamisole on anxiety-like behavior in the LDB test. The results indicate that treatment with levamisole significantly prolonged the latency to enter the dark compartment ($p=0.025$, $r=0.500$) (Figure 1A). Additionally, the number of transitions significantly decreased with levamisole administration ($p=0.021$, $d=1.132$) (Figure 1B). Conversely, no change was observed in the time spent in the light ($d=0.418$) and dark compartments ($d=-0.418$) following a four-week levamisole treatment ($p=0.363$ for both) (Figure 1C-D).

The Effects of Levamisole on Anxiety-Like Behavior in the OFT Test: Figure 2A-G displays the results of the OFT. Levamisole treatment did not modify the number of entries into the central area ($p=0.782$, $r=0.062$), the total number of crossed lines ($p=0.250$, $d=0.532$), the number of fecal pellets ($p=0.336$, $d=0.442$), the total number of rearings ($p=0.367$, $d=0.414$), and the duration of grooming ($p=0.187$, $d=0.614$) (Figure 2A-E). However, animals that received levamisole spent more time in the central area ($d=-0.944$), which was accompanied by less time in the peripheral area of the open field apparatus ($d=0.944$) ($p=0.049$ for both, Figure 2F-G, respectively).

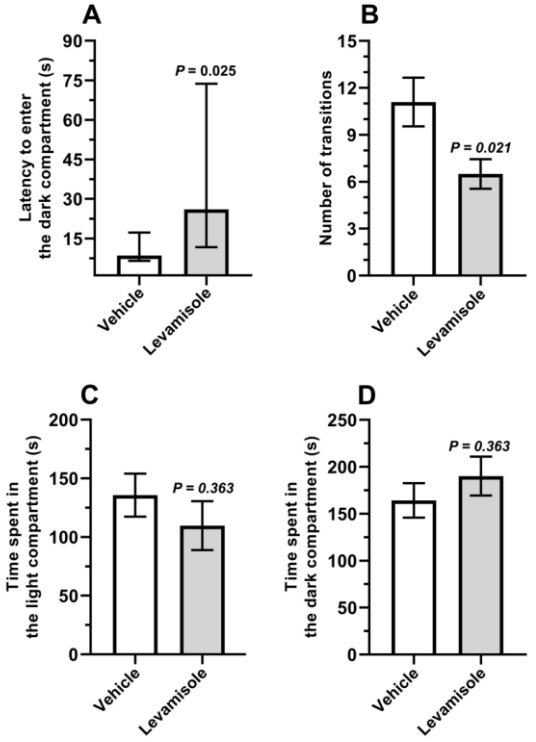

Figure 1. Effects of levamisole in the LDB test. **(A)** the latency to enter the dark compartment, **(B)** the number of transitions, **(C)** the time spent in the light compartment, and **(D)** the time spent in the dark compartment. Statistical differences in latency to enter the dark compartment were determined using a Mann-Whitney U test, and data are presented as median (interquartile range). A student's t-test was used for other parameters, and data are presented as mean ± S.E.M.

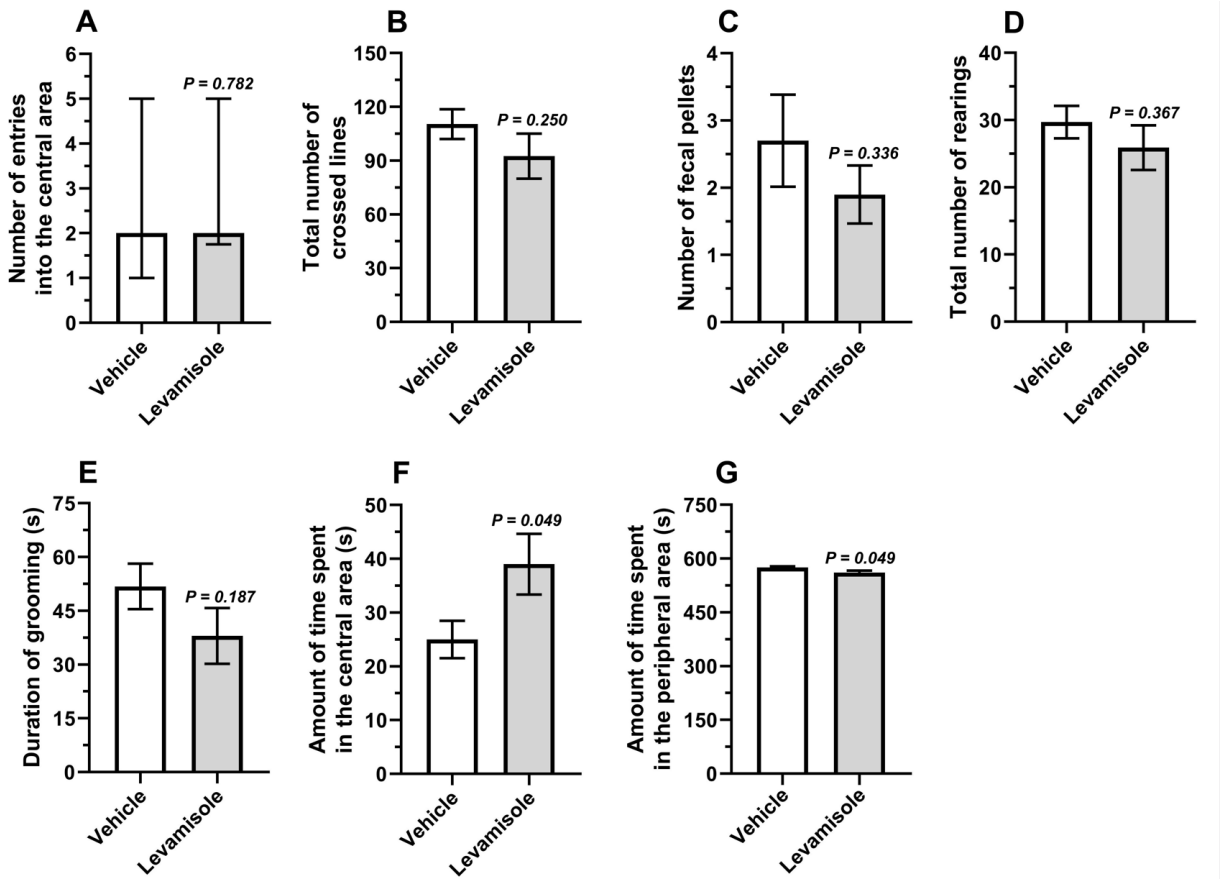

Figure 2. Effects of levamisole in the OFT test. **(A)** the number of entries into the central area, **(B)** the total number of crossed lines, **(C)** the number of fecal pellets, **(D)** the total number of rearings, **(E)** the duration of grooming, and **(F)** the amount of time spent in the central area, **(G)** the amount of time spent in the peripheral area. Statistical differences in the number of entries into the central area were determined using a Mann-Whitney U test, and data are presented as median (interquartile range). A student's t-test was used for other parameters, and data are presented as mean ± S.E.M.

Discussion

The LDB test has been used to determine the anxiolytic- and anxiogenic-like effects of drugs in rodents (20). It has several advantages, including being rapid and not demanding the training of animals, as well as the deprivation of water or food (33). The time spent in each compartment of the LDB apparatus reflects attraction or aversion, depending on whether it is illuminated or dark, respectively (34). Meanwhile, the decreased number of transitions between compartments indicates an anxiogenic phenotype (35). Moreover, prolongation of the latency to enter the dark compartment is an index of anxiolytic-like effect. It is suggested as one of the safest indicators of this effect in the LDB test (36).

In the current study, levamisole has been shown to enhance the latency to enter the dark compartment while interestingly attenuating the number of transitions in the LDB test. The time spent in the light or dark compartment remained unchanged. Taken together, these findings suggest a partial anxiolytic-like profile in the LDB rather than a uniformly anxiolytic pattern. The prolonged latency suggests a delay in the initial transition to the dark compartment, yet the reduced transition number may also reflect reduced exploratory behavior, altered risk-assessment behavior, or changes in shuttling behavior that are not exclusively attributable to anxiety.

Importantly, the effects of anxiolytic drugs on the number of transitions in the LDB test are controversial: some studies report an increase (37, 38), while others show no change (39). Moreover, the transitions between compartments and locomotion decreased at the same time, suggesting that the drug may have a sedative effect and indicating a drug-induced depression of general activity (28). Conversely, the enhanced number of transitions without an increment in locomotion has been reported to reflect anxiolytic activity. When any drug raises the number of transitions and locomotion, it is considered a general motor stimulant and is accepted as a possible anxiolytic (33). However, this interpretation is not fully supported because there is no change in the total number of crossed lines in the OFT. The reason for this paradoxical situation may be due to several factors, including experimental design (i.e., pharmacological settings, route of administration, and dose regimen) (27).

The OFT is a standard neophobic paradigm of anxiety (27). The OFT is primarily useful for observing and assessing the efficacy of candidate anxiolytic drugs (40). In this paradigm, animals are allowed to explore an open field freely enclosed by walls (41). Anxiolytic drugs have been shown to enhance time spent in the central area and reduce thigmotaxis (the tendency to stay close to walls), thereby decreasing the time spent in the peripheral area and exhibiting reduced anxiety-like behavior (40). The number of entries into the central area is another measure of an anxiety state (27) and increases with anxiolytic-like effect (42). The total number of crossed lines and rearings is generally used as a marker of locomotor activity. However, they are also an index of exploration and anxiety. The high levels of these behaviors point to increased locomotion and exploration and indicate a decrease in anxiety levels (32). In the current study, the number of entries into the central area, the total number of crossed lines, the number of fecal pellets, the total number of rearings, and the duration of grooming did not change with levamisole treatment. However, the time spent in the central area increased, while the time spent in the peripheral area decreased in the levamisole group. This pattern suggests that levamisole may have altered spatial preference in the center rather than increasing exploratory transitions into the center. In other words, central avoidance may have been reduced without a clear increase in approach frequency. This dissociation between center time and center entries may reflect changes in risk-assessment or decision-making strategies, whereby animals enter the center with similar frequency but remain there longer. Overall, the OFT findings indicate a limited anxiolytic-like effect, reflected mainly in altered spatial preference rather than a generalized increase in exploratory activity.

Levamisole can inhibit MAO-A (6), a finding confirmed by our previous study (25). MAO-A is a dominant enzyme in the rat brain compared to MAO-B (43). MAO-A mainly degrades adrenaline, NA, and 5-HT (15). Consistent with this, it has been noted that MAO-A deficiency leads to increased 5-HT levels in the brains of mice (44). 5-HT is closely associated with various neuropsychiatric conditions, particularly anxiety disorders (45), although the relationship between 5-HT and anxiety is complex and not solely determined by absolute transmitter levels. Levamisole was reported to alter the metabolism of 5-HT in particular brain regions of rats (46). Accordingly, it is plausible that monoaminergic pathways—potentially involving serotonergic mechanisms and/or other MAO-A–related substrates—could contribute to the behavioral pattern observed here. However, this mechanistic interpretation remains speculative because MAO-A activity and expression, as well as regional monoamine levels, were not assessed in the present study.

Reviewing the literature, most studies on MAO-A inhibition have primarily assessed the acute effects of compounds with MAO-A inhibitory activity—encompassing MAOIs, antidepressants, and non-antidepressant agents—on OFT and LDB outcomes. In contrast, the present study evaluated chronic levamisole exposure, which is an important distinction when interpreting behavioral findings, as acute and chronic regimens can yield partially divergent profiles due to differences in pharmacodynamics and behavioral adaptation. Given the renewed clinical interest in levamisole in the context of COVID-19, clarifying its potential central and behavioral effects may be relevant for a more complete understanding of its overall pharmacological profile. In particular, when a drug gains broader clinical attention beyond its traditional indications, defining possible neurobehavioral effects can help contextualize its benefit–risk profile and guide future mechanistic studies. In two acute treatment studies, moclobemide, a reversible inhibitor of the MAO-A, increased the time spent in the light compartment but did not alter the number of transitions in the LDB test. In the OFT, it attenuated the number of rearings but not ambulation (i.e., horizontal activity) (47), suggesting a selective modulation of exploratory components rather than a generalized change in locomotion. Notably, in a subsequent acute treatment study, the same agent (at the same dose as in the previous study, 10 mg/kg), moclobemide again increased the time spent in the light compartment, but, in contrast to the earlier study, also increased the latency to enter the dark compartment and the number of transitions in the LDB test (36), indicating that even within the same compound and dose, LDB outcomes—particularly transitions—may vary across experimental conditions. Consistent with this variability, in another acute study, diphenyl diselenide, which has MAO-A inhibitory activity, also increased the time spent in the light compartment and the latency to enter the dark compartment in the LDB test, but the number of transitions between compartments did not change. Importantly, it also did not change the number of crossed lines and rearings in the OFT (48), supporting the notion that some MAO-A–related compounds can produce anxiolytic-like shifts in approach/avoidance measures without clear effects on general activity indices. Extending these observations to another MAO-A–inhibitory compound, a recent study, J147, a derivative of curcumin, another MAO-A inhibitor, increased the time spent in the central area in different brain areas but did not alter the number of rearings in the OFT (49), again pointing to a partially selective profile in which center-related measures change in the absence of robust exploratory alterations.

Compared with these compounds, levamisole produced a partially overlapping but not identical behavioral profile. Unlike established MAO-A inhibitors, levamisole was not developed as a classical centrally acting MAOI and appears to have a broader pharmacological profile. Similar to several MAO-A inhibitors, levamisole increased latency to enter the dark compartment in the LDB test and increased time spent in the central area in the OFT, findings compatible with an anxiolytic-like effect. However, levamisole reduced the number of transitions in the LDB test, whereas previous studies with better-characterized MAO-A inhibitory agents generally reported either no change or an increase in this parameter. This difference suggests that

levamisole does not fully reproduce the behavioral pattern of conventional MAO-A inhibitors. Moreover, most previous studies assessed acute treatment, whereas the present study evaluated chronic exposure. Accordingly, comparisons with established MAO-A inhibitors should be made cautiously, and the effects of levamisole may not be explained solely by a classical MAO-A inhibitory mechanism.

Regarding the studies investigating the effects of levamisole on anxiety-like behaviors, there are very few studies on locomotor activity, not directly about anxiety-like effects. Overall, the findings vary by dose and experimental context. In acute levamisole treatment at 1 mg/kg, locomotor activity was reported as unchanged in one study (5), whereas another study observed a transient increase (50). At 5 mg/kg, acute treatment increased locomotor activity, whereas at 10 mg/kg, no change was observed (5). In a protocol most comparable to the present study in terms of dose and route (2 mg/kg by gavage daily for 10 days), levamisole reduced total distance in the OFT (48), whereas a subchronic study reported no locomotor change, consistent with our findings (52). Taken together, these data suggest that levamisole-related locomotor outcomes may depend on dose, strain, and exposure duration, raising the possibility of a non-linear dose–response relationship and indicating that results obtained at 2 mg/kg may not necessarily generalize to other doses.

Several limitations of the present study should be considered when interpreting the findings. First, an a priori power analysis was not performed. Although the sample size (n=10 per group) was determined based on group sizes commonly used in previous experimental animal studies, the study may still have been underpowered to detect subtle between-group differences. Therefore, non-significant findings should be interpreted with caution. In addition, multiple behavioral outcomes were analyzed without formal correction for multiple comparisons. Given the nature of the study, this approach may be acceptable; however, the absence of such correction may increase the risk of type I error, particularly when interpreting borderline significant findings. Additionally, only a single dose of levamisole (2 mg/kg) was evaluated; therefore, the dose–response relationship and the pharmacodynamic profile of levamisole with respect to anxiety-like behaviors could not be determined. Third, the behavioral results across LDB and OFT were not uniformly convergent, indicating a mixed profile that should be interpreted cautiously. Moreover, because the OFT was conducted on the same day immediately after the LDB test, potential carryover effects (e.g., stress, habituation, or learning-related effects from the first test) cannot be ruled out. Finally, mechanistic endpoints were not assessed; brain MAO-A activity/expression, regional brain monoamine levels, and peripheral or central biochemical measures were not evaluated. Future studies integrating behavioral testing with molecular and neurochemical assessments in relevant brain regions are warranted to elucidate the underlying pathways.

In conclusion, the present study suggests that chronic levamisole treatment is associated with a mixed behavioral profile in adult male rats, showing an anxiolytic-like component in specific measures without clear changes in several indices of general locomotor activity. To our knowledge, the current study is one of the first studies to investigate the effects of chronic levamisole exposure on anxiety-like behaviors in male rats. Further studies incorporating direct neurochemical and molecular assessments, together with multiple dose groups, are needed to clarify the underlying mechanisms, including whether MAO-A–related monoaminergic pathways contribute to these behavioral effects.

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